



# STUDIES ON HEAVY METAL AND HETEROCYCLIC COMPOUNDS CONTENT IN MILKFISH (CHANOS CHANOS) FRIED IN USED COOKING OIL

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**Keywords:** fried milk fish; reused frying oil; nickel; 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP).

This study is aimed to analyze the content of heterocyclic aromatic amine compounds along with heavy metals (Ni, Pb, Hg and Cd) in milkfish fried in used cooking oil. Chemical components were identified using gravity chromatography with Gel 60 as the stationary phase and purified by column Sephadex LH20, and checked by UV spectrometry, IR, and HPLC. The compound PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine) was found to be formed is thought to be caused by the influence of cooking oil. By atomic absorption spectrophotometer nickel (Ni) was found to have an average concentration of  $1.6075 \pm 0.2405$  mg kg<sup>-1</sup>. The role of nickel compounds in milkfish genotoxicity is suspected as a trigger effect on PhIP compound.

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## INTRODUCTION

Some studies have shown that heterocyclic aromatic amines are formed during maturation process of protein containing foods such as meat and fish at high temperatures. Heterocyclic aromatic amines (HAA) were classified as endogenous compounds which are carcinogenic in animals, and capable of inducing and then developing into cancer.<sup>1</sup> Mortality of the Iceland population is very high due to gastric cancer in relation to consumption of smoked fish in their diet.<sup>2</sup> One example of HAA compounds found by Ito et al,<sup>3</sup> is 2- amino-1 methyl-6- phenylimidazo[4,5-b]pyridine (PhIP) as a carcinogen that induces lymphomas in mice, colon and breast tumors in female mice, and prostate tumor male rats.<sup>3</sup> HAA and PhIP are mutagenic even in small amounts and can be absorbed from food and are extensively activated by N-hydroxy derivatives, which are genotoxic by the enzyme cytochrome P 4501A.<sup>4</sup>

Carcinogenic HAA compounds are produced during the pyrolysis of creatine in muscles with amino acids from protein.<sup>5</sup> Heterocyclic aromatic amines are formed in a simulated mixture of free amino acids, creatine, and glucose from beef or chicken on heating at 200 °C.<sup>6</sup> During cooking meat 17 different HAA compounds were identified as possible reason for risk of cancer in humans.<sup>7</sup>

The process of maturation of meat or fish by using direct fire can causes producing smoke containing polycyclic aromatic hydrocarbons (PAH) from fats drop onto the hot fire which can get attached to the surface of the food. These compounds are known to play a role in contributing to the inception of cancer. Baking meat produces PAHs which can also cause cancer by damaging DNA.<sup>8</sup>

Approximately 35 % cancer is caused from food each year.<sup>9</sup> In Indonesia, 182,000 women are diagnosed with breast cancer and 43,300 of them die in each year.<sup>8</sup>

Fish in South Sulawesi is a main menu which is generally cooked with spices, baked or fried.<sup>10</sup> In 2003, found that 93.9 % of respondents having breast cancer generally consumed more fried fish with using cooking oil. Used cooking oil is actually a vegetable oil that has peroxide bonding, and contains epoxides and other carcinogenic and mutagenic compounds.<sup>11</sup> It is, therefore, likely that the fish fried in cooking oil contains PhIP or 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine compound.

## MATERIALS AND METHODS

Milk fish from a farming area in South Sulawesi was randomly mixed, cleaned of scales and entrails and then washed. Fish was fried in used cooking oil at a temperature of 150-165 °C until lightly browned.

Column chromatography with gravitational cross sections 2.5 and 1.25 x 50 cm x 30 cm was used. Agilent 8453 UV-Visible Spectrophotometer, Thermo Nicolet Avatar 360 FTIR infra red Spectrophotometer, HPLC (LC 10 ATP Shimadzu) and atomic absorption spectrophotometer (Varian spectra Aso and Shimadzu AA-6200) were used.

Silica gel 60 (E.Merck, 0.2-0.5 mm), Sephadex TMLH-20, NaOH (E.Merck), anhydrous Na<sub>2</sub>SO<sub>4</sub>, chloromethane (Univar), Methanol (Univar), Aqua and acetic acid (E.Merck).

## Analysis of Heavy Metals

25 grams of milkfish collected from a pond were fried in used cooking oil and placed in a preheated oven at 375°C till it were converted to ash. It was cooled and treated with 2-3 drops of concentrated HNO<sub>3</sub>. The acidified ash was then

placed into the surface and heated again to white ash. The white ash was cooled and treated with 5 drops of 3 N HCl, filtered into 100 mL volumetric flask and then dipped in distilled water. Levels of Ni, Pb were determined at 235.6 nm and 217 nm, respectively using AAS.

### Isolation and Identification of Chemical Components<sup>12</sup>

100 gram of fried milkfish was added to 300 ml of 5 % NaOH in a 2.5 liter beaker and the pH was adjusted to 12 with 30 % NaOH. The mixture was stirred continuously while adding 500 grams of silica gel till the resulting mixture became homogeneous. It was left for few minutes before introducing into the chromatography column (70 cm x 6 cm). Dichloromethane was then allowed to pass through the column with a speed of 5-10 ml min<sup>-1</sup> for extracting neutral and alkaline fractions. After removing the organic phase with a rotary evaporator, it was dried by adding anhydrous Na<sub>2</sub>SO<sub>4</sub>. The residue was dissolved in 100 ml of HCl (0.1 N) to remove neutral components. Water solution was made up to pH 11 with 30 % NaOH, and the alkaline phase was extracted with 100 ml dichloromethane. The process was repeated 5 times. The organic phase, after being dried by addition of anhydrous Na<sub>2</sub>SO<sub>4</sub>, was extracted and the solvent was evaporated with a rotary evaporator. The residue was dissolved in 10 ml of methanol.

Alkaline extract was dissolved in methanol and 300-400 µL was injected into a glass column (40 cm x 9 mm) filled with Sephadex LH 20 resin. Operating conditions were as follows: solvent A: Water, solvent B: methanol containing 0.1 % acetic acid. The spectrum of each fraction was checked spectrophotometrically. The fractions were collected and then lyophilized on a freeze dryer, then identified UV, IR and HPLC. The results are shown in Figure 1, Figure 2 and Figure 3.

## RESULT AND DISCUSSION

### Analysis of Heavy Metals by AAS

Samples were obtained from milkfish marketed by Makassar Fish Eagles, and were analysed for Pb, Ni, Hg and Cd content using atomic absorption spectrophotometry. Only metallic nickel was found to be present. The average nickel content in the samples was found to be 1.6075±0.2405 mg kg<sup>-1</sup> (Table 1) and did not exceed the threshold value allowed by the Great Hall of the POM, which is 2 mg kg<sup>-1</sup>.<sup>13</sup>

**Table 1.** Nickel content found in fried milkfish samples

Samples	Weight, g	Uptake, mg	Content, mg kg <sup>-1</sup>	Average, mg kg <sup>-1</sup>
1	25	0.0055	1.37	
2	25	0.0056	1.39	
3	25	0.0054	1.34	
4	25	0.0072	1.84	1.6075
5	25	0.0074	1.89	
6	25	0.0071	1.81	

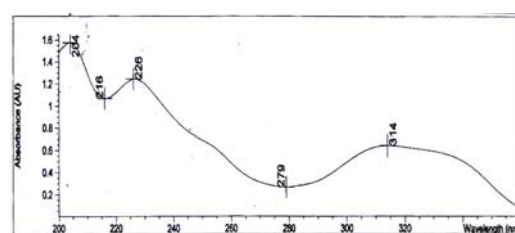
Description: 1-6 is looping sampling

The presence of nickel in an average of 1.6075 mg kg<sup>-1</sup> amount in fish could increase the intensity of gene mutations, because Ni compounds are carcinogenic that are either directly or indirectly bound to DNA. Ni levels were found are remained below the threshold value which is allowed by the Great Hall of the POM, but the long exposure can cause accumulation and ultimately an effective dose can be achieved that can cause mutations. Nickel metal analysis was conducted on the raw fish. Thus the existing contamination is thought to have come from farms which are likely to have compounds of nickel metal. Metal is thought to lead to the induction of gene mutations, adding to the effect of compound HAA<sup>14</sup> found that nickel compounds can increase the potential genotoxic effects.

Nickel subsulfide (Ni<sub>3</sub>S<sub>2</sub>) is mutagenic and nickel chloride (NiCl<sub>2</sub>) can raise hydroxylation reaction of 2-deoxyguanosin (dG) of DNA which contribute to genotoxic and carcinogenic properties.<sup>1,5</sup> Nickel also plays an important role in the effect of oxidative stress and it has a potential as genotoxic.<sup>16</sup>

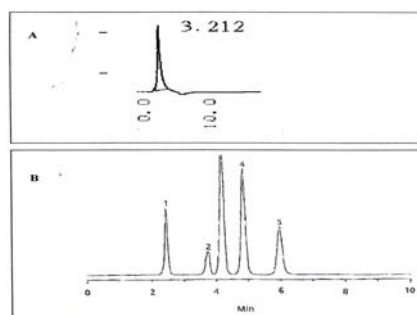
### Analysis results of PhIP

Powdered fried milk fish was mixed with cooking oil and its chemical components were isolated following the method of Gross gives results in the form of a white powder which point is 302-304 °C. White powder was then identified by spectrophotometry, HPLC and UV and IR. The resulting spectra is shown in Figures 1, 2 and 3.



**Figure 1.** UV spectrum of PhIP isolated.

UV spectrum shows peaks (maximum) at  $\lambda_{max}$  at 204, 226, and 314nm, while the library value of PhIP is 203, 226, and 315 nm. The peak of the other heterocyclic amine compound such as AC (2-amino-3,4-dimethylimidazo[4,5-f]quinoline) peak occurs at 215, 257, and 337 nm, and that of MeIQx (2-amino-3,8-dimethylimidazo[4,5-f]quinoxalin) peaks are at 210, 264 and 336 nm. These data support the opinion that the detected compound is PhIP.



**Figure 2.** HPLC retention time curve PhIP compound (A) and some of the HAA compounds Instruments Data Bank (B). Note: 1 = AC, 2 = PhIP, 3 = IQ, 4 = MeIQ, 5, MeIQx

The analysis of HPLC retention times showed that the isolated compound of fried milkfish is PhIP. Other heterocyclic aromatic amines as AC (2-amino-9H-pyrido[2,3-b]indole, 2,4 min), IQ (2-amino-3-methylimidazo[4,5-f]quinoline, 4 min), 2-MeIQ (2-amino-3,4-dimethylimidazo[4,5-f]quinoline, 8 min) and finally MeIQx (2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline, 6 min) retention times proved to be different.

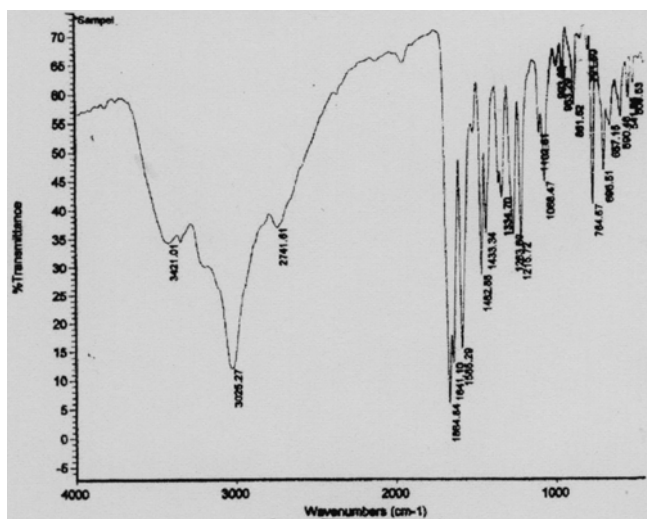


Figure 3. IR spectrum of isolated PhIP

The peaks found in the IR spectrum of isolated PhIP can be seen in Table 2.

The peak at  $3421\text{ cm}^{-1}$  and at  $1250\text{ cm}^{-1}$  in the IR spectrum of the isolated PhIP indicates the presence of  $\text{NH}_2$  groups (primary amines) and the peaks at  $3025.27\text{ cm}^{-1}$  and  $1641.10\text{ cm}^{-1}$  show the presence of aromatic C-H. The peak at  $2742\text{ cm}^{-1}$  belongs to N- $\text{CH}_3$ . The peaks at 1433 and 1335 indicate the presence of -CH and - $\text{CH}_3$ . The peak at  $1585\text{ cm}^{-1}$  belong to an -N=N- group, while the peaks at 1665, 1433 791 and  $657\text{ cm}^{-1}$  indicate the presence of pyridine ring.<sup>17</sup>

Milkfish obtained from several ponds contained nickel compounds - known carcinogens- can even strengthen the genotoxic effects of benzo[a]pyrene<sup>14</sup> and raise the hydroxylation reaction and deglycolization of 2-deoxyguanosin which can contribute to the genotoxic and carcinogenic effects of such metal compounds.<sup>15</sup>

The spectra suggested the presence of the suspected compound PhIP or 2-amino-6-phenylimidazo-1-metil-[4,5-b]pyridine. The UV spectrum showed a cluster of chromophores with spectral peaks at 204.226 and 314 nm corresponding to the known spectrum of PhIP at 203.226 and 315 nm). HPLC showed a retention time of 3.212 min (PhIP 3.60 min) and looks different from the other HAA compounds. IR spectra also showed the presence of an aromatic group, primary amine group, pyridine ring and -CH, - $\text{CH}_3$  and =N- $\text{CH}_3$  groups. The results obtained confirmed that the isolated compound is the suspected PhIP.

Table 2. Numbers of wave IR spectral peaks by intensity

Wave numbers, $\text{cm}^{-1}$	Intensity
1664.84	5.811
3025.27	11.998
1641.10	12.978
1585.29	15.516
1462.88	28.555
1215.72	34.180
3421.01	34.307
1263.69	35.634
1433.34	36.018
2741.61	36.911
764.57	40.715
1334.70	41.929
1068.47	44.727
696.51	46.659
1102.61	53.057
657.15	54.345
590.46	55.829
881.52	57.958
541.95	59.228
509.53	61.756
953.29	62.476
993.32	64.681
791.50	67.466

## CONCLUSION

1. The analysis results showed the presence of nickel in fish which strengthens the genotoxic effects of heterocyclic aromatic amine compounds.

2. The analysis results showed the presence of carcinogenic 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP).

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