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Research article

Heterocyclic amines in commercially available ready-to-cook and ready-to-eat foods in Chattogram, Bangladesh

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ABSTRACT

As part of survey of the heterocyclic amine (HCA) contents of available ready-to-cook (meat ball, sausage, bologna, chicken salami, and chicken-samusa) and ready-to-eat (shawrma, beef shikkabab, chicken chap, chicken tikka, fried chicken) foods were collected and were analyzed for heterocyclic amines PhIP (2-Amino-1-methyl-6-phenylimidazo [4,5-b]pyridine) using Ultra High Performance Liquid Chromatography. Results indicated the detectable PhIP in ready to eat samples but not in the ready to cook samples. The highest PhIP concentration was registered in fried chicken (88.3 ng/g), followed by chicken tikka (71.44 ng/g), shawrma (45.52 ng/g), chicken chap (35.17 ng/g) and beef shikkabab (0.16 ng/g). The amounts of heterocyclic amines measured in these ready-to-eat foods revealed that samples prepared for laboratory studies are representative of commonly consumed restaurant samples might pose health hazard and demands further attention.

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1. INTRODUCTION

Our diet consists of mixture of organic and inorganic substances that not only provide substance but also causes, modulate and prevent human diseases. These days, eating habits are changing gradually all over the world due to change in life style, which leads more people forced rely on ready to eat foods manufactured in the food industry and on fast food chains and restaurants. The quality of food, from the nutritional, microbial safety point of view and sensory aspects depend on a range of variables from farm-to-fork, including the quality of the raw material, processing techniques, packaging and cooking. The mainpurpose of industrial food processing is to provide safe and high-quality food as demanded by the consumer (Moskowitzet al., 2009; Luning and Marcelis, 2009). The conduction of thermal processing in an appropriate way is the key to ensure safe food supply in many cases, also with enhanced nutritional functionality respect to the starting raw material (Van Boekelet al., 2010). For example, processed meat products have been transformed through salting, curing, fermentation, smoking, or other processes to enhance flavor or improve preservation. These include fried meat, canned meat and meat-based preparations and sauces. But biogenic amines such as polycyclic aromatic hydrocarbons and heterocyclic amines (HCAs), also known as

carcinogen, generate in a wide range of foods from the reaction of creatine, amino acids, and sugars in meat and fish during cooking at high temperatures or with the food in direct contact with a flame or a hot surface, as in barbecuing or pan-frying (Skog et al., 1998) and these amines draw increasing attention in recent days since these may show mutagenic activity (Cheon-Ho, et al., 2008). Previous studies reported that extracts of roasted horse meat caused carcinogenic effects, when applied to mouse skin (Windmark, 1939); polycyclic aromatic hydrocarbons on the surface of well-done charcoal-broiled steaks, apparently formed through pyrolysis of fat dripping into flames and being absorbed by the food (Lijinsky and Subic, 1964); the presence of mutagenic activity was found in the charred surface of beef and fish, broiled over a naked flame or charcoal (Nagao et al., 1977; Sugimura et al., 1977). Commoner et al. (1978) showed that mutagen was formed in meat also under normal domestic cooking conditions and even by boiling beef extracts. Since then, a series of highly mutagenic compounds has been isolated and identified from pyrolysates of amino acids or proteins and from cooked protein-rich foods. Some of the food mutagens have been synthesized and have been shown to be carcinogenic in long-term animal studies (Sugimura and Wakabayashi, 1990). Various experimental studies showing that HCAs were mutagenic, and carcinogenic to non-human primates (Sugimura et al., 2004), it has been hypothesized that high HCA intake may be associated with an increased risk of cancer at several sites. In particular, the role of HCA has been most extensively studied for colorectal cancer, following findings that red and processed meats increase the risk of this disease (AICR, 2007). This might reflect the difficulties in assessing exposure since there is only limited information on the levels of these compounds in foods. Clarification of the role of HCAs in the etiology of human cancer requires the accurate assessment of HCA exposure. Yet information on the HCA content of meat and fish in Bangladesh has not been available.

The objective of the present study, therefore, was to detection of common HCA (PhIP) in frequently consumed commercially available ready-to-eat and ready-to-cook meat products in Bangladesh using Ultra High Performance Liquid Chromatography (UHPLC). The findings of this study will create a new avenue consolidating our understanding in risk exposure assessment and cancer prevention in the context of Bangladesh.

2. MATERIALS AND METHODS

Location and period of study

The study area of this research was Khulshi, Chattogram, Bangladesh over the period of six months from January 2018 to November 2018 in the laboratory of the Poultry Research and Training Center (PRTC) of Chattogram, Department of Applied Food Science and Nutrition, Chattogram Veterinary and Animal Sciences University (CVASU), Bangladesh.

Collection of samples

Ten types of processed meat products samples were purchased from different super-shops, restaurant and departmental stores of Khulshi area in Chattogram. Two categories of samples were selected (i) ready to cook (n=5 types) and (ii) ready to eat (n=5 types). Each type of food consists of 3 samples. Ready to cook food consists of meat ball, sausage, bologna, chicken salami, and chicken-samusa and ready to eat food consists of shawrma, beef shik-kabab, chicken chap, chicken tikka, and fried chicken. Samples were collected in aluminum-foiled box and stored at -20℃ until further analysis.

Chemicals and instruments

All chemicals, reagents, and solvents were either HPLC grade, and water was purified using ultra-pure water purification system (EVOQUA WATER TECHNOLOGIES). All solutions were filtered through a 0.2 µm syringe filter before injecting into the HPLC system. Acetonitrile, n-hexane and methanol (HPLC grade) were obtained from Sigma Aldrich (Milwaukee, WI) and Merck (Darmstadt, Germany). The 2-amino-1-methyl-6-phenyli-midazo [4,5-b]pyridine (PhIP) was purchased from Toronto Research Chemicals (Downview, Canada). Solid phase extraction cartridge (Strata-X-C Polymeric Strong Cationic Exchanger) 60 mg/3mL was purchased from Phenomenex (Torrance, California).

For the analyses of the extracts, C-18 column (50×4.6 mm 2.6 µm particle size) was used. The column was conditioned at oven temperature 23 ºC during 5 min using as eluent a 50% 30 mM Ammonium-formate buffer adjusted to pH 2.8 and 50% acetonitrile. The flow rate of the eluent was 0.5 ml/min and the detection wavelength was 316 nm with diode array detector (DAD) for PhIP. For the analysis of the samples the injection volume of extract was 2 µl/15µl. The retention time was 4.6 mins± 0.04 min.

Preparation of stock and buffer solutions

Stock solutions of PhIP were prepared dissolving 10 mg of standard in 10 mL of methanol and were stored at 4 ºC, protected from light and used within 1 month. The stock solutions of PhIP were further serially diluted to construct a calibration curve which was built in the range 0.01- 50 mg/L according to the limit of detection (LoD) and to the limit of quantification (LoQ). 30mM aqueous solution of ammonium formate was prepared by mixing appropriate weight (0.945g) in 500 ml water and filtered (0.45µm) before use.

Sample preparation

The ready-to-cook samples were cooked in a household cooking environment by pan-frying temperature. Cooked samples were left to cool down to room temperature (23 ºC) before further processing was done on them. Before extraction, the meat product samples were chopped into small portions and were blended into a fine homogenous powder with a laboratory blender (Jaipan®).

PhIP extraction& purification

Various extraction methods have been explored to simplify the sample treatment and to improve the recoveries. The extraction method based on acid extraction and one-step SPE purification was established in earlier researches, which include the one proposed by the author in 2014 (Ozdestan et al., 2014; Yan et al., 2014) where higher recoveries for all HCAs were obtained. In this study, a pretreatment method based on acetonitrile extraction and single step SPE purification was used to detect PhIP simultaneously in meat products using HPLC with DAD detector. The performance of the proposed method was validated in terms of its methodology and applicability in several commercial meat products. A total of 20 g mashed sample was accurately weighed into a 50 mL centrifuge tube. The extraction was carried out with 20 mL of acetonitrile and 15 mL of hexane. The tubes were then shaken vigorously for 1 minute. The samples were centrifuged with an Eppendorf Centrifuge 5810 R system operating at 3800 rpm for 10 minutes at 23ºC. The acetonitrile supernatant was transferred into a 50 mL centrifuge tube. Strata-X-C SPE cartridge was conditioned with 3 mL of methanol followed by 4 mL of ultra-pure water. The preconditioned cartridge was loaded with 10 mL of the acetonitrile extract. The cartridge was then washed with 3 mL of 0.1 M HCl followed by 3 mL of methanol. PhIP was eluted from the cartridge with 3 mL of 0.2 M ammonia-methanol solution. The eluent was filtered through a 0.22 µm syringe filter into an auto-sampling vial before injection into the Ultra High- Performance Liquid Chromatography (UHPLC) analysis system.

Calibration curves were constructed for five concentration levels from 0.05 to 0.25 mg/ml of PhIP stock solution. Standard solutions were filtered with a 0.22 µm filter before injection into the UHPLC system. The calibration curves were constructed by plotting peak area ratios of corresponding standard solutions against their theoretical concentrations.

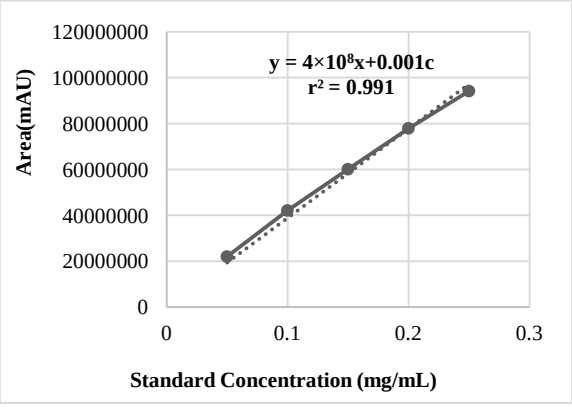

Figure 1. Calibration curves of PhIP standard solutions

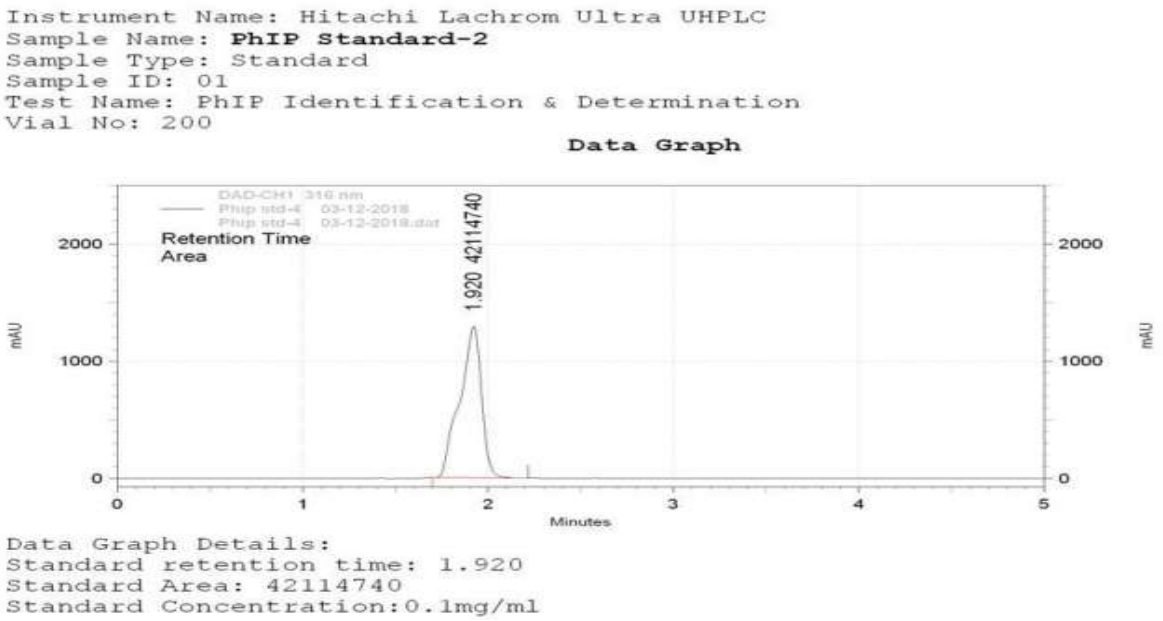

Figure 2. Ultra high-performance liquid chromatogram of the mixture of standard at 316 nm. The retention time of PhIP is 1.9 minutes containing standard solution 0.1 mg/mL.

Method validation

International Council for Harmonization of Technical Requirements for Pharmaceuticals of Human Use guide lines were followed for the validation of the method. In this regard linearity of the analytical method, limit of detection and limit of quantification were studied.

Linearity

Linearity was determined by constructing the calibration curves for standard solutions at 0.05 to 0.25 mg/ ml. Each concentration of standard solutions was analyzed using peak area ratios of 0.1 mg/ml. The calibration curves were linear in the range. The coefficients of determination (r^2) were 0.991.

Limit of detection (LoD)

A signal-to-noise ratio (S/N) of 3 was for estimating L0D. Peak-to-peak noise around the analyte retention time was measured, and subsequently, the concentration of the analyte that would yield a signal equal to certain value of noise to signal ratio was estimated. The noise magnitude was measured by auto-integrator of the instrument. LoD can be calculated from calibration graph by the formula, LoD = $3\times S_{xy}/a$, where S_{xy}=standard deviation of the response and a=slope of the calibration. LoDwas calculated by Excel 2010.The estimated LoD value was 0.02 ng/g.

Limit of quantification (LoQ)

A signal-to- noise ratio (S/N) of 10 was used for estimating LoQ. LOD can be calculated from calibration graph by the formula, LoD = $10\times S_{xy}/a$, where S_{xy}=standard deviation of the response and a=slope of the calibration. LoD was calculated by Excel 2010.The estimated LoQ was 0.08ng/g.

Quantification of PhIP

PhIP quantification was done by the external standard calibration method. Linear regression equations were used to calculate the unknown concentration of PhIP in samples which was expressed in nano gram (ng) per gram (g) of samples.

Statistical analysis

Data were expressed as the mean ± standard deviation of at least three independent experimental samples using Microsoft Excel 2010 software. Experiments were statistically analyzed by one-way analysis of variance (ANOVA). Statistical comparisons were conducted using SPSS version 20 (Statistical Package for the Social Sciences), and significance was accepted when $p<0.05$. Data

acquisition, data handling and instrument control were performed by Empower 2154 Software v1.0.

3. RESULTS

Food mutagen (PhIP) was detected by UHPLC using diode array detection method with detectable peaks at high resolution in the chromatogram. PhIP concentrations in ready-to-cook samples, (Meat ball, Chicken Sausage, Bologna, Chicken Salami, and Chicken Samusa) fried by pan frying, were not detected in chromatogram (data not shown).PhIP concentrations in ready-to-eat samples were detected in chromatogram. Table 1 depicts the detection status of ready-to-cook sample. Unknown concentrations of PhIP in meat product sample were determined with reference to the calibration equation.

4. DISCUSSION

In this study, the most abundant food mutagen, PhIP concentrations in various commercial meat products in Bangladesh were detected, and levels were much higher in ready-to-eat samples. PhIP concentrations in ready-to-cook meat products were not found. This general pattern is coincided with previous studies (Sinha et al., 1998; Skog et al., 1997), although concentration varied by food type and cooking methods. The formation of PhIP may be dependent on the type of meat. In the present study, PhIP levels were significantly higher in chicken than in beef. Previous studies reported PhIP levels 480 ng/g for grilled chicken breast without skin and bone (Sinha et al. (1995), and 30 ng/g for beef steak (Sinha et al., 1998), respectively. Although the pattern was similar, PhIP levels in the very well-done fried chicken in the present study were substantially higher, and comparatively the lowest values were detected for beef shik-kabab. The highest mean concentration of PhIP in fried chicken was 88.26 ng/g, the lowest mean concentration of PhIP in beef shik was 0.163ng/g.PhIP in fried chicken as an example, since it is generally the most mass abundant HCA measured in cooked meats, reported values range from 2.3 to 46.9 ng/g in Spain (Busquets et al., 2004); 0.2 to 2.1 ng/g in Canada (Klassen et al., 2002); 1.3 to 11.1 ng/g in Austria (Ristic et al., 2004) and non-detectable levels to 1.44 ng/g in the United States (Knize et al., 1998). It should be noted that the cooking conditions, sampling techniques, and methods of analysis varied somewhat among these surveys, however similar variation in values for other meat types are reported in these studies. Chicken products when subjected to deep frying generated the highest amount of PhIP 88.3 ng/g. The two high-temperature cooking methods (deep-frying, grilling/barbecuing) produce varying levels of PhIP. Deep frying appears to produce more PhIP. The reason may be length of time needed for the meat to reach the same internal temperature or some other cooking-related factor. The cooking surface temperature was significantly higher in deep frying and grilling as cooking methods. It might have predicted that PhIP concentration would be higher as a consequence. These data provide important information in estimating food mutagen exposure and will facilitate investigation of the role of food mutagen in the etiology of cancer in Bangladesh. The result might therefore have been affected by sampling variation and should be interpreted with caution. In addition, although comparison of PhIP concentrations across published studies is informative, consideration should be given to differences between studies in food samples, cooking and analytical methods. This study has shown that a slightly modified form of the procedure recommended by Toribio et al. (1999) is

Table 1. Heterocyclic amines (PhIP) in different ready-to-eat meat based processed restaurant food products

Sample Type	Range(ng/g)	PhIP Concentration (ng/g)	P-value
Beef ShikKabab	0.142-0.197	0.16±0.03	0.000
Chicken Chap	29.28-37.63	35.17±5.12	
Shawrma	45.33-45.68	45.52±0.18	
Chicken Tikka	65.38-74.46	71.44±5.25	
Fried Chicken	87.81-88.56	88.26±0.40	

applicable for the identification and quantification of less polar HCAs in meat samples. The extraction efficiency of the analytes from real samples, however, shows large matrix-to-matrix variations. It is therefore necessary to use the standard addition method to quantify HCAs. Diode array detection offers low detection limits in the 0.0266 ng/g. The results added new data on the detection of food mutagen (PhIP) in commercially available meat products in Bangladesh. The result revealed that high concentrations of PhIP are present in most of the ready-to-eat food tested. Their carcinogenic and mutagenic potential justifies future studies with the aim to improve existing methods for the determination of the PhIP and explore the possibilities offered by diode array detection to optimize the method to make them applicable in a variety of food matrices. The simultaneous analysis of multiple mutagenic HCAs in meat products is becoming more important. Therefore, a simple, fast, and more accurate pretreatment method based on acetonitrile extraction was proposed in this study and applied to the detection of 10 meat commodities. Thus, the proposed method could be a good choice when a variety of HCAs are expected to be determined simultaneously in meat products. The detection of food mutagen (PhIP) in commercially available meat products is important because there is significant carcinogenic risk of exposure to cancer by these compounds. Dietary preferences and food preparation variations can greatly influence individual exposures. This area of research contributes a great opportunity in cancer etiology, the chance to evaluate a class of carcinogens in human population. It is needed to conduct more research study on detection of PhIP as well as other food mutagen in various food products in Bangladesh. Besides this, future research with techniques such as cDNA micro-arrays and proteomics may provide further insight into the critical genes affecting susceptibility to HCA carcinogenesis (Hockley et al., 2008; Hong et al., 2015; Hebels et al., 2009; Okudaira et al., 2013).

5. CONCLUSION

Heterocyclic amine (PhIP) was found in ready-to-eat samples but not in the ready-to-cook samples. PhIP was found the highest in Fried chicken followed by Chicken tikka, Shawarma, Chicken chap, Beef Shik-Kabab, respectively. Further study is warranted with more samples size with food processing techniques.

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