**Identification and sensory evaluation of flavour enhancers in Japanese traditional dried herring (*Clupea pallasii*) fillet**

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**Abstract**

Flavour-enhancing components of dried herring fillet (migaki-nishin in Japanese) were isolated and evaluated for their effects on sensory perception. Sensory evaluation revealed that addition of dried herring fillet water-soluble extracts to Japanese noodle soup significantly ($P < 0.05$) enhanced the soup flavour characters such as thickness, mouthfulness and continuity. The extracts were fractionated by dialysis and chromatography. Fractions containing flavour enhancers were isolated by sensory perception. Results from instrumental analyses showed that the *kokumi* flavour enhancers in dried herring fillet were creatine and creatinine.

***Keywords***: Herring; taste; *kokumi*; creatine; creatinine; sensory evaluation

## 1. Introduction

Dried herring (*Clupea pallasii*) fillet (DHF, Japanese, migaki-nishin) is a traditionally popular food item in Japan due to its remarkable flavour-enhancing properties. In particular, addition of DHF to noodle soup enhances flavour characters, such as thickness, mouthfulness and continuity. These flavour characters are often called *kokumi* in Japanese. Compounds added to enhance existing flavour characters, such as thickness, mouthfulness and continuity of a food, without imparting a typical taste and aroma of their own, have been described as *kokumi* flavour enhancers (Ueda, Sakaguchi, Hirayama, Miyajima, & Kimizuka, 1990; Ueda, Yonemitsu, Tsubuku, Sakaguchi, & Miyajima, 1997).

The flavour of fish and shellfish principally originates from extractive components (Konosu & Yamaguchi, 1982). In general, most flavour components of foods are water-soluble. These include nucleotides, amino acids, peptides, organic acids and bases, and inorganic ions. Other than amino acids, peptides and nucleotides, creatine, creatinine and lactate were found to be taste-active components in dried skipjack (Fuke & Konosu, 1991). Lactate, succinate, creatine, creatinine and hypoxanthine were also recognised as taste-active

in stewed beef juice (Schlichtherle-Cerny & Grosch, 1998).

It is well known that amino acids and peptides contribute to the taste of a wide variety of foods. The taste quality produced by amino acids and peptides has also been described as *kokumi* that has translated as "rich thick taste" (Kawajiri, 1999). It is reported that pyrazines and some peptides, generated together in certain foods during the process of boiling or aging for a long period, form *kokumi* (Ogasawara, 2003). Moreover, Shima, Yamada, Suzuki, and Harada (1998) have identified to be *N*-(1-methyl-4-hydroxy-3-imidazolin-2,2-ylidene) alanine in beef broth as a "brothy taste" modifier, whereas glutathione enhanced the flavour characteristics of thickness, mouthfulness and continuity when added to an umami solution (Ueda et al., 1997). Recently, it has been reported that the addition of *γ*-glutamyl peptides to a savoury matrix, e.g. sodium chloride and monosodium glutamate solutions or chicken broth, significantly decreased the detection thresholds and remarkably enhanced mouthfulness, complexity, and long lastingness of the savoury taste (Dunkel, Köster, & Hofmann, 2007).

Preliminary experiments showed that addition of DHF water-soluble extracts to Japanese noodle soup (JNS) enhanced the soup flavour characters, such as thickness, mouthfulness and continuity, and enhancement of these flavour characters increased with drying time (Shah, Tokunaga, Kurihara, & Takahashi, 2008). However, to the best of our knowledge, compounds responsible for the characteristic flavour-enhancing effects of DHF have not been identified. Therefore, this study was aimed to identify the flavour-enhancing compounds of DHF and to evaluate their effects on sensory perception.

## 2. Materials and methods

### *2.1. General*

Field desorption mass spectra (FD-MS) were determined on a JEOL JMS-SX102A mass spectrometer (JEOL Ltd., Japan). $^{1}H$ and $^{13}C$ nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AMX-500 NMR spectrometer (Bruker, Rheinstetten, Germany) at

500 MHz and 126 MHz, respectively. Deuterium oxide ($D_2O$) was used as solvent and 3-(trimethylsilyl)propionic-2,2,3,3-$d_4$ acid (TSP-$d_4$) was used as an internal standard.

*2.2. Materials and chemicals*

DHF was obtained from a fishery processing company, in Hakodate, Japan. Herring (*C. pallasii*) was captured at the coast of Kamchatka Peninsula, Russia, in October 2006, and kept frozen until it was processed. Upon arrival at the factory, herring was thawed, gutted, washed and then filleted for drying. Herring fillets were dried using huge electric fans. Room temperature and relative humidity were maintained at approximately 14 °C and 45%, respectively. After drying, DHF was randomly sampled for analysis. The DHF was comprised of 54.4% crude protein, 39.1% total lipid, and 4.3% ash on a dry weight basis. All chemicals used were of analytical or HPLC grade and were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan), Sigma (St. Louis, MO), and Amersham Pharmacia Biotech (Uppsala, Sweden).

*2.3. Preparation of water-soluble extracts (WSE)*

DHF was cut into small pieces and freeze-dried. The freeze-dried DHF (20 g) was then defatted using *n*-hexane and homogenised with a 10-fold volume of de-ionised water. The homogenate was centrifuged at 10,000 × g for 20 min at 4 °C and supernatant was collected. Resulting supernatant was extracted with ethanol (final concentration, 80%) to remove high molecular weight compounds, followed by centrifugation and then filtration. After evaporation and further freeze-drying of the filtrate, lyophilised powder was obtained as the WSE of DHF (1.37 g, yield = 6.85%). In parallel, freeze-dried DHF (20 g) was boiled in a 10-fold volume of de-ionised water for 30 min and then homogenised. The homogenate was centrifuged at 10,000 × g for 20 min at 4 °C and supernatant was obtained. After ethanol extraction and centrifugation, the obtained clear supernatant was freeze-dried to give the

WSE of boiled DHF (1.78 g, yield = 8.90%).

*2.4. Fractionation of the WSE of DHF*

The WSE of DHF was dissolved in de-ionised water, and then the solution was dialysed against de-ionised water using 1000 and 5000 Da cut-off membranes (Spectrum Laboratories, Inc., California, USA) and finally freeze-dried. Three fractions were obtained, namely fraction I (<MW 1000 Da), fraction II (MW 1000–5000 Da), and fraction III (>MW 5000 Da).

*2.5. Gel filtration chromatography (GFC)*

The fraction II (MW 1000–5000 Da) was dissolved into de-ionised water (100 mg/ml) and, then applied onto the top of a water-cooled glass column (2.2 × 85.0 cm, Amicon Corporation, Lexington, MA, England) filled with slurry of Sephadex G-25 (Amersham Pharmacia Biotech, Uppsala, Sweden). The elution was achieved at a flow rate of 0.3 ml/min at 4 °C with de-ionised water as the mobile phase to allow sensory evaluation of the recovered fractions. The eluting solution was collected in 4 ml fractions. The UV absorbance of each fraction was measured at 220 nm using a Hitachi U-2000 spectrophotometer (Hitachi Ltd., Tokyo, Japan). The fractions were combined into five fractions F1–F5 on the basis of elution profile. The individual fractions were freeze-dried and stored at –50 °C prior to use.

*2.6. Separation by high-performance liquid chromatography (HPLC)*

Fraction F3, thus obtained, was analysed on a Hitachi 655A HPLC system (Hitachi Ltd., Tokyo, Japan). Chromatographic separation was performed with a TSK-GEL G2000SW semi-preparative column (10 $\mu$m; 7.5 × 300 mm, Tosoh Corp., Tokyo, Japan). The mobile phase consisted of an aqueous solution of 80% acetonitrile containing 0.1% trifluoroacetic acid. Elution was conducted at a flow rate of 1.0 ml/min and at room temperature. The

eluting solution was monitored with UV absorbance at 214 nm. The obtained fractions were then freeze-dried and stored at –50 °C until used for sensory evaluation and chemical analysis.

*2.7. Identification of the flavour enhancers in fraction F3-2*

A fraction thus obtained by rechromatography, F3-2, was further rechromatographed by a RP-HPLC system using an LC-10AT*VP* Shimadzu liquid chromatograph (Shimadzu Corp., Japan). Chromatographic separations were performed with an ODS column (Mightysil RP-18, Kanto Chem. Co., Tokyo, Japan) either in analytical (5 $\mu$m; 4.6 × 250 mm, flow rate 0.4 ml/min) or semi-preparative scale (5 $\mu$m; 10 × 250 mm, flow rate 1.0 ml/min). A linear gradient of acetonitrile from 10% to 20% for 20 min in water was used as mobile phase. Fractions F3-2-A and F3-2-B were isolated as flavour enhancers. Subsequent MS and NMR experiments of the isolates of RP-HPLC fractions F3-2-A and F3-2-B led to the identification of the *kokumi* components as creatine and creatinine, respectively.

*F3-2-A (Creatine)*: HRFDMS, *m/z* 132.0785 ($MH^+$, calcd for $C_4H_{10}N_3O_2$, 132.0774); $^1$H NMR (500 MHz, $D_2O$), δ 3.98 (2H, s), 3.05 (3H, s); $^{13}$C NMR (126 MHz, $D_2O$), δ 177.0, 160.0, 56.4, 39.8.

*F3-2-B (Creatinine)*: HRFDMS, *m/z* 114.0673 ($MH^+$, calcd for $C_4H_8N_3O$, 114.0668); $^1$H NMR (500 MHz, $D_2O$), δ 4.06 (2H, s), 3.05 (3H, s); $^{13}$C NMR (126 MHz, $D_2O$), δ 191.9, 172.2, 59.2, 33.0.

*2.8. Sensory evaluation*

Sensory evaluation was carried out by adding WSE and all other fractions (obtained by various fractionation steps) to JNS, following the method of Ueda et al. (1997) with slight

modification. JNS was prepared according to Shah, Tokunaga, Kurihara, and Takahashi (2009). It was diluted with six volumes of distilled water and then subjected to sensory evaluation. The WSEs of DHF and boiled DHF, and the three dialysed fractions obtained from the WSE of DHF were dissolved in JNS at a concentration of 0.10%. The lyophilised GFC and HPLC fractions were dissolved in JNS at concentration of 0.05% and 0.01%, respectively. After addition of test samples to JNS, the solution was heated to 60 °C in a water bath. About 50 ml of test and control solutions were served in opaque disposable plastic cups at the same time. Panel members were instructed to put an adequate volume in the mouth, and then to expectorate. The panelists were asked to judge the intensities of the test samples using a scale of 1–7, where three points were assigned to the control solution. Scoring was done on the basis of saltiness, umami, thickness, mouthfulness and continuity. Sensory evaluation was performed in the separated sensory booths. The panel was composed of three to five trained assessors (ages between 26 and 37 years) from the Food Research and Development Laboratory, Kirin Kyowa Food Co. Ltd., Ibaraki, Japan. All the panelists were trained in sensory experiments with various concentrations of commercial flavour enhancers at regular intervals for at least 2 years and were, therefore, familiar with the techniques and rating scales applied. Moreover, the members of the sensory panel had extensive experience in tasting and agreed on the intensities of saltiness, umami, thickness, mouthfulness and continuity in JNS.

### *2.9. Statistical analysis*

Statistical analysis was performed using Microsoft Office Excel 2003. The student's *t*-test was carried out to determine significant differences between test samples and the control. Trends were considered significant when the means of compared sets differed at $P < 0.05$.

## 3. Results and discussion

### *3.1. Sensory characteristics of water-soluble extracts*

The WSE had no basic taste but acquired a faint aroma in distilled water at a concentration of 0.10% (data not shown). However, addition of the WSE of DHF to JNS resulted in a pronounced effect of enhancing the soup flavour intensities of thickness, mouthfulness and continuity, compared to the WSE of boiled DHF, whereas no significant ($P > 0.05$) effect was observed on the saltiness and umami (Fig. 1). Sensory evaluation results suggest that *kokumi* flavour enhancers are naturally produced in DHF. Therefore, the WSE of DHF was used for further sensory-guided fractionation to identify the flavour enhancer.

The WSE of DHF was fractionated into three fractions by dialysis. Addition of these three fractions to JNS showed no significant ($P > 0.05$) influence on basic taste such as saltiness and umami (Table 1). However, addition of fraction II to JNS significantly ($P < 0.05$) increased the intensities of thickness and continuity of the soup flavour compared to the control. Addition of fraction III significantly ($P < 0.05$) enhanced mouthfulness intensity, while fraction I did not influence the soup flavour. From these results, it appears that fraction II (MW 1000–5000 Da) might contain at least some of the flavour enhancers. It has been reported that a peptide fraction (MW 1000–10,000) obtained from beef soup stock increased mildness and mouthfulness when added to beef soup (Ishii et al., 1995). Ogasawara, Yamada, and Egi (2006) also reported that peptide (MW 1000–5000 Da) products of Maillard reactions contain key substances that give characteristic flavour (mouthfulness and continuity) of *miso* ripened for extended periods.

### *3.2. Chromatographic separation of fraction II*

Fraction II (MW 1000–5000 Da) was further fractionated using gel filtration chromatography. The eluting fractions were separated into five fractions F1–F5 (Fig. 2). Fractions F4 and F5 were recovered in small amounts, therefore only three fractions F1–F3

were subjected to further analysis. The flavour-enhancing effect was predominant in F3, followed by F2, whereas F1 had practically no influence on the soup flavour (Table 2). Fraction F3 showed significant ($P < 0.05$) enhancement of thickness and continuity of the JNS compared to control.

Fraction F3 was divided into four fractions, from F3-1 to F3-4 on semi-preparative HPLC, as shown in Fig. 3. The taste profiles of JNS containing the fractions from F3-1 to F3-4 are shown in Table 3. Addition of those individual fractions to JNS had no significant influence on basic taste qualities, such as saltiness and umami, compared to the control. However, fraction F3-2 significantly ($P < 0.05$) enhances the intensities of mouthfulness and continuity of the JNS compared to the control. Furthermore, sensory evaluation showed that, not only mouthfulness and continuity, but also thickness were more clearly recognised by the panelists in fraction F3-2 than in other fractions.

*3.3. Isolation and identification of flavour enhancers in fraction F3-2*

Fraction F3-2 was further fractionated by RP-HPLC, to afford compounds F3-2-A and F3-2-B (Fig. 4). The MS and NMR data revealed that the structure of flavour-enhancing compounds of F3-2-A and F3-2-B were creatine (Hanstock, Rothman, Prichard, Jue, & Shulman, 1988) and creatinine (Yamada, Akahori, & Matsuda, 2009), respectively. The identification of both compounds was further confirmed by comparison of spectroscopic and chromatographic data with those obtained for the corresponding reference compound. Although creatine and creatinine have already been identified as taste-active compounds in dried skipjack (Fuke & Konosu, 1991), Vietnamese fish sauce (Park, Watanabe, Endoh, Watanabe, & Abe, 2002), and stewed beef juice (Schlicherle-Cerny & Grosch, 1998), the flavour-enhancing activity of these compounds has not so far been reported.

Although molecular weight of creatine and creatinine are 131.13 and 113.11 Da, respectively, surprisingly, these two compounds were found in fraction II (MW 1000–5000

Da). To determine the possible reasons for this, we dialysed a solution of authentic creatine and creatinine using a MW 1000 Da cut-off membrane. After dialysing these compounds against de-ionised water, it was found that the dialysis tube retained (i.e. >MW 1000 Da) 58% and 8% of creatine and creatinine, respectively. Moreover, creatine has positive and negative ions; aggregation of molecules in solution can therefore be expected. Thus creatine and creatinine were isolated from fraction II (MW 1000–5000 Da).

*3.4. Sensory evaluation of creatine and creatinine*

To confirm the roles of creatine and creatinine as *kokumi* flavour enhancers, food grade creatine and creatinine were added to the JNS and presented to the trained sensory panelists. Weight percentages of creatine and creatinine, of fraction F3-2-A and F3-2-B, were calculated from representative peak areas and the ratio of those were approximated as 95% and 5%, respectively. Test samples were added to the JNS at a concentration of 0.01%. Sensory evaluation results revealed that the addition of creatine and creatinine to the JNS significantly ($P < 0.05$) increased the intensities of thickness, mouthfulness and continuity of the JNS compared to the control, while no significant effect was observed on basic taste qualities, such as saltiness and umami (Fig. 5). Comparing the flavour profiles of creatine and creatinine with that of the HPLC fraction F3-2 (Table 3) demonstrated that there was no significant ($P > 0.05$) difference between the individual flavour characteristics, thus indicating that creatine and creatinine might have made a contribution to *kokumi* enhancement of DHF. It has been reported that addition of sulphur-containing components, such as, *trans*-*S*-propenyl-L-cysteine sulfoxide and γ-glutamyl-*trans*-*S*-propenyl-L-cysteine sulfoxide, to an umami solution gave a characteristic *kokumi* flavour (Ueda, Tsubuku, & Miyajima, 1994). The multivalent taste-modulating Maillard reaction product, alapyridaine, in beef broth, enhances sweetness and umami characters (Ottinger & Hofmann, 2003), and, most recently, γ-L-glutamyl dipeptides have been identified as key *kokumi* molecules,

enhancing mouthfulness and complex taste continuity of matured Gouda cheese (Toelstede, Dunkel, & Hofmann, 2009).

## 4. Conclusion

Flavour-enhancing compounds, creatine and creatinine, were isolated and identified from water-soluble extracts of dried herring fillet. Sensory evaluation showed that creatine and creatinine enhanced the characteristic flavours of thickness, mouthfulness and continuity of the JNS, thus indicating that creatine and creatinine might be the *kokumi* flavour enhancers of dried herring fillet.

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**Figure Captions**

Fig. 1. Taste profile of Japanese noodle soup (JNS) treated with water-soluble extracts (WSE) of dried herring fillet (DHF).

(–) JNS with a score of three for control, (–o–) JNS treated with the WSE of DHF, (---) JNS treated with the WSE of boiled DHF.

Fig. 2. Gel filtration elution profile of fraction II.

Fig. 3. HPLC chromatogram of fraction F3.

Fig. 4. RP-HPLC chromatogram of fraction F3-2.

Fig. 5. Taste performance of creatine and creatinine in Japanese noodle soup (JNS) by sensory evaluation.

(---) JNS with a score of three for control; (−▲−) JNS treated with a combination of creatine/creatinine (95/5).

**Table 1**

Taste profile of Japanese noodle soup (JNS) containing water-soluble fractions obtained by stepwise dialysis [a]

| Attribute | Fraction I | Fraction II | Fraction III |
|---|---|---|---|
| Salty | 3.1 ± 0.54 | 3.0 ± 0.71 | 3.4 ± 1.14 |
| Umami | 3.3 ± 0.45 | 4.1 ± 0.89 | 4.0 ± 0.79 |
| Thickness | 3.7 ± 0.84 | 4.7 ± 0.67[*] | 4.0 ± 0.79 |
| Mouthfulness | 3.7 ± 0.83 | 4.7 ± 0.97 | 4.7 ± 0.84[*] |
| Continuity | 3.6 ± 0.89 | 4.9 ± 0.54[*] | 4.6 ± 1.08 |

[a] Data are expressed as means ± standard deviation (n = 5). Taste intensity scored on a 1–7 point scale where three points were assigned to the score of JNS.

[*] Asterisks indicate significant difference from control at $P < 0.05$.

**Table 2**

Taste profile of Japanese noodle soup (JNS) containing fractions obtained by gel filtration chromatography [a]

| Attribute | F1 | F2 | F3 |
|---|---|---|---|
| Salty | 4.0 ± 0.71 | 4.25 ± 0.43 | 4.38 ± 0.41 |
| Umami | 3.75 ± 0.83 | 4.0 ± 0.71 | 4.38 ± 0.65 |
| Thickness | 3.5 ± 0.87 | 4.13 ± 0.54 | 4.5 ± 0.50[*] |
| Mouthfulness | 3.5 ± 0.87 | 4.5 ± 0.5[*] | 4.38 ± 0.65 |
| Continuity | 3.25 ± 0.43 | 3.63 ±0.65 | 4.75 ± 0.83[*] |

[a] Data are expressed as means ± standard deviation (n = 4). Taste intensity scored on a 1–7 point scale where three points were assigned to the score of JNS.

[*] Asterisks indicate significant difference from control at $P < 0.05$.

**Table 3**

Taste profile of Japanese noodle soup (JNS) containing fractions from F3-1 to F3-4 [a]

| Attribute | F3-1 | F3-2 | F3-3 | F3-4 |
|---|---|---|---|---|
| Salty | 3.0 ± 0 | 3.0 ± 0 | 3.0 ± 0 | 2.83 ± 0.29 |
| Umami | 3.0 ± 0 | 3.0 ± 0 | 3.17 ± 0.29 | 3.17 ± 0.29 |
| Thickness | 3.17 ± 0.29 | 4.33 ± 1.04 | 3.67 ± 0.76 | 3.33 ± 0.58 |
| Mouthfulness | 3.0 ± 0 | 3.83 ± 0.29[*] | 3.5 ± 0.5 | 3.5 ± 0.87 |
| Continuity | 3.67 ± 0.76 | 4.17 ± 0.76[*] | 3.67 ± 0.76 | 3.33 ± 0.58 |

[a] Data are expressed as means ± standard deviation (n = 3). Taste intensity scored on a 1–7 point scale where three points were assigned to the score of JNS.

[*] Asterisks indicate significant difference from control at $P < 0.05$.

Fig. 1 (Shah et al.)

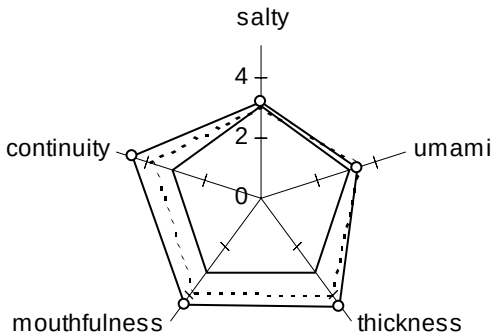
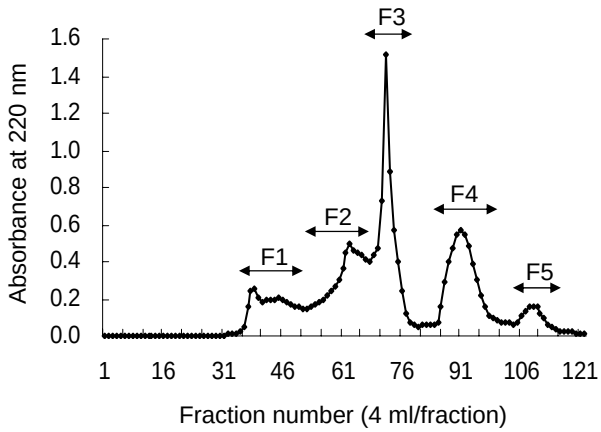


Fig. 2 (Shah et al.)



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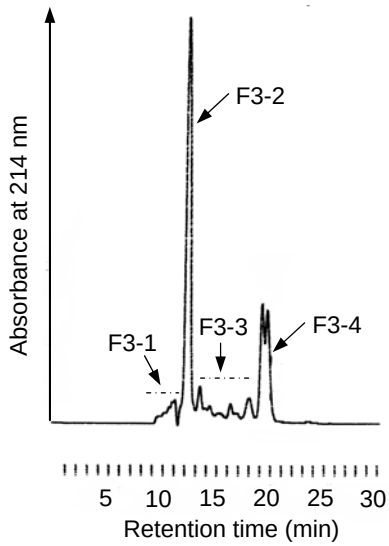

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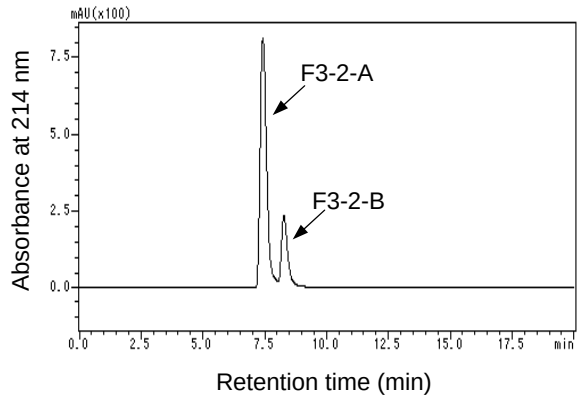

Fig. 5 (Shah et al.)