

Research Article

Appraisal and Validation of a Method (MVU-AAS) Used for the Detection of a Toxic Metal Mercury (Hg) in Poultry Feed Available in Bangladesh

Hossain MM^{1*}, Hannan ASMA¹, Kamal MM¹,
Hossain MA² and Quraishi SB³

¹Quality Control Laboratory, Department of Livestock Services, Bangladesh

²Department of Dairy and Poultry Science, Chattogram Veterinary and Animal Sciences University, Chattogram, Bangladesh

³Analytical Chemistry Laboratory, Atomic Energy Centre, Bangladesh Atomic Energy Commission, Dhaka, Bangladesh

*Corresponding author: Hossain MM, Quality Control Laboratory, Department of Livestock Services, Bangladesh

Received: June 09, 2022; Accepted: July 05, 2022;

Published: July 12, 2022

Abstract

Toxic metal mercury (Hg) in poultry feed might affect seriously on the environment and human being. So continual or gradual analyses of toxic metal contents in poultry feedstuff are of prime significance for assessing the feed quality and consumer safety. In this study, a method named Mercury Vapor Unit Atomic Absorption Spectrometry (MVU-AAS) was used to analyze the content of Hg in poultry feed in compliance with the Council Directive 333/2007/EC, Commission Decision 657/2002/EC. The metal Hg was analyzed from the feed sample in the laboratory by MVU-AAS (Model: AA-7000 Shimadzu, Japan) and the absorbance was read at 253.65 nm wavelength. The method was confirmed and validated by complying with the international guidelines and assessing a number of criteria say system suitability, precision studies, linearity check, uncertainty measurement and recovery % etc.. The results revealed that the coefficient of variation (CV%) for system suitability and precision was <10% for the metal (Hg) detected in this study. The linearity of the calibration curves was excellent ($r^2 > 0.998$) at various concentrated levels. The limits of detection (LoD) value in feed was 0.007 mg/Kg and the limit of quantification (LoQ) value in feed sample was found 0.023 mg/Kg. The values of instrumental detection limit (IDL) and instrument quantification limit (IQL) were 0.045(μg/l), and 0.15(μg/l), respectively. The recovery (%) found for the metal was 92.29 %. The overall CV% of repeatability and reproducibility ranged from 3.27 % to 5.92 %. The value ranges from 12 to 16 was observed for the measurement uncertainty (%) of Hg in this study. The proposed validation criteria indicate that it is a reliable method that can be used easily for the routine analysis of toxic metal (Hg) content in poultry feed sample.

Keywords: MVU-AAS; Method Validation; Complete Feed; Toxic Element (Hg); Poultry

Introduction

Poultry farming is an emerging industry in Bangladesh. Poultry farming basically commercial poultry say modern chicken firm has become a very dominant and lucrative business in the developing country like Bangladesh. Modern chickens (e.g. broiler, layer, parent stock) are reared under intensive condition with special type of feeding care and management practices. Complete feed or balanced diet or mixed diet is necessary for the rapidly growing modern chicken. A good number of different sort of feedstuffs is required to formulate the complete feed i.e. used for the feeding of the modern chicken. The raw materials used for the preparation of poultry feed come from different sources or origin. Most of these feedstuffs are derived mainly from the plant sources grown in the soil. Apart from this, feeds used by the poultry are also come from animal (bone meal, fish meal, meat and bone meal) and synthetic sources (vitamin mineral premix). Poultry feeds either it be natural or artificial sourced, liable to be affected by the contents of heavy metals. It is reported that the feed resources can carry a good number of heavy metal contaminants and toxins that mainly come from anthropogenic and natural sources [1-3]. So it seems better to monitor any probable propagation of toxic

metals into the food chain through the finished products rather than the various raw materials used for the preparation of ready-made feeds [4]. The feedstuffs might be adulterated and contaminated by different ways from growing to supply the birds. For example, meat and bone meal procured from contaminated animals, fish meal collected from contaminated water, vitamin minerals premix supplementation to poultry ration etc., can transfer heavy metals to poultry feed, as we know that poultry diet is composed of various sources of raw materials together [5].

Feed plays an important role for the optimum production of meat chicken. Feed is the single variable unit and the major cost in poultry production is the feed i.e. 65 to 70 %. Poultry industry can use the by-products released from the tannery for the diet formulation of poultry as a cheap sources of raw materials for the reduction of feed cost. Few report stated that the wastages retrieved from the industries, particularly tannery are liable to cause a great problems for the human being and environmental pollution by heavy metal contamination in food chain [6]. The availability of these wastes in poultry feed and meat could warrant further research to determine the extent of contamination of heavy metal pollutants.

Feed and food quality, food security and feed safety are emerging issues in Bangladesh. Quality feed is required to assure food safety and to enhance the productivity of poultry. For this reason, poultry farmers, or feed integrators, use very often many feed additives, enzymes, drug, growth promoters, feed supplements (vitamin mineral premix), antibiotics and so on in their formulated diet to boost the quality of feed, which result in increased weight gain, prevent diseases, and increased concentration of mineral elements added in the poultry diet. We see heavy metals are ubiquitous, stable, and not degradable in the nature. So, poultry feeds, whether plant or animal sourced or fortified with many additives or by special manufacturing processes, have been reported to be influenced by the content of heavy metals in the feed.

In view of above, it has no doubt that feed quality is the main determinant factor for achieving optimum production in the farm animals. Sustainable poultry production is not possible at all without supplying quality feed to the birds. So it is crystal clear that poultry feeds contain wide range of toxic metals, and their exposure can be understood through after ingestion of these feedstuffs by the birds. The presence of many heavy metals say mercury (Hg), chromium (Cr), cadmium (Cd), lead (Pb), and arsenic (As), are found in the poultry feedstuffs. We attempted herein this study to establish a validation method by the detection of Hg in poultry feed. The Hg is one of the ten hazardous metals or chemicals posing a great threat to public health designated by [7]. It is reported that continual exposure of metal Hg in body tissue can damage the renal tubules [8].

We see that fishmeal is commonly used as animal protein source in poultry feed, which is considered as the most common source of Hg for the farmed animals [9]. Comparatively very few data available on the speciation of Hg in fish meal. The Hg is found in the environment in various forms such as metallic, inorganic and organic. As a result of mining, smelting, industrial activities, combustion of fossil fuels, this Hg (elemental and inorganic) is released into the air, which later accumulates in soil and water. Methyl mercury is the most active form which bioaccumulates and biomagnifies along the food chain, and causes toxicological concern [9]. The long time consumption of this inorganic Hg causes health hazard. About all of the Hg present in the foliage originated from the atmosphere [10].

When the consumption of heavy metals goes beyond the maximum permissible limit causes hazards to both animals and humans [11]. Besides, the undegradable nature of these metals causes bioaccumulation and toxicity in food chain and poses a severe health risk on environment. The useful tool is the feeding strategy to reduce health hazard through the food chain [12]. In this regard, detection of heavy metals, establishing and validating new method is very important for assuring food safety, feed quality and public health. The appraisal of heavy metal levels in poultry feeds might be an useful tools with respect to food safety and environmental sustainability. So it should be compulsory to check the heavy metal contents in the poultry feedstuffs routinely and regularly for the periodic monitoring, examination, and inspection of food safety. Heavy metals entering into food chain through different sources must be properly monitored for planning and implementation of mitigation strategies.

To create national policy, planning on reduction strategy, and maintaining food safety for the safeguarding of the people, the data

or reliable information regarding the metal contamination in poultry feed are still scarce in the country like Bangladesh. Some previous studies were also done to detect the toxic level of Hg in poultry feed [13-14], However, the findings of those studies are still contradictory, inconsistent and questionable to apply for the present poultry enterprises due to continual changes of genetic constitution and rearing strategies of poultry [15]. For this reason, a method (MVU-AAS) has been developed to detect the toxic element mainly Hg in poultry feed. This method appears to be very faster, precise, accurate, sensitive, selective, and economical [16]. The poultry feed quality can be tested routinely and regularly by the farmers, if the method is implemented and validated globally. Hence, the developed method will help to identify the toxic elements which has a great application in laboratory analyses of poultry feed component [17]. Besides, poultry industry, feed miller company and poultry integrators would be greatly benefitted from this research. The aim of the study was to quantify the metal Hg in poultry feed samples used in the different poultry farms of Bangladesh using a validated analytical method.

Materials and Methods

Ethical Study

The study had no dealings with live animals, so ethical approval is not necessary in this study.

Study Area and Experimental Period

Quality Control Laboratory was used to conduct all laboratory analyses for this study. The lab was located at the Savar, Dhaka, Bangladesh. The study was performed during November 2021 to April 2022.

Tools, Chemicals and Reagents

A sophisticated laboratory furnished with all necessary scientific tools and equipment was used in this study. An atomic absorption spectrophotometer (Model: AA-7000, Shimadzu, Japan) equipped with graphite furnace (GFAAS, FAAS 7000), HVG, MVU-AAS 7000 and an auto sampler (ASC 7000) were used. For Hg (253.65 nm, 10 mA and slit 0.7 nm), a hollow cathode lamp was used and operated according to the company instructions. Chemicals namely nitric acid (HNO₃, 69%), hydrogen peroxide (H₂O₂, 30%), 2M HCL solution, 10% stannous chloride solution, 1:1 sulfuric acid: water solution, mercury absorption solution and standards of Hg were used (Sigma Aldrich, Germany) for Standard calibration curve and sample preparation. The digestion process was done in Microwave Acid Digestion System (Ethos Easy Milestone). The bulk standard solutions were made daily by dilution of respective metals stock standard solutions, using 1% (w/w) (HNO₃, 69%). Auto sampler was used for the working standard solutions. Deionized water (18 MΩ/cm) produced using an E-pure system (Thermo Scientific, USA) was used to prepare all the solution as well as to clean and wash all containers and glassware prior to use. For environmental and lab personnel safety Mercury absorption solution was used.

Sample Preparation & Analytical Procedure

Poultry feed was prepared with the conventional feed ingredients locally available in Bangladesh following the standard as prescribed by the NRC [18]. After that, the samples were grinded by mill type grinder and put in air sealable bag before undergoing lab analysis. For the digestion of samples, approximately 1.0 gm of feed sample

was weighed and digested with 8 ml of HNO_3 (69%) and 2 ml of H_2O_2 (30%) in acid pre-washed Teflon vessels. The digestion procedure was done by Microwave acid digestion system (Ethos Easy Milestone). After digestion, added 20 ml stannous chloride and 20 ml 1:1 sulfuric acid: water solution, then it was diluted 150 ml final volume with deionized water. The reference material analytical blanks were also prepared with each batch of digestion set. All samples were prepared in triplicate. Diluted samples and the standard solution were separately put into a set of fresh sample vessels for mercury vapor unite atomic absorption spectrophotometer (MVU-AAS Shimadzu AA-7000). Final heavy metal (Hg) was measured at 253.65 nm wavelength, respectively.

Preparation of Spiking Solution for Hg

In digestion vessel about 1 g (1000 mg) sample was taken, later added 8 mL of Nitric acid (69%) and 2 mL of H_2O_2 (30%), 0.6 mL (600 μL) from 1000 ppb Hg solution and digested this solution. After digestion, it was added 20 ml stannous chloride and 20 ml 1:1 sulfuric acid: water solution then it was diluted 150 ml final volume with deionized water.

Criteria for the Validation of Proposed Method

The parameters for the validation of the proposed method in this study followed were, linear range, limits for detection and quantification, accuracy (%), precision checks and degree of uncertainty measurement [19, 20]. The validation criteria were assessed following the guidelines of Commission Decision [19, 21].

Linearity: Standard mixtures of Hg were prepared and a linear equation was established for each metals by plotting the absorbance's versus the concentrations to measure linearity. Three calibration curves were obtained on three consecutive days with a specified standard concentration of each metal. Linearity was calculated by running aqueous standard solution of each metals at final concentrations of 0.25, 0.50, 1.0, 2.5, 5.0 and 10.0 $\mu\text{g/mL}$ for Hg. The slope, intercept and r^2 values were retrieved from linear regression and correlation method.

Recovery percentage: Three set of spike samples (0.05, 0.1 and 0.15 mg/kg) have been prepared and each set replicated seven times. Sample reading was taken by measuring two times. For the estimation of recovery, an accurate amount of each metal three concentration levels (20.05, 0.1 and 0.15 mg/kg mg/kg for Hg was added to approximately 1.0 g of blank matrix powder, and then the matrix blank was digested and analyzed for recovery using the formula: recovery (%) = (amount obtained/amount spiked) \times 100.

Limit of detection (LOD), limit of quantification (LOQ), instrument quantification limit (IQL), and instrument detection limit (IDL): The lowest qualitative and quantitative concentrations for the tested linearity range were calculated for each metal according to the guidelines of ICH2000. Both LOD and LOQ were calculated using the expression: $k \times \text{S.D}/b$, where $k=3.3$ for the LOD and 10 for the LOQ, S.D= The standard deviation of the intercept, and b =Slope of the calibration curve tested for linearity. detection limit (IDL) and Instrument quantification limit (IQL) were calculated using following formula: IDL = 3s and IQL = 10s; where, s= standards deviation.

Statistical Analysis

All the data were statistically analyzed using the statistical software [22]. The means and standard deviations of the metal concentrations in samples were calculated. Finally, one-way ANOVA was used to compare the level of heavy metal residue in poultry feed samples.

Results

The result of linear regression data of the metal mercury (Hg) in poultry feed samples was demonstrated (Table 1). The value for the regression coefficient (r^2) obtained in this study was 0.998 for Hg. The overall recovery % for the metal (Hg) detected in poultry feed samples was 92.20 % (Table 2). The result of IDL for Hg was 0.045 $\mu\text{g/L}$, while the value for IQL was 0.15 $\mu\text{g/L}$, respectively. The result of method LoD was found to be 0.007 whereas the value for LoQ being 0.023 mg/Kg, respectively (Table 3). The precision studies were performed in this experiment by measuring the repeatability and reproducibility of data shown in Tables (4, 5). The CV% of the repeatability was 3.27% and reproducibility was found 5.92% measured for the Hg, respectively (Tables 4, 5). The value of measuring uncertainty (MU) for the metal was 12 to 16 %, Lower uncertainty values indicate higher accuracy.

Discussion

Chicken meat is considered as one of the most consumable food item in Bangladesh and many parts of the world, because it is cheaper in cost, easily available and supply of essential nutritional contents to the consumer world [23]. So poultry industry in Bangladesh is flourishing steadily. The main tool to boost up the poultry industry in Bangladesh is the supply of quality feed to the chicken. Modern meat chickens feed on balanced diet entire the rearing time. Lately, the diet of poultry is being formulated with the many raw materials namely fish meal that can transfer heavy metal, particularly mercury (Hg), to the poultry diet in undesirable levels, when it is sourced from contaminated water [5, 23].

However, it is obvious from the linearity result obtained in this study that it has meet the acceptance level i.e $r^2=0.995$. The data showed that the r^2 value (0.998) found in this study is greater than acceptance level (0.995), and it implies that the result is very good as

Table 1: Linear regression data of mercury (Hg) in poultry feed sample.

Metal	Linear range ($\mu\text{g/L}$)	Calibration with aqueous standard solution		
		Slope \pm SD ($\mu\text{g/L}$)	Intercept \pm SD	Regression coefficient (r^2)
Hg	0.25 --10	0.0278 \pm 0.0007	0.005451 \pm 0.0155	0.9988

Table 2: The e recovery (%) of data for the analyses of Hg in poultry feed.

Metal	Spiked analyte concentration (mg/L)	Calculated analyte concentration (n=7) (mg/Kg)	Recovery (%) (n=7)	Overall recovery % (n=21)
Hg	0.05	0.049	98.67	92.20
	0.10	0.086	85.91	
	0.15	0.138	92.01	

Table 3: The results of Instrument detection limit (IDL), instrument quantification limit (IQL), method limit of detection (LoD) and limit of quantification (LoQ) of Hg in poultry feed.

Metal	Parameters			
	IDL in solution ($\mu\text{g/L}$)	IQL in solution ($\mu\text{g/L}$)	Method LoD for feed (mg/kg)	Method LoQ for feed (mg/kg)
Hg	0.045	0.150	0.007	0.023

Table 4: Repeatability precision data for the determination of Hg in poultry feed.

Metal	Day	Spike concentration (mg/Kg)	Overall mean concentration (n=21) (mg/Kg)	SD	CV%	Overall CV (%)
Hg	1 to 3	0.05	0.049	0.002	3.30	3.27
	1 to 3	0.10	0.09	0.002	1.90	
	1 to 3	0.15	0.14	0.01	4.62	

Table 5: Reproducibility precision data for the determination of Hg in poultry feed.

Metal	Days	Spike concentration (mg/Kg)	Overall mean concentration (n=21) (mg/Kg)	SD	CV%	Overall CV%
Hg	1 to 3	0.05	0.05	0.00	5.06	5.92
	1 to 3	0.10	0.09	0.01	8.73	
	1 to 3	0.15	0.14	0.01	3.97	

the liner relationship found herein is accurate and authentic [24]. Our result is agreed with the report of previous investigators [25-26] who found similar results for the validation of heavy metals data for this parameter in milk and fish feed samples.

We see that the acceptable level for the recovery % should be within the range of – 20 % to + 10 %. Our recovery % was 92.20 % for the detection of Hg in poultry feed samples. In this regard, we can say that our analytical value of recovery % has obviously meet the standard level or criteria. Recovery percentage refers to how much extraction can be obtained by analytical process while conducting sample testing in the lab [27]. Though no verified data or reference materials for poultry feed sample are available, the recovery % was done to justify the accuracy of the method [24].

Form the result, it is clear that the methods LoDs and LoQs in feed samples were found to be lower for the metal than the highest range as reported by the Regulations (EC) No. 1275/ [28], and it has matched properly according to Regulation 333/ 2007/EC [19]. The LoD and LoQ are measured to detect the performance or efficacy of an instrument or an analytical method.

The repeatability and reproducibility data (CV%) found less than 5% in our analytical values of the study. The acceptable value for these parameters is considered 10% [29]. Therefore, it can be stated that the method exhibited a good repeatability and reproducibility precision based on the values obtained in this study. The analytical precision was determined according to the commission regulation of EC [21]. The precision is normally assessed by CV%, and it denotes the proximity of compromising data [30].

Measuring uncertainty (MU) calculation is necessary for each and every measurement according to ISO/IEC17025 [31], because it checks the errors and omission occurred while conducting any assay. The reproducibility data can be used for the estimating of MU [32]. However, despite the uncertainty or limitations, the method we developed here for the detection of toxic metal in feed sample is very simple, precise, accurate, reliable and cost effective as well. Thus, the proposed method can be used for routine analyses or simultaneous detection and quantification of heavy metal which has a great implication in laboratory examination of poultry feed sample across the globe [17]. Our results are also agreed with the previous investigators who stated similar type of remarks regarding methods validation for the fish feed [25], milk [26] and detection and assessment of heavy metals in other food stuffs [12, 17, 33, 34, 35, 36].

Conclusion

From an overview of the result obtained in this study revealed that, all the criteria for method validation (e.g linearity, repeatability, reproducibility), accuracy or recovery percentage, LoD and LoQ, uncertainty) have meet the international standard or acceptable level. So it can be suggested from the findings that the method can be validated for the determination of toxic metal (Hg) in poultry feed samples. The findings reveal that the contents found in the poultry feedstuffs were deemed lower than the prescribed values as denoted by the Council Directive 2002/32/EC.

Competing Interests

The authors declare that the study has no competing or contradictory or conflicting issues or interests.

Acknowledgements

The authors are thankful to the fund and facility provided by QC Lab, DLS, Dhaka, Bangladesh.

References

- Sarkar MM, Hossain MM, Rahman MM, Rahman SME. Effect of feeding urea molasses block on the productive and reproductive performances of Black Bengal does. *Bangladesh J AgrilUniv*. 2008; 6: 39-46.
- Rahman SME, Islam MA, Rahman MM, Oh DH. Effect of cattle slurry on growth, biomass yield and chemical composition of maize fodder. *Asian-Aust. J. Anim. Sci.* 2008; 21(11): 1592-1598.
- Hossain, Hossain M, Hashem MA, Bhuiyan K. Organic beef cattle production pattern at Shahjadpur upazilla of Sirajgonj district in Bangladesh. *Bangladesh Journal of Animal Science*. 2016; 45(1): 25-30. doi:10.3329/BJAS.V45I1.27484.
- Islam MS, M Azizul Islam Kazi, MAS Hossain, MM Ahsan, MA Hossain, AMMM. Propagation of Heavy Metals in Poultry Feed Production in Bangladesh. *Bangladesh J. Sci. Ind. Res.* 2007; 42(4): 465-474.
- Sadeghi A, M Hashemi, F Jamali-Behnam, A Zohani, H Esmaily, Dehghan AA. Determination of Chromium, Lead and Cadmium Levels in Edible Organs of Marketed Chickens in Mashhad. *Iran J Food Quality and Hazards Contrl.* 2015; 2: 134-138.
- Ansar A, Durham J. Heavy Metals in Poultry Products in Bangladesh: A Possible Death Threat to Future Generations. *Journal of Social and Political Sciences*. 2019; 2(1). doi:10.31014/AIOR.1991.02.01.53.
- World Health Organization (WHO)(2010) .Action is needed on chemicals of major public health concern. [http://www.who.int/ipcs/features/10chemicals_en.pdf?ua=1\(2010\)](http://www.who.int/ipcs/features/10chemicals_en.pdf?ua=1(2010)), Accessed Apr 2018.
- Varsha M, N Madaan, A Mudgal, R.B. Singh, S Mishra. Effect of toxic metals on human health. *OpenNutraceuticals J.* 2010; 3 : 94-99.
- European Food Safety and Authority (EFSA). Opinion of the Scientific Panel on Contaminants in the Food chain on a request from the European Commission on mercury as undesirable substance in feed, *The EFSA J.* 2008; 654: 1-76.
- Ericksen JA, Gustin MS, Schorran DE, Johnson DW, Lindberg SE, Coleman JS. Accumulation of atmospheric mercury in forest foliage. *Atmos. Environ.* 2003; 37:1613-1622.
- Li Y, McCrory DF, Powell JM, Saam H, Jackson-Smith D. A survey of selected heavy metal concentrations in Wisconsin dairy feeds. *Journal of dairy science*. 2005; 88(8): 2911-2922. doi:10.3168/JDS.S0022-0302(05)72972-6.
- Korish MA, Attia YA. Evaluation of Heavy Metal Content in Feed, Litter, Meat, Meat Products, Liver, and Table Eggs of Chickens. *Animals : an Open Access Journal from MDPI*. 2020; 10(4): 727. doi:10.3390/ani10040727.
- Ansari MS, Britton WM. Effect of dietary selenium and mercury and 203Hg

- metabolism in chicks. *Poultry science*. 1974; 53(3): 1134-1137. doi:10.3382/PS.0531134.
14. Al-Fayadh H, Mehdi AW, Al-Soudi K, Al-Khazraji AK, Al-Jiboori NA, Al-Muraib S. Effects of feeding ethyl mercury chloride to chickens. *Poultry science*. 1976; 55(2): 772-779. doi:10.3382/PS.0550772.
 15. Kim JH, Park GH, Choi HS, Ji SY, Kil DY. Determination of the Toxic Level of Dietary Mercury and Prediction of Mercury Intake and Tissue Mercury Concentrations in Broiler Chickens Using Feather Mercury Concentrations. *J. Appl. Poult. Res*. 2019; 28(4): 1240-1247.
 16. Zhong W, Ren T, Zhao L. Determination of Pb (Lead), Cd (Cadmium), Cr (Chromium), Cu (Copper), and Ni (Nickel) in Chinese tea with high-resolution continuum source graphite furnace atomic absorption spectrometry. *Journal of food and drug analysis*. 2016; 24(1): 46-55. doi:10.1016/j.jfda.2015.04.010.
 17. Hosain MZ, Islam SMS, Kamal MM. Development of a rapid and reliable high-performance liquid chromatography method for determination of water-soluble vitamins in veterinary feed premix. *Veterinary World*. 2021; 14: 3084-3090. doi:10.14202/vetworld.2021.3084-3090.
 18. National Research Council. *Nutrient Requirements of Poultry*. 9th revised edition National Academic Press, Washington, DC. 1994.
 19. European Commission. Regulation (EC) No. 333/2007 of 28 March 2007 laying down the methods of sampling and analysis for the official control of the levels of lead, cadmium, mercury, inorganic tin, 3-MCPD and benzo(a) pyrene in foodstuffs. 2007. p. L 88/29–L 88/38.
 20. Eurachem. *The fitness for purpose of analytical methods*. Teddington: LGC; 1998. 309 EURACHEM/CITAC. Quantifying uncertainty in analytical measurement. UK: LGC; 2012.
 21. European Commission (2002b) Directive 2002/32/EC of the European Parliament and of the Council 338 of 7 May 2002 on undesirable substances in animal feed. p. L 140/10–L140-21.
 22. Minitab. *Minitab Statistical Software User Guide 2: Data Analysis & Quality Tools*. Minitab Inc., State College, P.A. USA. 2000.
 23. Shah AQ, Kazi TG, Baig JA, Afridi HI, Kandhro GA, Khan S, et al. Determination of total mercury in chicken feed, its translocation to different tissues of chicken and their manure using cold vapour atomic absorption spectrometer. *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association*. 2010; 48(6): 1550-1554. doi:10.1016/j.fct.2010.03.023.
 24. Ertas OS, Tezel H. A validated cold vapour-AAS method for determining mercury in human red blood cells. *Journal of pharmaceutical and biomedical analysis*. 2004; 36(4): 893-897. doi:10.1016/J.JPBA.2004.08.014.
 25. Ullah AKM, Atique MA, Maksud SR, Khan LN Lutfa, Shamshad B. Quraishi Development and validation of a GF-AAS method and its application for the trace level determination of Pb, Cd, and Cr in fish feed samples commonly used in the hatcheries of Bangladesh. *J Anal Sci and Technol*. 2017; 8(4): 15.
 26. Hossain MM, Hannan ASMA, Kamal MM, Hossain MA, Zaman S. Development and Ratification of a Precise Method (GF-AAS) Used for the Determination of Poisonous Metal Lead (Pb) in Dairy Cow Milk Sample Commonly Available in the Market of Bangladesh. *Austin J Anal Pharm Chem*. 2022; 9(1): 1142.
 27. FDA. *Bioanalytical Methods Validation guidance for Industry*, U. S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research (CDER), Center for Vet. Medicine (CVM), 2018; 1-41.
 28. European Commission (2013) Regulation (EC) No. 1275/2013 of 6 December 2013 amending Regulation (EC) No. 2002/32/EC on undesirable substance in animal feed. 2013. p. L328/86–L328/92.
 29. European Commission.(2002a) Decision of 12 August 2002 implementing Council Directive 335 96/23/EC concerning the performance of analytical methods and the interpretation of results. *Off. J. Eur. Communities*.;657/EC: L 221/8–L 221/36.
 30. Jeevanaraj P, Hashim Z, Elias SM, Aris AZ, Total mercury (THg), lead (Pb), cadmium Cd) and arsenic (As) in hair samples: method validation and quantification among women at reproductive age 341 in Selangor. *Int J.Sci. Basic Appl Res*. 2015; 24(4): 332–347.
 31. ISO/IEC 17025 (2005) General requirements for the competence of testing and calibration 307 laboratories. 2005.
 32. Nordtest Report TR 537. *Handbook for calculation of measurement uncertainty in environmental laboratories*. 2nd ed.; 2004.
 33. Rashid M, Sarker M, Khatun H, Sarker N, Ali MY, Islam M. Detection of heavy metals in poultry feed, meat and eggs. *Asian-Australasian Journal of Food Safety and Security*. 2018; 2(1): 1-5. doi:10.3329/aajfss.v2i1.55893.
 34. Rawar E, Rohman A. Validation of Atomic Absorption Spectrophotometry and Mercury Analyzer for Analysis of Cadmium, Lead and Mercury in Red Chili Pepper. *Asian Journal of Agricultural Research*. 2016; 10(1): 65-71. doi:10.3923/AJAR.2016.65.71.
 35. Hossain MM, Hannan ASMA, Kamal MM, Hossain MA, Zaman S. Evaluation of Broiler Meat through Detection of Poisonous Metals (Cr, Cd) available in Bangladesh. *Austin J Anal Pharm Chem*. 2022; 9(2): 1145.
 36. Hossain MM, Hannan ASMA, Kamal MM, Hossain MA. Detection of heavy metals and evaluation of beef procured from the different market of Dhaka in Bangladesh. *European J. Food Sci. Technol*. 2022, 10 (2):1-10.