Original paper

Supercritical Fluid Extraction of “Koku” Enhancing Compounds from Fish and Fishery by-Products

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The potential of supercritical carbon dioxide (SC-CO₂) extraction of “koku” enhancing compounds from dried herring fillet (“Migaki-nishin” in Japanese) and sturgeon liver was explored. “Koku” enhancing compounds from a water-soluble extract and a mixed water miscible SC-CO₂ extract were isolated and evaluated for their effects on sensory perception. Results showed that only select compounds were found in the mixed water miscible SC-CO₂ extract compared to the complicated composition of the water-soluble extract. A notable feature was that the mixed water miscible SC-CO₂ extract contained “koku” enhancing compounds, e.g., nicotinamide, glycerol and creatine. This study revealed for the first time that these compounds were extracted by SC-CO₂ extraction and that nicotinamide enhances “koku”. We also extracted “koku” enhancing compounds from sturgeon liver using the SC-CO₂ extraction technique. Instrumental analysis revealed that the sturgeon liver contained relatively large amounts of nicotinamide and glycerol. Thus, it is suggested that this technique might be applicable to the extraction of select compounds from fish and fishery by-products.

Keywords: “koku”, supercritical carbon dioxide (SC-CO₂), nicotinamide, glycerol, creatine, sensory evaluation

Introduction

Dried herring (*Clupea pallasii*) fillet, called “Migaki-nishin” in Japanese, is a traditional popular food item in Japan due to its remarkable flavor-enhancing properties. It is widely used as an ingredient in savory dishes, including noodles. In particular, the addition of dried herring fillet to noodle soup enhances flavor characteristics such as mouthfulness and continuity. The flavor

characteristic that encompasses the sensation of mouthfulness and taste continuity is known as “koku” in Japanese. It has been reported that the addition of a dialyzed water-soluble fraction (MW 1000 – 5000 Da) of “Migaki-nishin” to Japanese noodle soup enhanced “koku” (Shah *et al.*, 2010). Furthermore, it was reported that pyrazines and some peptides, generated together in certain foods during the process of boiling or aging for extended periods,

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form “koku” (Ogasawara, 2003). It is also known that some γ -glutamyl peptides such as glutathione enhance “koku” (Ueda *et al.*, 1997; Kawajiri, 1999; Dunkel *et al.*, 2007; Toelstede *et al.*, 2009).

The flavor of fish and shellfish principally originates from extractive components (Konosu and Yamaguchi, 1982). In general, most flavor components of foods are water-soluble, and include nucleotides, amino acids, peptides, organic acids and bases, and inorganic ions. Conventionally, flavor components from foods are recovered by the water extraction method. However, this method has a number of drawbacks, such as low selectivity, high energy costs, and the possible loss of volatile compounds during water removal. On the other hand, the high solubility and mass transfer rate makes extraction with a supercritical fluid very attractive. Additionally, supercritical fluid extraction has better transport properties than liquids and can easily diffuse through complex matrices.

Supercritical fluid extraction (SFE), which uses carbon dioxide (CO_2) as the extractant, is one of the methods used to extract compounds from natural products. Supercritical CO_2 is a suitable and safe extractive solvent for foods because it is non-flammable, non-explosive, inactive, non-toxic, cost-efficient, readily available, easily removed and has a relatively low critical temperature (304.25 K) and critical pressure (72.9 atm or 7.39 MPa) (Herrero *et al.*, 2006). Therefore, it is highly appropriate for obtaining pure extracts from natural products. In addition, the SFE technique is known to extract useful compounds selectively, and this technology has already been applied in the manufacturing of, e.g., hops extract (Hubert and Vitzthum, 1978), caffeine-free coffee beans (Peker *et al.*, 1992), and extracts from spices (Díaz-Maroto *et al.*, 2002; Roy *et al.*, 1996; Simándi *et al.*, 1998) and medicinal plants (Hamburger *et al.*, 2004). Moreover, because CO_2 is a non-polar solvent, SFE is mainly used to extract non-polar compounds. Therefore, we attempted to reveal the mixed water miscible polar compounds in extracts obtained by SFE from fish and fishery by-products and its flavor properties when water was used as an entrainer.

Materials and Methods

Materials Dried herring fillets (“Migaki-nishin” in Japanese) were obtained from a fishery processing company in Hakodate, Japan. Herring (*C. pallasii*) was captured off the coast of Kamchatka Peninsula, Russia between October 2007 and 2009 and kept frozen until processed. Upon arrival at the factory, the herring was thawed, gutted, washed and then filleted for drying. Herring fillets were dried for 10 days using huge electric fans. Room temperature and relative humidity were maintained at approximately 14°C and 45%, respectively. Sturgeon (*Huso huso* $\times$ *Acipenser ruthenus*) liver was a gift from the Laboratory of Aquaculture Biology, Faculty of Fisheries Sciences, Hokkaido University, Hakodate, Japan. Dried herring fillets and sturgeon liver were stored at -50°C until used.

Chemicals All chemical reagents and solvents used were of analytical or HPLC grade.

Preparation of water soluble extracts (WSE) from “Migaki-nishin” The skin and small bones were removed from the dried herring fillets, which were then comminuted and freeze-dried. After that, the moisture content of sample was adjusted to 20% v/w by the addition of water. Ten grams of sample was added to ten times its volume of water, and extraction was carried out at an extraction temperature of 30°C and extraction time of 3h. The extract was separated with *n*-hexane and water. The water layer was collected and then filtered. After evaporation and further freeze-drying of the filtrate, the lyophilized powder was obtained as the WSE. The WSE was stored at -50°C until used.

Supercritical carbon dioxide extraction from “Migaki-nishin” A laboratory-scale SC- CO_2 extraction system (Model SC- CO_2 2080 plus; JASCO Corp., Tokyo, Japan) was used in this study. Samples were prepared as above. Ten grams of sample was placed in the SC- CO_2 extraction cell. Then, the extraction cell was put into a thermostatic oven. The extraction was carried out under the following conditions: extraction temperature, 30°C; fluid pressure, 25 MPa; fluid flow rate, 3 mL/min; and extraction time, 3h. The extract was separated in a separatory funnel using *n*-hexane and water. The water layer was collected, freeze-dried and stored at -50°C until used.

Thin Layer Chromatographic (TLC) analysis The composition of the extracted samples was determined using a commercial silica gel 60F₂₅₄ (Merck, KGaA, Darmstadt, Germany) thin layer chromatography (TLC) plate, with a single step development system consisting of *n*-butanol:acetic acid:water (4:1:2, v/v/v). The plate was sprayed with 50% H_2SO_4 and heated at around 150–160°C for 15 min to visualize the spots. Then, the extracted compounds of samples were compared on the TLC plate.

Fractionation by Hydrophilic Interaction Liquid Chromatography (HILIC) The extracted samples were dissolved in 80% acetonitrile, then filtered through a 0.45 μm filter and subjected to HILIC analysis. A PU-2089 high performance liquid chromatograph was used for HILIC (JASCO), equipped with a TSK-GEL Amide-80 column (10 μm , 7.8 $\times$ 300 mm; Tosoh Corp., Tokyo, Japan). The separation conditions were as follows: a linear gradient of acetonitrile (80% to 45%) for 20 min, followed by 45% acetonitrile for an additional 10 min, at a flow rate of 2.0 mL/min. The eluted solution was monitored using a UV detector at a wavelength of 210 nm (UV-2070; JASCO), an Alltech ELSD 2000 evaporative light scattering detector (ArtisanTM Technology Group, Deerfield, IL, USA), and a photo-diode array detector (MD-2010; JASCO). Individual fractions were collected using a collecting trigger. The obtained fractions were then freeze-dried and stored at -50°C until used for sensory evaluation and chemical analysis.

MS (Mass Spectrometry) analysis For ESI-MS analyses, a Bruker DALTONICS micro TOF-HS focus (Bruker, Rheinstetten, Germany) was used. Methanol was used as the solvent.

NMR (Nuclear Magnetic Resonance) analysis For ¹H and ¹³C NMR analyses, a Bruker AMX-500 NMR Spectrometer (Bruker) was used. Tetradeuteromethanol was used as the solvent.

Sensory evaluation The “koku” enhancing activities of individual extracted fractions were evaluated for effects on sensory perception using Japanese noodle soup, following the modified method of Ueda *et al.* (1997). Japanese noodle soup was prepared according to the method described by Shah *et al.* (2009), diluted with six volumes of distilled water and used as the control solution. Test samples were dissolved in the Japanese noodle soup at a concentration between 0.03 to 0.3% (w/v). After addition of the test samples to the soup, the solution was heated at 60°C in a water bath. Approximately 20 mL of the 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 to 7, where “3” was assigned to the control solution. Only two of the authors were involved in the preliminary sensory evaluation of the extracts and in this case error bars are not shown.

The “koku” enhancing activity of the corresponding authentic food grade chemical compound (nicotinamide) was evaluated using Japanese noodle soup, *miso* soup and “Shiokara” (“akazukuri shiokara”). Commercial products of *miso* soup and “Shiokara” were purchased from a local market. Test samples were dissolved in Japanese noodle soup, or *miso* soup or “Shiokara” at a concentration between 0.03 to 0.3% (w/w). The 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 to 7, where “3” was assigned to the control solution. Scoring was done on the basis of saltiness, umami, “koku”, mouthfulness, and continuity. Sensory evaluations were performed in separate sensory booths. The panel consisted of six trained assessors (3 men and 3 women; ages between 26 and 37 years) from the Food Research and Development Laboratory, MC Food Specialties Inc., Ibaraki, Japan. All panelists had extensive experience in tasting and agreed on the intensities of saltiness, umami, “koku”, mouthfulness, and continuity in Japanese noodle soup, *miso* soup or “Shiokara”.

Application of moisture adjusted SC-CO₂ extraction to fishery by-products Sturgeon liver was also comminuted and freeze-dried; the moisture content was adjusted by the addition of water. Lipid extraction was also carried out using *n*-hexane, and lipid content was adjusted by the addition of extracted lipids. Ten grams of moisture and lipid content-adjusted samples were put into the SC-CO₂ extraction cell. Then, the extraction cell was put into a thermostatic oven. Extractions were carried out using varying conditions, e.g., moisture content, lipid content, extraction temperature, fluid pressure, and fluid flow rate.

Statistical analysis Statistical analysis was performed using

Microsoft Office Excel 2003. A student’s *t*-test was carried out to determine significant differences between the test sample and control. Trends were considered significant when the means of compared sets differed at *P* < 0.05.

Results and Discussion

Comparison of extracts obtained from “Migaki-nishin” The number of spots appearing on normal phase TLC was compared between the water-soluble and mixed water miscible SC-CO₂ extracts. Compared to the water-soluble extract, the number of visible spots on the TLC of the mixed water miscible SC-CO₂ extract was very limited, as shown in Fig. 1a. Sensory evaluation of these two extracts was performed to evaluate their “koku” enhancing effect on Japanese noodle soup. Results showed that both extracts exhibited “koku” enhancing properties (Fig. 1b). For this reason, it was considered that the SC-CO₂ extraction method specifically extracted the “koku” enhancing compounds from “Migaki-nishin”.

Identification of the specifically extracted “koku” enhancing compounds To identify the “koku” enhancing compounds, the mixed water miscible SC-CO₂ extract was then analyzed using HILIC (Fig. 2). Figure 2 shows five clearly separated peaks detected on ELSD. Based on the elution profile, 5 fractions were collected manually and subjected to sensory evaluation. Sensory evaluation revealed that fractions F2-F5 contain “koku” enhancing compounds. Therefore, these fractions were analyzed using

Fig. 1. Normal phase TLC analysis of extracts from “Migaki-Nishin” (a). “Koku” score of Japanese noodle soup containing individual extracts (b).

Developing solvent, *n*-butanol:acetic acid:water (4:1:2, v/v/v).

A: Water extract; B: SC-CO₂ extract

“Koku” was scored as the intensity of the test samples using a scale of 1 to 7, where “3” (dotted line) was assigned to Japanese noodle soup. Test samples were dissolved in Japanese noodle soup at a concentration of 0.1% (w/v). Error bars are not shown because the sensory evaluation was performed by only two panelists.

Fig. 2. HILIC elution profiles of the mixed water-miscible SC-CO₂ extract obtained from “Migaki-nishin” (Upper). “Koku” score of Japanese noodle soup containing the individual fractions (Lower).

Detection at 210 nm (Upper) and ELSD detection (Middle). Column, TSK-GEL Amide-80 (7.8 × 300 mm); separation conditions consisted of a linear gradient of acetonitrile (80% to 45%) for 20 min, followed by 45% acetonitrile for another 10 min, at a flow rate of 2.0 mL/min.

“Koku” was scored as the intensity of the test samples using a scale of 1 to 7, where “3” (dotted line) was assigned to Japanese noodle soup. Individual fractions were dissolved in Japanese noodle soup at a concentration of 0.1 % (w/v). Error bars are not shown because the sensory evaluation was performed by only two panelists.

Fig. 3. Effect of nicotinamide on the taste performance of “Shiokara”.

Nicotinamide was added to “Shiokara” at a concentration of 0.1%. Sensory evaluation was scored on a scale of 1-7 points, where 3 points (dotted line) was the score given to “Shiokara” (control).

(n = 6; **P* < 0.05 vs. control; ***P* < 0.01 vs. control)

Fig. 4. Determination of optimum conditions for the extraction of “Koku” enhancers from sturgeon liver.

(A) Effect of fluid pressure and temperature on yield. (B) Effect of fluid moisture and fat content (%) on yield. (C) Effect of fluid speed and extraction on yield.

“O” indicates optimum points.

¹H-NMR, ¹³C-NMR, and ESI-MS to identify the “koku” enhancing compounds. The prominent peaks appearing in the individual fractions were identified as nicotinamide, glycerol, and creatine (Fig. 2). The fraction F5 peak was not identifiable because of its insolubility in tetradeuteriomethanol.

The “koku” enhancing potential of nicotinamide Although glycerol and creatine are currently recognized as “koku” enhancers of many foods (Gawel *et al.*, 2007; Fuke and Konosu., 1991; Park *et al.*, 2002; Schlichtherle-Cerny and Grosch, 1998; Shah *et al.*, 2010), the “koku” enhancing activity of nicotinamide has not been reported to date. Therefore, the “koku” enhancing effect of nicotinamide was evaluated in “Shiokara”, which is a dish in Japanese cuisine made from squid, consisting of small pieces of squid meat in a brown viscous paste comprising the animal’s

heavily salted, fermented viscera. Among the Japanese noodle soup, *miso* soup and “Shiokara” test samples, the “koku” enhancing effect of nicotinamide was the most prominent in “Shiokara” when the additive concentration of nicotinamide was 0.1%, followed by *miso* soup and Japanese noodle soup (data not shown). Sensory evaluation revealed that nicotinamide enhanced all the palatability factors, including “koku” in “Shiokara” (Fig. 3). Since *miso* can also be broadly classified as a fermented food, nicotinamide might enhance “koku” in most fermented foods. Further, this may be one of the reasons why the palatability of “Shiokara” was enhanced by the addition of squid liver, because liver tissue usually contains nicotinamide.

Application of moisture adjusted SFE on fishery by-products Sturgeon is highly prized for their eggs, which are processed into caviar. Besides caviar, sturgeon muscle and even the scales have recently begun to be utilized. However, liver continues to be discarded because it is highly perishable and contains relatively large amounts of heavy metals. As in the study using moisture-adjusted SFE to obtain “koku” enhancing compounds specifically from fishery by-products, sturgeon liver was chosen as a test sample. This was based on the presence of nicotinamide in the liver and the perception of sturgeon as a specialty product.

To extract “koku” enhancing compounds from sturgeon liver using the SFE technique, various conditions were examined for optimum yield. The optimum conditions for the extraction of “koku” enhancing compounds from sturgeon liver were: extraction temperature of 55°C, fluid pressure of 30 MPa, fluid flow speed of 3 mL/min, extraction time of 6 hrs, sample moisture content of 30%, and sample fat content of 20% (Fig. 4). The obtained extract was then analyzed using HILIC-ELSD, and showed four main peaks (Fig. 5). Of those, the fraction F2 peak was identified as nicotinamide based on the retention time and $\lambda_{\max}$ of authentic nicotinamide. The fraction F3 peak was identified as glycerol based on the retention time and ¹H-NMR (double doublet and quintet signals were observed between 3.50 to 3.65 ppm).

In this study, hydrophilic “koku” enhancing compounds were found in the mixed water miscible SC-CO₂ extracts obtained by the SFE technique. The compounds were identified as nicotinamide, glycerol and creatine, which were confirmed as “koku” enhancing substances in Japanese noodle soup, *miso* soup and “shiokara”. This SFE technique was then successfully applied to the specific extraction of hydrophilic “koku” enhancing compounds from sturgeon liver, a fishery by-product. The naturally occurring nicotinamide and glycerol might be useful in enhancing the “koku” of instant foods. Moreover, this SFE technique might be applicable to the extraction of selective compounds from fishery by-products.

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Fig. 5. HILIC elution profile of the mixed water-soluble fraction of SC-CO₂ extract obtained from sturgeon liver. Detection at 210 nm (Upper) and ELSD detection (Lower). Column, TSK-GEL Amide-80 (7.8 × 300 mm); separation conditions consisted of a linear gradient of acetonitrile (80% to 45%) for 20 min, followed by 45% acetonitrile for another 10 min, at a flow rate of 2.0 mL/min.

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