〈Original Article〉

Metabolomic approach using ultra-high-performance liquid chromatography-mass spectrometry: Quantitative amino acid analysis and characterization of four species of marine fish, and a cuttlefish

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Summary Metabolomics enables us to detect not only the targeted metabolites but also the non-targeted metabolites. Therefore, it becomes an effective new analytical method for biological materials. This paper reports on the verification of this analytical method based on the metabolomic approach using ultra-high-performance liquid chromatography-mass spectrometry. The feasibility of this metabolomic approach was tested for analyzing four species of marine fish and a cuttlefish. It was found that: 1) twenty-four kinds of amino acids were quantified as fast as 10 min.; 2) those amino acids were discriminated with some characteristic metabolites being found simultaneously using chemometrics; and 3) the cuttlefish was different in higher amino-acid content and metabolic profile compared with the fishes. As a result, this metabolomic approach is found to be useful for multi-component analysis of various kinds of seafood species, and it may be applicable for other biological materials such as blood serum.

Key words: Seafood, Amino acid, Metabolites, Chemometrics, Multi-component analysis

1. Introduction

Seafood is an important food source and contains many useful components, such as the omega-3 fatty acids docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) and amino acids, which have attracted attention as healthy food constituents[1-3]. Therefore, the evaluation of these components is necessary for understanding the nutritional significance and efficacy of seafood. The characterization of these seafood components may further demonstrate the added value, quality, and effective use of seafood[4, 5]. In particular, free amino acids in seafood are fundamental components for nutrition, taste[6, 7], and quality control[8].

Various methods to quantify amino acids are available: high-performance liquid chromatography

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(HPLC) is the main method but is time consuming and difficult to use for evaluating several components simultaneously. However, amino acids have recently been analyzed using mass spectrometry (MS) coupled to HPLC[9, 10] or ultra-high-performance liquid chromatography (UHPLC)[11]. LC-MS technique allows a simple sample preparation without derivatization of amino acids and high specific sensitivity, and it is suitable for multiple targets. Furthermore, mass spectrometry provides high specificity and sensitivity with simultaneous evaluation of several components as well as amino acids.

Metabolomics in combination with principal component analysis (PCA) and partial least-squares discriminant analysis enables us to detect not only targeted metabolites but also non-targeted metabolites. In other words, the metabolomic approach may discover unique metabolites and thus generate new hypotheses because the change in the metabolite is combined with the phenotype directly[12-14]. Metabolomics has attracted attention because of its use in discovering various biomarkers in the medical field[15, 16]. It is also a useful approach for discovering unknown ingredients without genome information. Therefore, this approach is ideal for comprehensively examining characteristic ingredients in food. For example, astaxanthin, carotenoid[17], and selenoneine[18] have recently been found to be strong antioxidants. Novel metabolites in seafood may therefore be discovered using the metabolomics approach.

The aim of the present study was to verify a metabolomic protocol to detect both free amino acids and characteristic metabolites in the field of bio-scientific samples, such as marine fishes and a cuttlefish using UHPLC-MS followed by PCA based on spectrally aligned data. As a result, this method appears to offer efficiency and versatility for detecting targeted metabolites and discovering non-targeted metabolites simultaneously.

2. Materials and methods

1) Chemicals and reagents

Acetonitrile, methanol, and chloroform were purchased from Nacalai Tesque (Kyoto, Japan). Formic acid, trichloroacetic acid (TCA), standards for amino acids (tryptophan, glutamine, asparagine, ANII-type amino acids solution and B-type amino acid solution) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Betaine and creatine were obtained from Sigma-Aldrich Japan (Tokyo, Japan). Octopine from Toronto Research Chemicals (Toronto, Canada). All chemicals used in UHPLC-MS analyses were analytical grade. Amino acid standard stock solutions were prepared at a concentration of 50 nmol/mL in 0.1 N HCl. Betaine, creatine and octopine were prepared at a concentration of 50 nmol/mL in distilled water. These solutions were stored at −80 °C until use.

2) Samples

Fillets (200-300 g weight) of red seabream (*Pagrus major*), yellowtail (*Seriola quinqueradiata*), bonito (*Katsuwonus pelamis*), spotted mackerel (*Scomber australasicus*) and cuttlefish (*Sepia lycidas*) were obtained from a local fish market. Raw fillets of fish were the products of Japan, and a frozen fillet of cuttlefish was from Sri Lanka. Muscle of back parts of fishes and body muscle of the cuttlefish for analysis were used for experiment.

3) Sample preparation

The sample was deproteinized using TCA solution with minor modification by the method of Yoshida et al[19]. Briefly, 1 g of sample (fresh weight) was homogenized in 10 mL of 2% TCA solution using a Multi-beads shocker (Yasui Kikai Corp., Osaka, Japan). After centrifugation at 9,600 g for 5 min at 4 °C, the supernatant was filtered with a glass fiber filter (GB-140: Advantec., Tokyo, Japan) and made up to 40 mL with distilled water. A 10-mL aliquot was placed in a new tube and then added to 10 mL of methanol and 10 mL of chloroform. The tube was mixed using a vortex mixer for 15 sec, centrifuged at 9,600 g for 5 min at 4 °C, and then the organic layer phase was removed. The aqueous phase was filtered through a 0.22-μm hydrophilic membrane (Millix LG 0.2 μm; Merck Millipore, Tokyo, Japan) and the filtered sample was diluted 50-fold with 0.2% formic acid in 50% acetonitrile. Ten microliters of the diluted sample

was used for UHPLC-MS analysis.

4) UHPLC-MS analysis

UHPLC-MS data were obtained using an Ultimate 3000 system coupled to an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Acclaim C18 column (150 × 2.1 mm, 3-µm particle size) (Thermo Fisher Scientific) was used for UHPLC separations, set at 40 °C at a flow rate of 0.2 mL/min. The mobile phase consisted of solvent A, 0.1% formic acid in water; and solvent B, 0.1% formic acid in acetonitrile. The linear gradient was 5-95% of solvent B in 4 min and then kept for 1 min. It then returned to 5% of solvent B in 0.1 min and remained there for 5 min for re-equilibration. MS analysis was performed using electrospray ionization in the positive ion mode. The MS conditions were as follows; spray voltage 3.0 kV, capillary temperature 325 °C, sheath gas flow rate 50 arbitrary units, auxiliary gas flow rate 15 arbitrary units, and scanning ions in mass range of *m/z* 60-350. Data were analyzed using Xcalibur software version 2.2 SP1 (Thermo Fisher Scientific).

5) Spectral alignment and multivariate analysis (Fig. 1)

Statistical analyses were performed to pre-aligned mass spectral data as metabolites to discriminate between four fish species and a cuttlefish to detect characteristic metabolites. The mass spectral data were spectrally aligned using SIEVE 2.0 (Thermo Fisher Scientific) and PCA was performed after the aligned data were loaded into SIMCA-P+Version12 (Umetrics; Umeä, Sweden).

3. Results

1) Targeted analysis of amino acids using UHPLC-MS

The amino acids as targeted metabolites were quantified using the external standards based on peak areas of the mass chromatogram. All samples were analyzed three times to provide an average and standard deviation.

Figure 2 shows mass chromatograms of the separation of a standard mixture of amino acids. Twenty-nine mass chromatograms were obtained by this method. Twenty-four amino acids can be measured unambiguously, while five amino acids marked by an asterisk are undistinguishable to other amino acids having the same masses by this method.

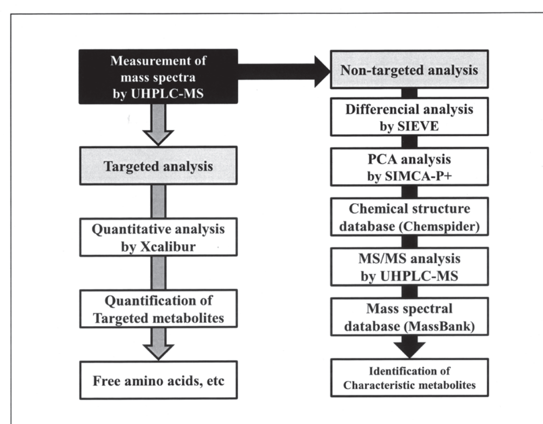

Fig. 1 Flow chart of metabolomic application of UHPLC-MS.

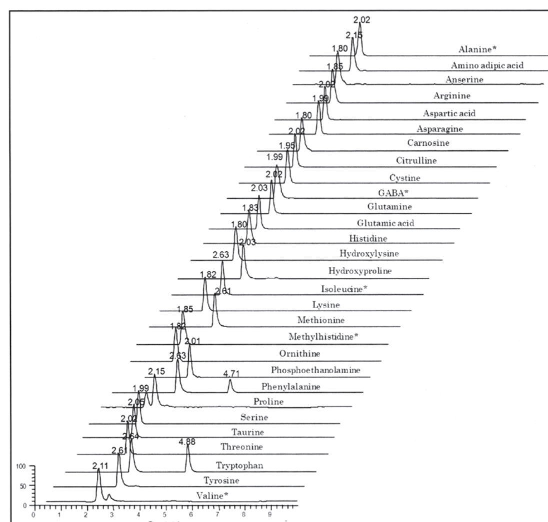

Fig. 2 Mass chromatograms of amino acid standard mixture.
Amino acids marked with an asterisk were detected as the total value of amino acids with the same mass. Alanine* is a mixture of alanine, sarcosine, and β-alanine. GABA* is a mixture of GABA, 2-amino butyric acid, and 3-amino butyric acid. Isoleucine* is a mixture of isoleucine and leucine. Methyl histidine* is a mixture of 1-methyl histidine and 3-methyl histidine. Valine* is a mixture of valine and betaine.

The calibration curve, the limit of detection (LOD), and the limit of quantitation (LOQ) are shown in Table 1. Four serially diluted concentrations (0.5, 0.25, 0.125 and 0.0625 nmol/mL) of the standard amino acid mixture were diluted in 0.2% formic acid in 50% acetonitrile. Standard curve and coefficient of determination were calculated using Xcalibur software version 2.2 SP1 (Thermo Fisher Scientific). It was found that the UHPLC-MS method was sensitive, with LODs and LOQs as low as 0.149 nmol/ml and 0.453 nmol/ml, respectively. As expected, metabolites having the same masses could not be separated by this method.

Table 2 shows the amino acid contents of the

Table 1 Calibration data for amino acids

Amino acids	**Calibration Curve** $Y = aX\ (10^5) + b\ (10^5)$	**LOD** (nmol/ml)	**LOQ** (nmol/ml)	R^2
Alanine*	Y = 7.59X+0.32	0.149	0.453	0.995
Aminoadipic acid	Y = 7.75X+0.07	0.015	0.046	0.992
Anserine	Y = 3.28X−0.14	0.034	0.103	0.990
Arginine	Y = 32.99X−0.96	0.011	0.035	0.998
Asparagine	Y = 13.76X+0.32	0.029	0.087	0.995
Aspartic acid	Y = 8.97X+0.09	0.029	0.089	0.993
Carnosine	Y = 4.24X −0.19	0.031	0.138	0.994
Citrulline	Y = 14.09X+0.02	0.025	0.075	0.995
Cystine	Y = 17.19X−0.33	0.034	0.104	0.992
GABA*	Y = 10.40X+0.58	0.123	0.371	0.990
Glutamine	Y = 15.87X+0.71	0.028	0.086	0.994
Glutamic acid	Y = 12.23X+0.28	0.043	0.131	0.990
Histidine	Y = 41.07X−0.57	0.013	0.380	0.997
Hydroxyproline	Y = 19.98X+0.28	0.040	0.120	0.993
Hydroxylysine	Y = 16.09X−0.42	0.015	0.046	0.997
Isoleucine*	Y = 36.02X+0.67	0.050	0.152	0.996
Lysine	Y = 15.55X−0.32	0.015	0.046	0.998
Methionine	Y = 27.30X+0.06	0.019	0.059	0.997
Methyl-histidine*	Y = 43.95X−1.28	0.009	0.027	0.998
Ornithine	Y = 14.59X−0.25	0.014	0.043	0.998
Phenylalanine	Y = 42.68X+0.51	0.024	0.072	0.997
Phosphoethanolamine	Y = 5.67X−0.07	0.008	0.026	0.995
Proline	Y = 25.38X+1.35	0.033	0.101	0.992
Serine	Y = 8.41X+0.26	0.049	0.150	0.986
Taurine	Y = 7.80X−0.02	0.012	0.035	0.993
Threonine	Y = 10.44X+0.16	0.048	0.147	0.990
Tryptophan	Y = 31.50X+0.62	0.006	0.018	0.999
Tyrosine	Y = 21.71X+0.03	0.023	0.070	0.997
Valine*	Y = 62.64X+0.59	0.034	0.104	0.997

Concentrations of standard amino acids used are 0.5, 0.25, 0.125, and 0.0625 nmol/mL. Amino acids marked with an asterisk were detected as the total value of amino acids with the same mass. Alanine* is a mixture of alanine, sarcosine, and β-alanine. GABA* is a mixture of GABA, 2-amino butyric acid, and 3-amino butyric acid. Isoleucine* is a mixture of isoleucine and leucine. Methylhistidine* is a mixture of 1-methylhistidine and 3-methylhistidine. Valine* is a mixture of valine and betaine.

samples with their main characteristic described as follows. Histidine was detected as the dominant amino acid in bonito (603.35 mg/100 g), spotted mackerel (521.34 mg/100 g), and yellowtail (339.84 mg/100 g). For red seabream, taurine, anserine, and alanine were the major amino acids with concentrations of 221.25 mg, 54.15 mg, and 46.63 mg/100 g, respectively. In cuttlefish, the most abundant amino acids were taurine, proline, alanine, and valine with concentrations of 916.51, 817.7, 430.98, and 259.46 mg/100 g, respectively. The resulting amino acid content was similar to the reported value[20, 21]. These results indicate that the UHPLC-MS method makes it possible to acquire data more rapidly with a higher degree of accuracy than by the conventional method based on HPLC.

2) Non-targeted analysis in four kinds of marine fishes and a cuttlefish using chemometrics

First, the result of principal component analyses (PCA) based on metabolites of four species of marine fish is shown in the figure 3. All mass spectra between *m/z* 60 and 350 were aligned and 104 characteristic metabolites were detected using SIEVE 2.0. The PCA parameters for the explained variation (R^2) and the cross-validated predictive ability (Q^2) were as follows: PC1: R^2 = 0.574, Q^2 = 0.400; PC2: R^2 = 0.220, Q^2 = 0.223. The first two PCA components (PC1 and PC2) explained 79.4% of the variation in the metabolic data, showing the split of the four species of marine fish into individual groups (Fig. 3A). Figure 3B represents the loading data corresponding to figure 3A and shows 8 dominant metabolites that the loading

Table 2 The average free amino acid contents quantified in four fish species and a cuttlefish

Amino acids	Red seabream	Yellowtail	Bonito	Spotted mackerel	Cuttlefish
Alanine*	46.63±1.05	26.71±0.30	39.24±0.35	44.4±0.54	430.98±7.39
Aminoadipic acid	ND	ND	ND	ND	9.19±0.58
Anserine	54.15±3.82	13.6±0.34	36.52±0.37	ND	ND
Arginine	5.11±0.18	3.53±0.14	4.28±0.07	2.89±0.06	95.01±0.93
Asparagine	ND	ND	ND	ND	2.18±0.08
Aspartic acid	ND	ND	ND	0.28±0.07	10.39±0.47
Citrulline	2.5±0.03	ND	ND	ND	1.27±0.06
GABA*	ND	ND	ND	ND	6.03±0.26
Glutamine	2.71±0.28	4.87±0.02	ND	ND	27.3±0.54
Glutamic acid	20.75±0.16	2.6±0.12	14.31±0.33	11.05±0.52	11.18±1.13
Histidine	9.3±0.1	339.84±8.13	603.35±20.95	521.34±19.92	5.1±0.34
Hydroxyproline	1.41±0.29	0.34±0.1	1.23±0.25	1.41±0.06	69.52±0.9
Isoleucine*	4.19±0.26	2.1±0.13	6.83±0.11	5.21±0.22	99.1±1.6
Lysine	31.54±1.19	12.91±0.33	26.87±1.3	5.44±0.17	25.21±0.38
Methionine	1.09±0.08	0.68±0.03	3.17±0.19	2.27±0.11	28.88±0.47
Ornithine	1.69±0.05	4.33±0.27	1.86±0.03	1.39±0.16	21.89±0.78
Proline	ND	ND	5.16±0.21	ND	817.7±4.94
Serine	2.18±0.2	2.7±0.3	1.24±0.14	1.71±0.17	49.05±1.81
Taurine	221.25±2.9	30.76±0.35	29.6±0.88	60.28±0.33	916.51±10
Threonine	2.48±0.1	0.88±0.08	4.23±0.13	2.69±0.05	65.53±1.68
Tryptophan	1.71±0.04	1.62±0.05	2.15±0.03	1.97±0.01	2.16±0.09
Tyrosine	1.45±0.09	0.96±0.05	2.97±0.15	3.89±0.11	5.81±0.16
Valine*	ND	ND	ND	ND	259.46±3.3

(mg/100g fresh weight)

All samples were analyzed three times and expressed as mean ± standard deviation. The amino acids marked with an asterisk were detected as the total value of amino acids with the same mass. Alanine* is a mixture of alanine, sarcosine, and β-alanine. GABA* is a mixture of GABA, 2-amino butyric acid, and 3-amino butyric acid. Isoleucine* is a mixture of isoleucine and leucine. Valine* is a mixture of valine and betaine.

values are more than 0.1 as inverted triangle symbols. The characteristic metabolites were labeled according to their mass values (*m/z*). The dominant peak in spotted mackerel, bonito, and yellowtail was histidine as *m/z* 156.0770. Taurine (*m/z* 126.0220), *m/z* 151.1440 and *m/z* 76.0756 were dominant peaks in red seabream.

Next, the result of PCA based on metabolites of four kinds of fish and a cuttlefish is shown in the figure 4. All mass spectra between *m/z* 60 and 350 were aligned and analyzed and 109 characteristic metabolites were detected using SIEVE 2.0. The PCA parameters for the explained variation (R^2) and the cross-validated predictive ability (Q^2) were as follows: PC1: R^2 = 0.663, Q^2 = 0.593; PC2: R^2 = 0.141, Q^2 = 0.085. The first two PCA components (PC1 and PC2) explained 80.4% of the variation in the metabolic data, showing the split of the four types of fish into individual groups (Fig. 4A). Figure 4B represents the loading data corresponding to figure 4A and shows 17 dominant metabolites that the loading values are more than 0.1 as inverted triangle symbols. Four species of marine fish were distinguished from each other, even though they belonged to the same family. As shown figure 4A, the cuttlefish belonging to Mollusca Cephalopoda was clearly distinguishable from the four fishes.

The dominant characteristic metabolites which were acquired in this study are shown in Table 3. The metabolites were compared by peak area ratio (%), and the masses (*m/z*) were experimental values. Nine components out of 19 metabolites were identified from the database, and the remaining 10 components were unknown metabolites. The metabolite of *m/z* 132.0770 in red seabream and the metabolite of *m/z* 247.1400 in cuttlefish were confirmed to be creatine

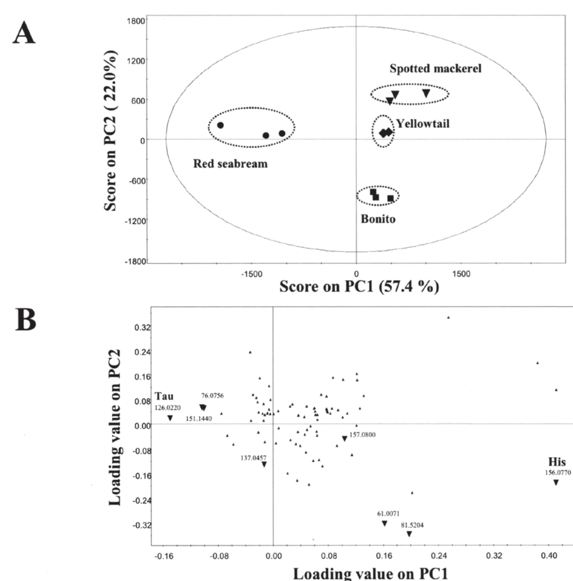

Fig. 3 PCA score plot and loading data of four fish species.
A: The PCA score plot was calculated, and the four groups of fish were visualized. All samples were analyzed three times. Individual data were shown in the figure.
B: PCA loading data from the PCA clearly shows several characteristic metabolites. Characteristic metabolites were labeled according to their mass values (*m/z*) with inverted triangle symbols.

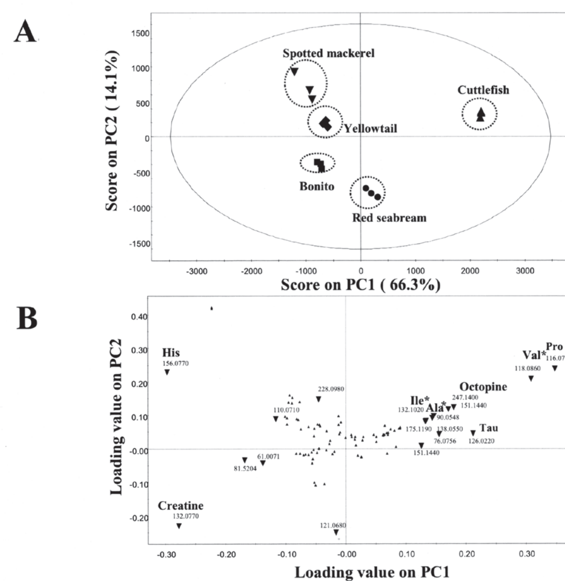

Fig. 4 PCA score plot and loading data of four fish species and a cuttlefish.
A: The PCA score plot was calculated, and the four groups of fish and a cuttlefish were visualized. All samples were analyzed three times. Individual data were shown in the figure.
B: PCA loading data from the PCA clearly shows several characteristic metabolites. Characteristic metabolites were labeled according to their mass values (*m/z*) with inverted triangle symbols.

and octopine by MS/MS analysis, respectively. As shown as Fig. 5, the product mass spectrum of *m/z* 132.07675 was *m/z* 90.05475, and the product mass spectra of *m/z* 247.14008 were *m/z* 70.06496, 112.08688, 116.07056, 130.09744, 142.08617, 158.09230, 175.11879, 188.09152, 201.13457, 229.12944, and 230.11343, as well as the creatine standard and octopine standard (data not shown). The elution time of *m/z* 132.07675 and *m/z* 247.14008 were the same as the elution time of creatine standard and octopine standard, respectively (data not shown). Consequently, creatine of all fish species except cuttlefish represented a major mass spectral peak. In cuttlefish, the major mass spectral peaks were *m/z* 116.0710, 118.0860, 247.1400, and 138.0550, corresponding to proline, valine, octopine, and unknown metabolite respectively.

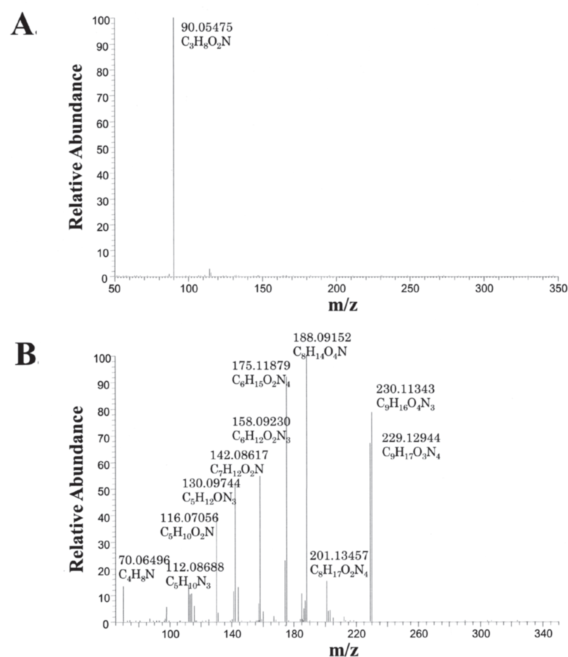

Fig. 5 Product mass spectra in the MS/MS analysis for non-targeted metabolites.
A: Product mass spectrum of *m/z* 132.07675 in red seabream extract.
B: Product mass spectrum of *m/z* 247.14008 in cuttlefish extract.

4. Discussion

Innovative approach and the data processing methodology are essential for various fields of analytical science to examine biological materials. A multi-

Table 3 The dominant characteristic metabolites of figure 3B and 4B

m/z	Rt	Metabolites	Red seabream	Yellowtail	Spotted mackerel	Bonito	Cuttlefish	Total peak area
61.0071	1.75	unknown	++	++	++	++	++	1.70×10^7
76.0756	2.01	unknown	++	+	+	−	+++	2.30×10^7
81.5204	1.69	unknown	++	++	++	+	++	2.90×10^7
90.0548	2.06	Alanine*	+	+	+	+	+++	1.30×10^7
110.0710	1.84	unknown	−	++	++	++	−	1.80×10^7
116.0710	2.13	Proline	−	−	−	−	++++	4.60×10^7
118.0860	2.11	Valine*	−	−	+	+	++++	3.10×10^7
121.0680	1.86	unknown	+++	++	−	++	−	6.00×10^6
126.0220	2.05	Taurine	++	+	+	+	+++	2.10×10^7
132.0770	2.09	Creatine	++	++	++	++	+	1.31×10^8
132.1020	2.63	Isoleucine*	+	+	+	+	++++	7.00×10^6
137.0457	2.57	unknown	++	++	+	++	++	8.00×10^6
138.0550	2.22	unknown	−	−	−	−	++++	1.00×10^7
151.1440	2.00	unknown	++	++	+	−	+++	8.00×10^6
156.0770	1.85	Histidine	−	++	++	++	−	1.03×10^7
157.0800	1.79	unknown	−	++	++	++	−	7.00×10^6
175.1190	1.85	Arginine	+	+	−	+	++++	5.00×10^6
228.0980	2.16	unknown	−	−	++++	−	−	3.00×10^6
247.1400	2.05	Octopine	−	−	−	−	++++	2.80×10^7

peak area ratio (%) : 0-1% − , 1-10% + , 10-50% ++, 50-80% +++, 80-100% ++++

The amino acids marked with an asterisk were detected as the total value of amino acids with the same mass. Alanine* is a mixture of alanine, sarcosine and β-alanine. Isoleucine* is a mixture of isoleucine and leucine. Valine* is a mixture of valine and betaine.

component evaluation is effective for understanding the nutritional significance and efficacy of foods including seafood. Until now, amino acid analysis has meant a long processing time with difficulty in evaluating multi-components simultaneously. However, the metabolomic approach using MS in combination with chemometrics was found to provide high sensitivity and evaluate targeted metabolites as well as non-targeted metabolites from all those detected.

In the present study, we examined whether this metabolomic protocol is suitable for quantification of free amino acids, and discrimination in metabolite variation comprehensively, using four species of marine fish and a cuttlefish. We chose red seabream, yellowtail, bonito, spotted mackerel and a cuttlefish as the popular seafood in Japan. From 2005 to 2009, those seafoods were reported to account for within a 15th of consumed seafood in Japan[22]. We quantified 24 amino acids as targeted metabolites in four species of marine fish and a cuttlefish at high sensitivity using UHPLC-MS. Additionally, these fishes could be classified despite closely-related species and we detected 19 characteristic metabolites simultaneously using chemometric analysis of the mass spectral data.

As targeted metabolites, histidine was the predominant amino acid in bonito, spotted mackerel, and yellowtail. Anserine[23] and taurine[24] as antioxidant ingredients were relatively high in red seabream as previously reported[20, 21]. On the other hand, non-targeted metabolites may be important ingredients: e.g. creatine was detected at high levels in spotted mackerel, red seabream, bonito, and yellowtail, whereas octopine was detected only in cuttlefish.

Octopine is known as an amino acid derivative and a specific component of marine invertebrates and agrobacteria[25]. Octopine was detected only in cuttlefish and may represent a unique metabolite marker for marine invertebrates. In fact, some non-targeted metabolites may be candidates for other markers for seafood (Table 3). For example, *m/z* 228.0980 and *m/z* 138.0550 were unknown metabolites; however, they were most abundant in spotted mackerel and cuttlefish, respectively. We were not able to classify yellowtail and spotted mackerel based on a PCA of their free amino acids (data not shown). However, a PCA based on non-targeted metabolites using UHPLC-MS could distinguish one species from another (Fig. 3A, 4A). This efficient method is highly sensitive and makes it possible to characterize four species of marine fish and a cuttlefish through an analysis based on targeted and non-targeted metabolites. This may be the basis for a high-quality system for evaluating and understanding the ingredients of seafood.

Seafood has attracted attention as a healthy food that includes many functional ingredients: e.g. creatine improves exercise capacity and octopine has a hypotensive effect[26, 27]. As sources of natural seafood decrease and fishing becomes limited, the characterization of seafood ingredients is important to promote the value and a stable supply of seafood. Recently, some amino acids in fish have been reported as quality indices for the maintenance, growth, immunity, behavior, and reproduction in metabolomics for fish[28]. In addition, amino acids are important for resistance to environmental stressors and pathogenic organisms; for example, the metabolomics approach has been used to determine the biomarkers of health in whale sharks[29].

Therefore, the characterization for seafood offered by the method presented here might prove useful for quality control of taste and functional ingredients. In the present study, we examined the metabolomics protocol using four species of marine fish and a cuttlefish. Further study using a variety of seafood samples is needed to establish a metabolomics protocol to characterize seafood. It might be applied to new guidelines for cultured fish, as well as for judging the nutritional value of underused seafood. Consequently, this metabolomics protocol, which is theoretically applicable to any type of biological materials such as blood serum/plasma, may become a versatile approach to test physiology, condition, and disease in the future.

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