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Analysis of Mutagenic Heterocyclic Amines in Cooked Food Samples by Gas Chromatography with Nitrogen-Phosphorus Detector

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Heterocyclic amines (HCAs) formed during heating (broiling, frying or grilling) of various proteinaceous foods such as meats and fishes are well known as potent mutagens in the Ames/*Salmonella* assay (Felton & Knize 1991; Eisenbrand & Tang 1993; Stavric 1994). These amines have also been found in various environments (Kataoka 1997). Up to the present, twenty-three heterocyclic amines have been isolated as mutagens and the structures of nineteen of them have been determined. Some heterocyclic amines have much higher mutagenic activity than typical mutagens/ carcinogens such as aflatoxin B_1, AF-2 and benzo[a]pyrene. Among these amines, Trp-P-1, Trp-P-2, AαC, MeAαC, Glu-P-1, Glu-P-2, IQ, MeIQ, MeIQx and PhIP have been verified to be carcinogenic in rats and mice, and IQ was also found to be carcinogenic in the monkey (Ohgaki et al. 1991; Eisenbrand & Tang 1993; Adamson et al. 1996; Sugimura et al. 1996). Furthermore, several case-control studies have reported positive associations between higher consumption of well-done red meat and risk of colon cancer (Probst-Hensch et al. 1997; Sinha et al. 1999 and 2001), breast cancer (Zheng et al. 1998; Sinha et al. 2000), lung cancer (Sinha et al. 1998) and gastric cancer (Ward et al. 1997; De Stefani et al. 1998). However, the association between the risk of cancer and dietary HCA intake has not sufficiently been clarified yet.

HCA contents in cooked meats are well investigated and evaluated contribution of meat dishes to the total intake of HCAs in population in Europe and America (Eisenbrand and Tang 1993; Byrne et al. 1998; Keating et al. 1999; Voskuil et al. 1999; Bogen and Keating 2001), but the data of those in cooked fish are limited (Skog et al. 1997; Felton et al. 2000; Toribio et al. 2000). In Japan, middle-aged and older people generally consume more fish than animal meat (Kobayashi et al. 1999), and chopped and stir-fried meat is the preferable cooking method. Therefore, it is important to grasp HCA contents in cooked fish in order to estimate dietary HCA intake levels and their major sources in Japan. Recently, we developed a selective and sensitive method for the determination of HCAs by gas chromatography with nitrogen-phosphorus detection (NPD-GC), in which these compounds are analyzed as their *N*-dimethylaminomethylene derivatives (Kataoka & Kijima 1997), and applied to the analysis of combustion smoke samples (Kataoka et al. 1998) and river water samples (Kataoka et al. 2000). In

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this paper, we investigated the presence and contents of HCAs in various fishes and meats cooked by common cooking procedure by using the NPD-GC method.

MATERIALS AND METHODS

In this study, the following ten HCAs were used for the analysis. 2-Amino-3-methylimidazo[4,5-*f*]quinoline (IQ) was purchased from Toronto Research Chemicals (Downsview, Canada). 2-Amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline (4,8-DiMeIQx), 2-amino-3,7,8-trimethylimidazo-[4,5-*f*]quinoxaline (7,8-DiMeIQx) and 2-amino-9*H*-pyrido-[2,3-*b*]indole (AαC) were purchased from Funakoshi Pharmaceutical Co. Ltd. (Tokyo, Japan). 3-Amino-1,4-dimethyl-5*H*-pyrido[3,4-*b*]indole (Trp-P-1) was purchased from Wako Pure Chemical Industries (Osaka, Japan). 3-Amino-1-methyl-5*H*-pyrido[3,4-*b*]indole (Trp-P-2), 2-amino-6-methyldipyrido [1,2-*a*:3′2′-*d*]imidazole (Glu-P-1) and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]-pyridine (PhIP) were kindly provided by Dr. H. Hayatsu, Professor Emeritus of Okayama University. 2-Amino-3,4,7,8-tetramethylimidazo[4,5-*f*]-quinoxaline (TriMeIQx; Funakoshi) was used as an internal standard (IS). Each HCA was dissolved in methanol to make a stock solution at a concentration of 0.1 mg/mL and used after dilution with methanol to the require concentration. N,N-Dimethylformamide dimethyl acetal (DMF-DMA) was purchased from Nacalai Tesque (Kyoto, Japan). Blue-rayon® was obtained from Funakoshi. All other chemicals were of analytical-reagent grade.

Food samples were purchased at local retail markets and were treated for analyses on the same day. Eight kinds of fresh or processed fishes including pacific saury ("sanma", raw), sardine ("iwashi", raw), horse mackerel ("aji", raw), mackerel ("saba", raw), salmon ("sake", raw), atka mackerel ("hokke", salted), eel ("unagi", seasoned) and semi-dried horse mackerel ("aji-hiraki", salted and semi-dried spilt) were grilled by 150-250°C oven for 8-20 min per both sides. These cooked fishes were divided into flesh and skin, and each part of several fishes was separately combined and used for analysis. In addition, cooking pan-fried beef, stir-fried pork and grilled chicken were used as meat sample. The cooking of these samples was done in domestic conditions.

Extraction of HCAs from food samples was carried out by modification of the previously reported method (Kataoka and Pawliszyn 1999). Each cooked sample (25 g) was chopped and ground by food processor, and then homogenized with 500 mL of 0.1 *M* HCl containing 67 ng of TriMeIQx (IS) by cooking mixer. After centrifugation at 3100 rpm for 30 min, the precipitate was re-extracted with 300 mL of 0.1 *M* HCl. To the combined supernatants were added trichloroacetic acid (TCA) at final concentration of 5% and then the mixture was stored at 4°C overnight. After centrifugation at 2070 x g for 30 min, the supernatant was neutralized with 6 *M* NaOH, and then removed insoluble materials by filtration. HCAs in this filtrate were extracted by Blue-rayon adsorption method (Hayatsu et

al. 1992). Two parts of 500 mg Blue-rayon® were added to the filtrate and shaken with a shaker set at 300 rpm. (left and right) for 20 min each time. After filtration with nylon mesh by suction, the combined Blue-rayon® was washed twice with 200 mL of distilled water and dried with a paper towel. Then the HCAs adsorbed on Blue-rayon® were eluted three times with 50 mL of MeOH-28% NH_3 (50:1). After evaporation to dryness of the combined eluate at 50°C under reduced pressure, the residue was dissolved in 2 mL of 0.1 *M* HCl and transferred to 10-mL Pyrex glass tube with a PTFE-lined screw-cap. After washing with 2 mL of n-hexane, the aqueous layer was adjusted to pH>10 with 28% NH_3 and then extracted twice with 2 mL of dichloromethane. After evaporation to dryness of the combined organic layer, the residue was dissolved in 0.2 mL of MeOH and used for derivatization of the sample.

Derivatization of HCAs was performed by the previously reported method (Kataoka & Kijima 1997; Kataoka et al. 1998 and 2000). An aliquot of the sample containing 0.5-10 ng of HCAs was pipetted into a 10-mL Pyrex glass tube with a PTFE-lined screw-cap. To this solution was added 10 ng of IS (if necessary) and 10 μL of DMF-DMA, and the mixture (0.2-0.5 mL) was heated at 100°C for 15 min without capping the tube. After evaporation to dryness, the residue was dissolved in 20-40 μL of ethyl acetate and then 1 μL of this solution was injected into the gas chromatograph.

GC analysis was carried out with a Hewlett-Packard 5890 Series II gas chromatograph equipped with an electronic pressure control (EPC) system, a split/splitless capillary inlet system, and a nitrogen-phosphorus detector (NPD). Two connected fused-silica capillary columns (J & W, Folsom, CA, USA) containing DB-17ht (10 m x 0.25 mm I.D., film thickness 0.15 μm) and DB-1 (10 m x 0.25 mm I.D., film thickness 0.25 μm), respectively, with a two-way press fit fused-silica tube were used. The operating conditions were as follows: column temperature, programmed at 10°C/min from 240 to 290°C, programmed at 20°C/min from 290 to 330°C and held at 330°C for 2 min; injection and detector temperature, 340°C. The inlet helium pressure controlled with EPC, was programmed at 4 kPa/min from 185 to 205 kPa, programmed at 10 kPa/min from 205 to 225 kPa and held at 225 kPa for 1 min. Make-up gas flow-rate: 30 mL/min; split ratio: 10:1. A chromatographic run (run made with no sample injected) data was subtracted from sample run data to remove baseline drift (usually caused by column bleed) using a single-column compensation function and then base-line corrected data was recorded on the chromatogram. The peak height ratios of HCAs and the IS were measured and the peak height ratios against the IS were calculated to construct calibration curves.

RESULTS AND DISCUSSION

Food samples were cooked by common cooking conditions in order to evaluate contribution of meat and fish dishes to the total intake of HCAs in Japanese, although the formation of HCAs is dependent on cooking time and temperature.

HCAs are present in foods at low parts per billion or less. Therefore it is not too much to say that the clean-up procedure for complex sample matrix greatly influences the reliability and accuracy of the HCA analysis. In order to achieve an efficient isolation and preconcentration of HCAs, we used the Blue-rayon® adsorption method developed by Hayatsu (Hayatsu 1992). Blue rayon®, rayon bearing covalently linked copper phtharocyanine trisulphonate as ligand, can selectively adsorb HCAs and other mutagens/carcinogens having polycyclic planar molecular structures, so that its batch-wise use is an effective means to concentrate HCAs in aqueous solution. The HCAs isolated by Blue-rayon® adsorption method could be easily converted into their *N*-dimethylamino-methylene derivatives by a previously reported NPD-GC method (Kataoka & Kijima 1997). Figure 1 shows the typical chromatograms obtained from cooked atka mackerel skin. NPD-GC method was selective and sensitive for HCAs, and cooked fish samples could be analyzed without any interference from coexisting substances, although some unknown peaks were observed on the chromatogram. Overall recoveries of Trp-P-1, IQ, MeIQx, 4,8-DiMeIQx and PhIP added to cooked atka mackerel skin by this technique were 90.6 ± 0.8, 56.2 ± 2.9, 66.8 ± 2.7, 96.3 ± 5.2 and 39.3 ± 4.4, respectively. The quantitation limits of HCAs in cooked fish samples were ca. 50 pg/g.

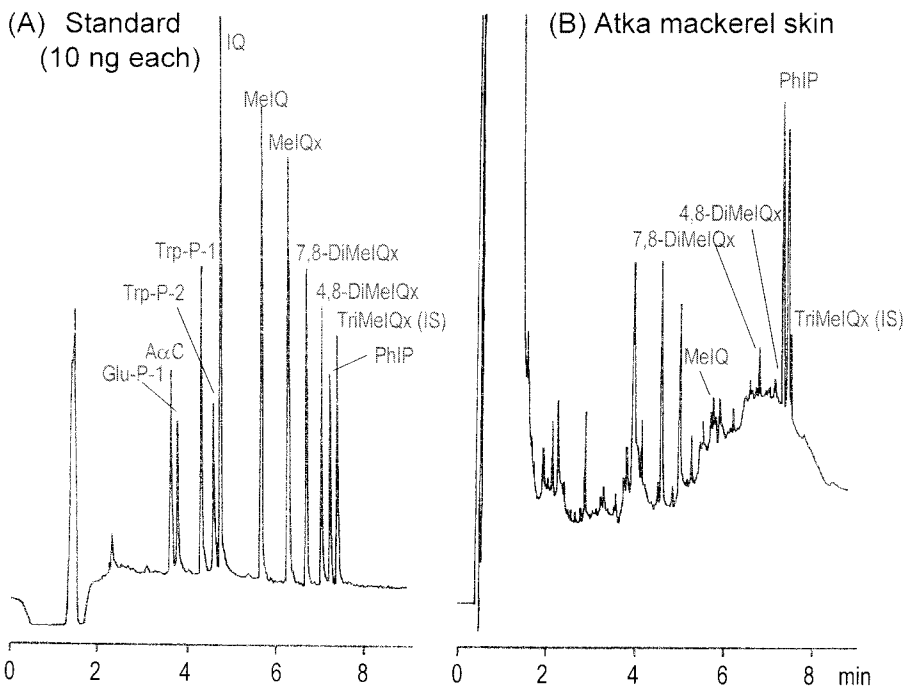

Figure 1. Gas chromatograms obtained from standard heterocyclic amines and cooked atka mackerel skin. GC conditions are given in the text.

Table 1. Heterocyclic amine contents in cooked food samples

Cooked food sample	Content[a] pg/g sample						
	Trp-P-1	IQ	MeIQ	MeIQx	7,8-DiMeIQx	4,8-DiMeIQx	PhIP
Pacific saury (skin)	ND[b]	194±37	424±87	492±87	ND	ND	1611±196
Pacific saury (flesh)	ND	ND	ND	191	ND	ND	333
Sardine (skin)	ND	ND	317±14	860±44	ND	961±19	816±79
Sardine (flesh)	ND	ND	ND	152	ND	ND	ND
Horse mackerel (skin)	216±26	140±58	ND	385±34	ND	413±34	2121±69
Horse mackerel (flesh)	ND	ND	ND	25	64	ND	160
Mackerel (skin)	ND	ND	375±52	588±70	749±90	1196±176	1595±218
Mackerel (flesh)	ND	ND	67	ND	ND	ND	226
Salmon (skin)	289±38	ND	ND	593±59	4142±233	ND	5928±802
Salmon (flesh)	ND	ND	180	99	ND	ND	294
Salted atka mackerel (skin)	ND	ND	435±57	ND	1606±550	427±62	5377±309
Salted atka mackerel (flesh)	ND	ND	98	ND	ND	ND	658
Seasoned eel (skin)	ND	ND	ND	ND	ND	ND	359±56
Seasoned eel (flesh)	ND	ND	319	ND	ND	ND	214
Semi-dried horse mackerel (skin)	278±37	ND	262±41	420±29	949±74	546±41	3682±331
Semi-dried horse mackerel (flesh)	ND	ND	70	ND	ND	ND	524
Chicken	527±96	ND	142±22	77±10	ND	ND	289±37
Pork	ND	ND	86±12	202±12	ND	ND	857±100
Beef (well-done)	ND	ND	94±12	198±71	ND	ND	634±75
Beef (Medium)	ND	ND	125±31	138±67	ND	ND	537±105

[a] Mean ± SD (n=3); fish flesh samples show single measurement result.

[b] Not detectable.

The HCA contents in various fishes and meats cooked by common cooking procedure are shown in Table 1. Although IQ, MeIQ, MeIQx, 4,8-DiMeIQx, Trp-P-1, Trp-P-2 and PhIP have been previously detected in cooked fishes, comparative studies show clear differences between fish species and types of preparation (Eisenbrand and Tang 1993). Among ten HCAs tested in this study, PhIP was detected at high concentration in most cooked food samples, but AαC, Glu-P-1 and Trp-P-2 were not detectable. Especially, the PhIP contents in fish skin were 2-20 times higher than those in fish flesh, although creatine, which is a precursor of PhIP, is mainly included in fish flesh. It is considered that the skin has contacted to the flesh, and the heating temperature is higher in skin than in flesh, and therefore burnt deposits containing HCAs are easy to produce in the part of skin. Furthermore, grilled fishes contained high concentration of HCAs in comparison with pan-fried meats. These results suggest that contribution of fish dishes to the total intake of HCAs is relatively high, because Japanese people generally consume more fish than animal meat in normal daily life.

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