RISK ASSESSMENT OF AFLATOXINS IN NUTS AND NUT PRODUCTS AMONGST ADULT POPULATION IN THE STATE OF PULAU PINANG

by

LEONG YIN HUI

Thesis submitted in fulfillment of the requirements

for the degree of

Doctor of Philosophy

June 2011

ACKNOWLEDGEMENTS

First, I am thankful to have Assoc. Prof. Rosma Ahmad as my main supervisor who was so helpful and provided valuable and critical comments on my thesis. I like to express my heartiest words of appreciation to my co-supervisor, Prof. Aishah Latiff, for her professional and constructive suggestions to my work. To my field supervisors, Dr. Nurul Izzah Ahmad and Dr. Lokman Hakim Sulaiman, I would like to express my gratitude to them for giving guidance in designing the field work and analysing the data. I also like to thank Madam Normaliza Abdul Manaf for the practical supervision when working at the Doping Control Centre. Appreciation also goes to Assoc. Prof. Noryati Ismail, who was the main supervisor up to the time of her retirement. Her guidance in designing the experiments is most valued.

To all my dear friends, colleagues from School of Industrial Technology and staff of Doping Control Centre, especially Dr. Gan, Hooi Ling, Kim Siew, Pui Nyuk, Thuan Chew, Wan Teik, Li Choo, and many others for sharing all these countless experiences. Thank you for your company.

Special thanks to IPS for supporting my postgraduate studies through the Fellowship program. Financial support of this work through research grants from Food Safety and Quality Division, Ministry of Health, Malaysia is also acknowledged. Also, thanks to Family Development, Ministry of Health for providing technical information on the food consumption survey and Department of Statistical Malaysia for helping in sampling design and methodology.

I am grateful to many of my assistants and medical staff of IPPT and Health Centre who helped and made the survey successful. Sincere gratitude also goes to all participants for their co-operation in the survey.

Last, but not least, deepest appreciation to my parents and family members for their never-ending support, encouragement, inspiration and understanding in embarking on the completion of this thesis.

To See Keat, for his love and everything.

LEONG YIN HUI

April, 2011

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Abbreviation	Caption
ADI	Acceptable daily intake
AF	Aflatoxin
ALARA	As low as reasonable achievable
ALP	Alkaline phosphatase
ALT	Alanine transaminase
Anti-HBs	Antibody to hepatitis B surface antigen
Anti-HCV	Antibody to hepatitis C virus
APCI	Atmospheric pressure chemical ionization
AST	Aspartate transaminase
BMD	Benchmark dose
BMDL	Benchmark dose lower confidence level
BMI	Body mass index
BMR	Benchmark response
bw	Body weight
CAST	Council for Agricultural Science and Technology
CDC	Centre for Disease Control and Prevention
CI	Confidence interval
CRM	Certified reference material
EB	Enumeration block
ECHA	European Chemicals Agency
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assay

EPA	Environmental Protection Agency
ESI	Electrospray ionization
FAO	Food and Agricultural Organization
FAPAS	Food Analysis Proficiency Assessment Scheme
FDA	Food and Drug Administration
FFQ	Food frequency questionnaire
FPIA	Fluorescence polarization immunoassay
GDP	Gross Domestic Product
GEMS	Global Environment Monitoring System
HBsAg	Hepatitis B virus antigen
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HPLC	High performance liquid chromatography
IAC	Immunoaffinity column
IARC	International Agency for Research on Cancer
IPCS	International Programme on Chemical Safety
IRMM	Institute for Reference Materials and Measurements
ISTD	Internal standard
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LC-MS	Liquid chromatography coupled to mass spectrometry
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LOAEL	Lowest observed adverse effect level
LOD	Limit of detection

LOQ	Limit of quantification
LQ	Living quarter
MANS	Malaysian Adult Nutrition Survey
MOE	Margin of exposure
MOH	Ministry of Health
MRM	Multiple reaction monitoring
MSPD	Matrix solid-phase dispersion
NIST	National Institute of Standard and Technology
NOAEL	No observed adverse effect level
OR	Odd ratio
OWLS	Optical waveguide lightmode spectroscopy
PHRED	Photochemical reactor for enhanced detector
POD	Point of departure
Pulau Pinang	Penang
RASFF	Rapid Alert System for Food and Feed
RIA	Radio immunoassay
RM	Reference material
RSD	Relative standard deviation
SFE	Supercritical fluid extraction
S/N	Signal to noise ratio
SPE	Solid-phase extraction
SPR	Surface plasmon resonance
TDI	Tolerable daily intake
TLC	Thin layer chromatography
TTC	Threshold of toxicological concern

TWI	Tolerable weekly intake
UNDP	United Nations Development Programme
WHO	World Health Organization

LIST OF APPENDICES

LIST OF PUBLICATIONS

Journal publication

Leong, Y.-H., Ismail, N., Latif, A.A. and Ahmad, R., (2010). Aflatoxin occurrence in nuts and commercial nutty products in Malaysia. Food Control, 21, 334-338.

Leong, Y.-H., Ismail, N., Latif, A.A., Normaliza A.M. and Rosma, A. Determination of aflatoxins in commercial nuts and nut products by using liquid chromatography tandem mass spectrometry. World Mycotoxin Journal, 4(2), 119-127.

Leong, Y.-H., Ismail, N., Latif, A.A., Nurul Izzah, A., Narazah, M.Y. and Nurul Ain, A.B., (2011). Nuts Consumption Pattern Among Malaysian Adults: A Socio-demographic and Dietary Behaviour Perspective. International Food Research Journal, 18(1), 319-328.

Leong, Y.-H., Rosma, A., Latif, A.A. and Nurul Izzah, A. Exposure assessment and risk characterization of aflatoxin B1 in Malaysia. (Mycotoxin Research, in press)

Oral presentation

Leong, Y.-H., (2009). Determination of aflatoxins B1, B2, G1 and G2 in commercial nut products by liquid chromatography-tandem mass spectrometry. Proceedings of Symposium of USM Fellowship Holders 2009, 14-15 November 2009, Penang. P. 180.

Leong, Y.-H. and Ismail, N., (2008). Assessment of aflatoxins: Towards development of stringent food safety system. The sixth Regional IMT-GT UNINET Conference 2008. Sustaining Natural Resources Towards Enhancing the Quality of Life Within the IMT-GT Zone, 28-30 August 2008, Penang. P. 49-52.

Leong, Y.-H., Ismail, N., Latif, A.A. and Nurul Izzah, A., (2010). Dietary exposure to aflatoxin from nuts in adult population from Penang, Malaysia. International Mycotoxin Conference MycoRed 2010. Global Mycotoxin Reduction Strategies: Asia and Pacific Rim, 1-4 December 2010, Penang. P. 119.

Poster presentation

Leong, Y.-H., Ismail, N., Latif, A.A. and Ahmad, N.I., (2010). Occurrence and risk assessment of aflatoxin B1 in Malaysia: application of the margin of exposure (MOE). The World Mycotoxin Forum 6th Conference, 8-10 November 2010, Noordwijkerhout, The Netherlands. P. 113.

Leong, Y.-H., Ismail, N., Latif, A.A. and Nurul Izzah, A., (2009). Assessment of exposure to aflatoxin contamination in nuts and nutty products among population of Penang state: Phase 1-Dietary survey. The 6th Food Science

and Technology Seminar. Transformation of Agro-Food Industry: Opportunities and Challenges, 14-15 February 2009, Sabah. P. 35.

PENILAIAN RISIKO AFLATOKSIN DALAM KEKACANG DAN PRODUKNYA DI KALANGAN POPULASI DEWASA DI NEGERI PULAU PINANG

ABSTRAK

Aflatoksin telah dikenalpasti sebagai ancaman yang paling serius kepada kesihatan awam di antara mikotoksin diketahui, disebabkan oleh kesan toksik yang tinggi dan karsinogenik mereka pada manusia dan haiwan. Dalam kajian ini, penilaian risiko aflatoksin dalam kekacang dan produknya di kalangan populasi dewasa di negeri Pulau Pinang telah dijalankan. Kajian ini melibatkan pengumpulan data pemakanan kekacang (kacang tanah, kacang jenis pokok dan produk mereka) oleh responden terpilih dari lima daerah di Pulau Pinang, analisis tahap pencemaran aflatoksin di dalam kekacang, dan penganggaran risiko atas pendedahan aflatoksin menggunakan pendekatan margin pendedahan (*margin of exposure*, MOE). Perbandingan dan pengesahan keputusan antara pendedahan dietari dengan penilaian biopenanda juga telah dijalankan. Seramai 364 subjek (129 lelaki dan 235 perempuan) telah menyertai kajian ini; 54.4% adalah orang Melayu, 64.6% adalah perempuan dan 76.1% adalah daripada Seberang Perai. Purata jumlah pemakanan kekacang sebanyak 0.77 g/hari untuk orang dewasa telah didapati tiada perbezaan khusus untuk jantina tetapi berbeza secara ketara ($P < 0.05$) di kalangan kumpulan etnik dengan pengambilan tertinggi oleh kaum India (0.49 g/hari). Kuih pau kacang adalah produk yang paling banyak diambil (median = 0.81 g/hari). Satu kaedah analisis kepastian untuk aflatoksin (B1, B2, G1 dan G2) dengan aflatoksin M1 sebagai piawai dalaman, menggunakan kromatografi cecair berganding dengan spektrometer massa telah

dibangunkan dan disahkan. Sampel diekstrak dengan metanol:air (60:40, v/v) dan teknik pembersihan dengan turus imunoafiniti. Aflatoksin telah dikesan dalam 73/128 (57%) sampel kekacang; julat tahap pencemaran daripada 0.40 – 221.61 µg/kg untuk AFB1 dan 0.33 – 273.63 µg/kg untuk jumlah aflatoksin dengan yang tertinggi dalam kacang tanah goreng. Aduk AFB1-lisin telah dikesan dalam 165/170 (97%) sampel serum. Anggaran dietari untuk pendedahan rendah dan tinggi adalah 0.36 dan 8.89 ng/kg bb/hari masing-masing, yang mencerminkan pengambilan AFB1 sebanyak 0.02 – 0.53 µg setiap hari. Anggaran pendedahan dietari ini adalah selaras dengan keputusan aduk AFB1-lisin (lingkungan 0.20 – 23.16 pg/mg albumin), yang mencadangkan satu nilai kiraan pengambilan sebanyak 0.01 – 0.60 µg AFB1 setiap hari. Dengan menggunakan pendekatan dos tanda aras (*benchmark dose*, BMD) dan MOE, nilai BMD pada selang kepercayaan lebih rendah 95% ($BMDL_{10}$) ialah 0.305 µg/kg bb/hari. Nilai MOE yang diperolehi (34 – 847) mencadangkan AFB1 perlu diberi perhatian demi kesihatan awam dan mungkin diberi keutamaan tinggi untuk tindakan pengurusan risiko. Kajian ini memberikan maklumat berguna tentang kejadian dan pendedahan semasa untuk aflatoksin dalam kekacang. Hal ini akan membantu kerajaan dalam rancangan pencegahan demi mengurangkan pengambilan aflatoksin secara dietari serta digunakan sebagai rujukan untuk perangkaan undang-undang baru.

RISK ASSESSMENT OF AFLATOXINS IN NUTS AND NUT PRODUCTS AMONGST ADULTS POPULATION IN THE STATE OF PULAU PINANG

ABSTRACT

Aflatoxins have been identified the most serious threat to public health among the known mycotoxins, due to their highly toxic and carcinogenic effect in human and animals. In this study, risk assessment of aflatoxins in nuts and nut products amongst adult population in Pulau Pinang State was conducted. This study involved collecting data on nuts (groundnuts, tree nuts and their products) consumption by selected respondents from five districts of Pulau Pinang, analysis of contamination levels of aflatoxins in nuts, and estimation of risk on aflatoxins exposure using the margin of exposure (MOE) approach. Comparison and verification of results between dietary exposure with the biomarker assessment was carried out. A total of 364 subjects (129 men and 235 women) participated in the study; 54.4% were Malays, 64.6% were female and 76.1% were from Seberang Perai. Average total nuts consumption of 0.77 g/day among adults was found to be no gender-specific difference but was significantly different ($P < 0.05$) among ethnic groups with the highest among Indians (0.94 g/day). *Kuih pau kacang* was the most consumed product (median = 0.81 g/day). A confirmatory analytical method for aflatoxins (B1, B2, G1 and G2) with the aflatoxin M1 as the internal standard, using liquid chromatography tandem mass spectrometry had been developed and validated. Samples were extracted using methanol:water (60:40, v/v) and immunoaffinity column clean-up technique. Aflatoxins were detected in 73/128 (57%) of the nuts samples; contamination levels ranged from 0.40 – 221.61 µg/kg for aflatoxin B1 (AFB1) and 0.33 – 273.63 µg/kg

for total aflatoxins with the highest in fried peanut. AFB1-lysine adduct was detected in 165/170 (97%) of serum samples. Estimated dietary for low and high exposures were 0.36 and 8.89 ng/kg bw/day, respectively, which reflected an intake of 0.02 – 0.53 μg AFB1 per day. This dietary exposure estimation was in line with the AFB1-lysine adduct results (ranged 0.20 – 23.16 pg/mg albumin), which suggested a calculated intake value of 0.01 – 0.60 μg AFB1 per day. Using the benchmark dose (BMD) and MOE approaches, the value of a BMD at 95% lower confidence interval ($BMDL_{10}$) was 0.305 μg/kg bw/day. The derived MOE values (34 to 847) suggested that AFB1 would be of public health concern and might reasonably be considered as a high priority for risk management actions. The present study provided useful information about the occurrence and current exposure of aflatoxins in nuts. This would help the authorities in the intervention planning in order to reduce the dietary intake of aflatoxins as well as used as a reference for drafting of new legislation.

CHAPTER 1 – INTRODUCTION

1.1 Background

Aflatoxins belong to a group of fungal toxins known as mycotoxins. The major classes of mycotoxins include the aflatoxins, fusarial toxins (trichothecences, fumonisins, zearalenone), ochratoxin A, and the ergot alkaloids. Among the known 400 mycotoxins, aflatoxins are the most hazardous to human health due to their highly toxic, carcinogenic, teratogenic, hepatotoxic and mutagenic characteristics (Pariza, 1996). The FAO has estimated that up to 25% of the world's foods are significantly contaminated with mycotoxins (WHO, 1999). This problem is of current concern and has received a great deal of attention since the last three decades because of the significant economic losses associated with their impact on human health, animal productivity and trade. These toxins cannot be entirely avoided or eliminated from foods and feeds by current agronomic practices and manufacturing processes and are considered unavoidable contaminants.

Aflatoxins pose a potential threat to food safety. As it is epidemiologically implicated as carcinogen in humans and an environmental contaminant which is widespread in nature, its possible chronic toxicity is therefore, of greater concern than acute toxicity. This toxin probably plays a role in the high incidence of liver carcinoma in the tropics as well as chronic hepatitis B and C. Aflatoxins are chemically stable in foods and resistant to degradation under normal cooking procedures. It is difficult to eliminate once it is being produced. Many gaps still remain in humans' knowledge about aflatoxins, resulting in the need for more descriptive and analytical epidemiological studies. An assessment to identify the

significance of aflatoxins exposure in terms of their toxicity or hazard in consumption of certain agriculture products is thus indispensable for this approach.

Malaysia is a tropical country with the average temperature of 28 – 31 ^{o}C and heavy rainfall throughout the year. Relative humidity is in the range of 70 – 80% during wet season and 50 – 60% during dry season. The warm and humid weather of Malaysia provide the optimal condition for the growth of certain strains of *Aspergillus* species of fungi, primarily by *Aspergillus flavus* and *Aspergillus parasiticus* and subsequently aflatoxins production. According to JECFA (1998), maize and peanuts and their products are the most important dietary sources of aflatoxins. In a survey of aflatoxins in foods and stored agricultural products in Malaysia, peanuts and their products were shown highly susceptible to mould and aflatoxins contamination (Mat Isa and Tee, 1984). In addition, seven cases of border rejection of nut products from Malaysia by United Kingdom and Greece has been reported by the Rapid Alert System for Food and Feed (RASFF) up to mid 2010 (RASFF, 2010). Due to the high risk potential, nuts and their products were selected in this study, considering their major dietary component as the snacks, sauces or as part of the ingredients of many dishes in the daily diet. To date, limited quantitative surveillance data on aflatoxins in our country is available even though the daily diet poses a potential health hazard and climatic conditions favour development of *Aspergillus* species and aflatoxins production.

In this study, a risk assessment on aflatoxins which involved exposure assessment and risk characterization has been performed in an attempt to project the particular risk in Pulau Pinang (Penang) population based on the evidence obtained. For the exposure assessment, it included the nuts and nut products consumption survey, determination of contamination levels of aflatoxins in nuts using liquid

chromatography tandem mass spectrometry (LC-MS/MS) as well as biomarker assessment. For the risk characterization, MOE was calculated using benchmark dose approach to quantify the health risk of aflatoxins in the study population, taking into the account that aflatoxins are both the genotoxic and carcinogenic substances. Furthermore, population risk for primary liver cancer in Penang region has also been estimated.

In the present work, biomarker assessment by analysing the aflatoxin B1-lysine adduct in human blood and the risk characterization of aflatoxins using the new approach of MOE are the two major components that have not been reported in Malaysia. These informations would be very useful in intervention planning to reduce human risk in a public health context. In addition, the occurrence of aflatoxins in different kinds of nuts and nut products is essential information needed by government or local authority for a better monitoring or prevention strategies planning. The results can also be used as a reference for drafting of new legislation, particularly for foodstuffs which are prone to contamination such as peanut butter, *satay* sauce, etc. At present, legal limits are only set for peanuts and milk in general in the Malaysian Food Act 1983 and Regulations 1985. So far, scientific work on natural toxicants has been less extensive than other chemical contaminants in food, notably residues of pesticides in food. With increasing knowledge of aflatoxins, more contribution can be made to eliminate the toxin or reduce its content in foods and feedstuffs to significantly lower levels as what a major global concern about.

1.2 Objectives

The aim of this research was to assess the aflatoxins exposure through the dietary intake of nuts and their products and subsequently quantify the risk posed to

Penang adult population. Due to this research was the very first study on the risk assessment of aflatoxins in Malaysia, therefore many limitation factors have been taken into account in designing the experiments. For this and other reasons like limited resources and time constrain, only adult population and one food group (nuts and nut products) were evaluated, rather than involving more vulnerable population of children and adolescents for a comprehensive study.

For this purpose, consumption survey on nuts, analysis of nuts samples using a validated LC-MS/MS method and determination of aflatoxin B1-lysine adduct levels in blood samples have been carried out. Consequently, the specific objectives of this study were:

i. To determine the consumption pattern of nuts and nut products among three main ethnic groups (Malays, Chinese and Indians) in Penang and their association with a variety of socio-demographic characteristics and behavioural factors (purchase place, expenditure, etc).
ii. To determine the incidence and contamination levels of aflatoxins (B1, B2, G1 and G2) in frequently consumed nuts and nut products by study subjects.
iii. To establish a relationship between dietary intake of aflatoxins and the amount of aflatoxin B1-lysine adduct levels detected in blood samples.
iv. To verify the estimation of risk obtained from the nuts consumption survey with the analysis of the corresponding biomarker in the blood samples of the study subjects.
v. To assess aflatoxins exposure and quantify the risk posed to population in Penang state.

CHAPTER 2 – LITERATURE REVIEW

2.1 Aflatoxins

Aflatoxins are a group of mycotoxins produced as secondary metabolites by fungi belonging to several *Aspergillus* species, mainly *A. flavus* and *A. parasiticus* (Groopman et al., 1988; Gourama and Bullerman, 1995; O'Riordan and Wilkinson, 2008). Over 400 mycotoxins are known and the major classes of mycotoxins are aflatoxins, trichothecences, fumonisins, zearalenone and ochratoxin A. The frequent occurrence of these food-borne toxins and their severe effects on animal and human health has been recognized (Hussein and Brasel, 2001). The aflatoxins are probably the most extensively studied mycotoxins. The name aflatoxin is an abbreviation of "a" for *Aspergillus* genus and "fla" for the species *flavus*, and the word "toxin" (meaning poison) (Ellis et al., 1991). Currently, more than 14 different types of aflatoxins have been identified and the naturally occurring aflatoxins are aflatoxin B1, B2, G1 and G2 (noted AFB1, AFB2, AFG1 and AFG2 respectively). AFB1 and AFB2 are produced typically by fungi belonging to *A. flavus* while AFG1 and AFG2 are produced by *A. parasiticus*. Aflatoxins were discovered in the 1960s when a series of outbreaks in poultry and fish occurred in different parts of the world. One of the famous outbreaks was the sudden death of more than 100,000 turkeys and other farm animals (turkey "X" disease) in UK where the cause was attributed to *A. flavus* contaminated peanut meal (Bennett and Klich, 2003). Aflatoxins occur mostly in tropical regions where hot and humid climates promote fungal growth. Poor harvesting practices, improper storage, and less than optimal conditions during transport and marketing can also contribute to proliferation of fungal and increase the risk of aflatoxins production (Bhat and Vasanthi, 2003).

2.1.1 Structure and toxicity

Aflatoxins show fluorescence under ultraviolet light and they are grouped into AFB1 or AFB2 for blue, and AFG1 or AFG2, for green, where number 1 or 2 refer to the chromatographic mobility (Bennett and Klich, 2003). Aflatoxins are a group of difuranocoumarin compounds and attached to either pentanone (AFB1 and AFB2) or a six-membered lactone (AFG1 and AFG2) (Figure 2.1). Aflatoxin M1 and M2 (AFM1, AFM2), are the metabolites of AFB1 and AFB2, respectively and are found in milk and milk products. AFB1 is the most toxic and most prevalent compound, followed by AFG1, AFB2 and AFG2 with decreasing toxicity. The carcinogenic potency of AFM1 is around 10-fold lower than that of AFB1.

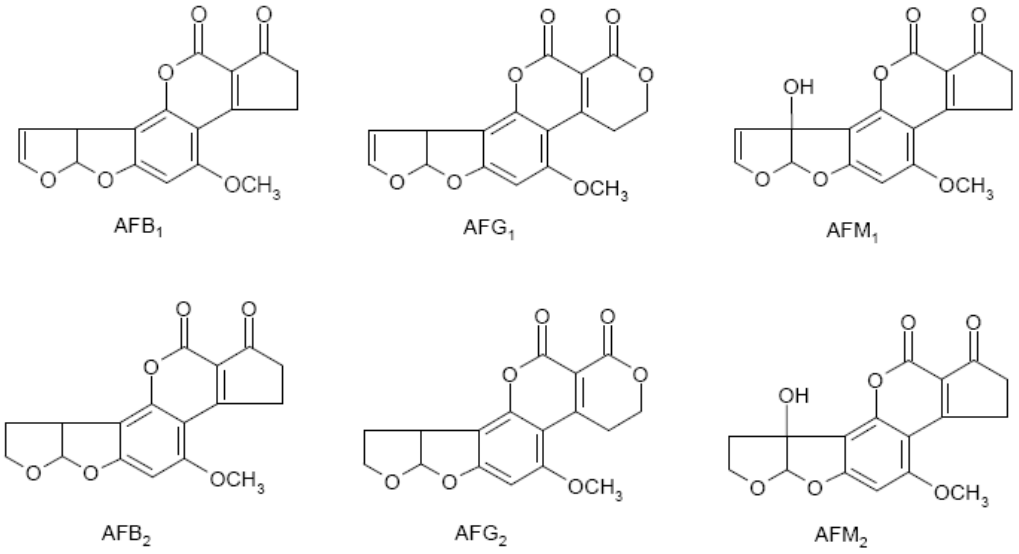

Figure 2.1 Chemical structures of major aflatoxins (AFB1, AFB2, AFG1, AFG2, AFM1 and AFM2).

2.1.2 Health effects

The diseases caused by aflatoxin consumption are collectively called aflatoxicosis. Acute aflatoxicosis results in death while chronic aflatoxicosis results

in cancer, immune suppression, and other pathological conditions (Hsieh, 1988). The symptoms of acute aflatoxicosis may include high fever, vomiting, hemorrhage, rapid progressive jaundice and swollen livers (Fung and Clark, 2004). Numerous studies have linked the incidence of primary hepatocellular carcinoma with the intake of aflatoxins, resulting in the classification of naturally occurring aflatoxins as Group one human carcinogen by the International Agency for Research on Cancer (IARC) (IARC, 1993). It has also been shown to produce DNA-adducts, gene mutation and chromosomal anomalies in humans and animals *in vivo* and *in vitro* (IARC, 1993). Their role in hepatocarcinogenesis often in conjunction with hepatitis B is well established (Wild and Hall, 1998; Wild and Turner, 2002). There is some evidence for associations with Reye's syndrome (encephalopathy with severe lesions in kidney and liver following influenza or varicella), Kwashiorkor (severe protein-energy malnutrition disease), and acute hepatitis (Wild and Hall, 1998). Moreover, the illness known as "cirrhosis of Indian childhood" is also partly due to an aflatoxin poisoning. Aflatoxins can cross the human placenta and the exposure in early life has been associated with impaired growth, particularly stunting (Gong et al., 2002; IARC, 2002).

In the studies on aflatoxicosis in Kenya (Ngindu et al., 1982; Azziz-Baumgartner et al., 2005) and India (Krishnamachari et al., 1975), staple foods contaminated with 5,000 ppb or above of aflatoxins were associated with fatality while daily consumption of foods with greater than 1,000 ppb was linked to aflatoxicosis. Based on the observed levels of contamination in the studies, the intake of total aflatoxins resulting in a risk of fatality can therefore be estimated to be greater than 1 mg/kg, or in excess of 20 μg/kg bw/day in adults (Wild and Gong, 2010). An acute outbreak of aflatoxicosis in Malaysia was reported (Chao et al.,

1991; Lye et al., 1995). The acuteness of the illness differed from previously reported outbreaks described in Kenya and India. The offending food was a Chinese noodle called "Loh See Fun" and the poisoning resulting in 13 deaths in children in the state of Perak, Malaysia during the 9-day Chinese Festival of the Nine-emperor Gods in 1988. It was reported that up to 3 mg of aflatoxin would have to have been present in the contaminated noodles using estimates from the Kenyan experience (Lye et al., 1995).

Among the farm animals, ingestion of aflatoxins contaminated feeds can lead to substantial loss in productivity and meat quality (Bonomi et al., 1994). Symptoms of acute aflatoxicosis in mammals include lethargy, ataxia, rough hair coat, and enlarged fatty liver (Nibbelink, 1986). For chronic exposures, the major symptoms are reduced intake and milk production, jaundice and decreased appetite (Nibbelink, 1986). Reduced growth rate is possibly the most obvious indication for chronic aflatoxicosis (Pier, 1992) and is related to disturbances in protein, carbohydrate and lipid metabolism (Cheeke and Shull, 1985).

2.1.3 Metabolism

Aflatoxin metabolism has been extensively studied in animals and humans and several comprehensive reviews have been published (Eaton and Gallagher, 1994; Guengerich et al., 1998; Wild and Turner, 2002). Aflatoxin is metabolized by liver cytochrome P450 enzymes after ingestion to form highly reactive 8, 9-epoxide which subsequently reacts with DNA to generate guanine adducts (Groopman et al., 1993; Walton et al., 2001, Bennett and Klich, 2003), or with serum albumin to generate lysine adducts (Sabbioni et al., 1990). Aflatoxin can be hydroxylated to other metabolites such as AFM1 and AFQ1. The pathways and consequences for aflatoxin

in animal metabolism are shown in Figure 2.2. The differences in susceptibility to aflatoxin across species and between persons depend largely on the fraction of the dose that is directed into the various possible pathways, with harmful "biological" exposure being the result of activation to the epoxide and the reaction of the epoxide with proteins and DNA. There is also evidence that the fractions that follow the different possible pathways are influenced by dosage, perhaps because of the saturation of the most chemically competitive processes (Eaton et al., 1993). Susceptibility to aflatoxin is greatest in the young, and there are very significant differences between species, individual of the same species (according to their differing abilities to detoxify aflatoxin by biochemical processes), and the sexes (according to the concentrations of testosterone). The toxicity of aflatoxin also varies according to many nutritional factors (Pier et al., 1985; Ayub and Sachan, 1997), and recovery from protein malnutrition is delayed by exposure to aflatoxin (Rogers, 1993; Adhikari et al., 1994).

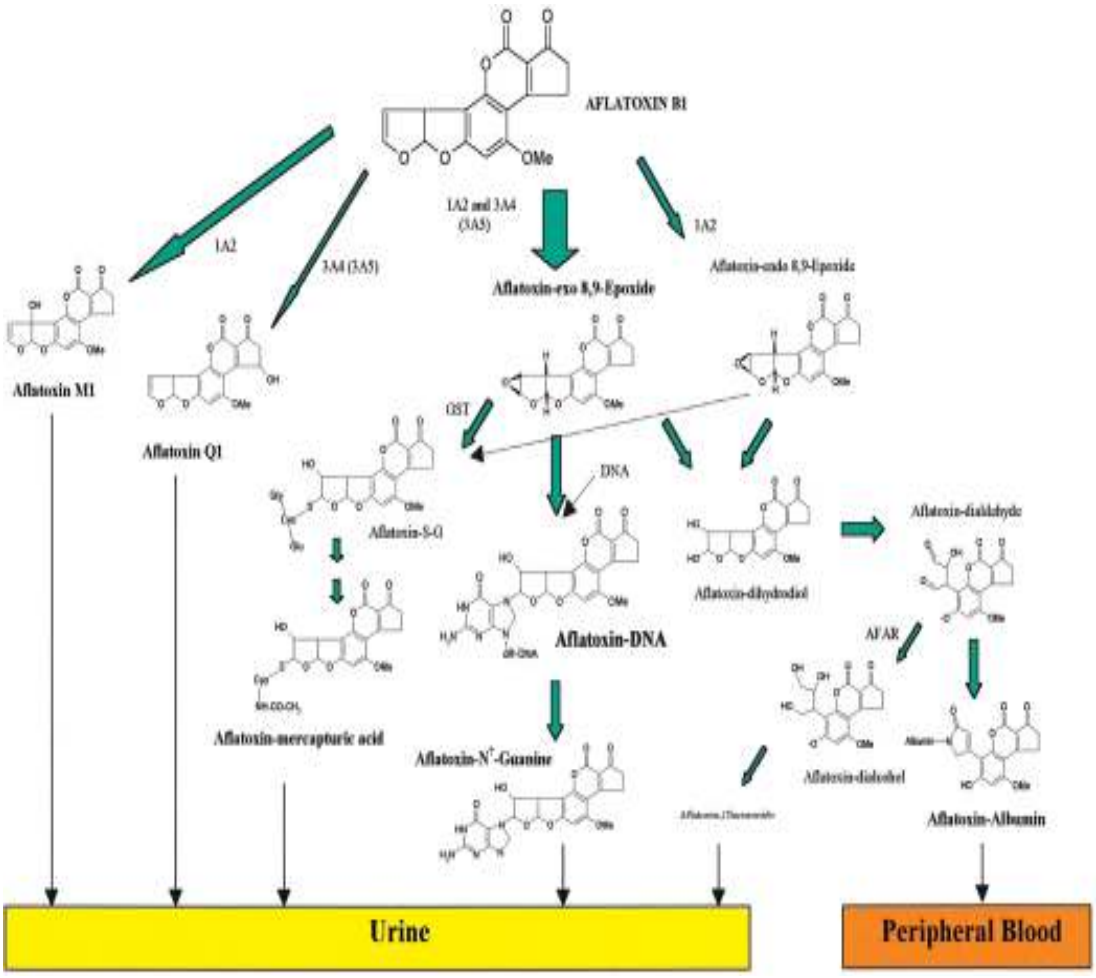

Figure 2.2 Principle metabolism of aflatoxin B1 leading to reactive metabolites and biomarkers. 1A2, CYP1A2; 3A4, CYP3A4; 3A5, CYP3A5; GST, glutathione S-transferase; AFAR, aflatoxin aldehyde reductase; Aflatoxin-S-G, aflatoxin-glutathione conjugate. (Wild and Turner, 2002, Mutagenesis 17(6), Figure 1, pg 472)

2.1.4 Occurrence in foodstuffs

A review of monitoring studies on the occurrence of aflatoxins in food products has demonstrated that aflatoxins are still being found frequently in food products at levels that are of significant concern for consumer protection (Stroka and Anklam, 2002). According to a European Union screening during the years 2000 – 2006, aflatoxins were detected in 26% of all analysed foods (EFSA, 2007a). Aflatoxins have commonly been found to contaminate a wide range of important

agricultural commodities, such as cereals (maize, sorghum, rice, wheat), spices (chillies, turmeric, black pepper), oilseeds (peanut, cottonseed), tree nuts (almond, pistachio, walnut) and milk (human and animals) (Bhat et al., 2010). High concentration of AFM1 in milk, between 28 and 1012 ng/kg, has been reported in a few European Union countries (Markaki and Melissari, 1997; Martin and Martin, 2000). AFM1 was detected in powdered milk, pasteurized milk, ultra-high-temperature treated milk, and other dairy-based products (Montagna et al., 2008; Ghazani, 2009; Shundo et al., 2009).

Occurrence of aflatoxins in several developed countries such as USA, Sweden, UK and others was reported in 1990s (Moss, 2002) and earlier (CAST, 2003) but there are considerably more evidence in developing countries. The significance of the aflatoxins problems in developing countries might be due to the less sophisticated of food handling or preservation technology as well as the unsatisfactory control or regulation over the food quality and safety than the developed contries. In addition, the fungi producing aflatoxins are prevalent in areas of high humidity and temperature, therefore crops in tropical and subtropical regions are more subject to contamination. The growth of fungi is influenced by several factors such as availability of nutrients, temperature, acidity/pH, moisture, water activity, and other associated factors (WHO, 1999). Table 2.1 shows a list of notification of aflatoxins in foods which have recently been reported by Rapid Alert System for Food and Feed (RASFF). The RASFF is running by European Food Safety Authority (EFSA) as a tool for information exchange on consignments of food and feed in cases where a risk to human health has been identified and measures such as withholding, recalling, seizure or rejection of the products concerned have been taken.

Table 2.1 Detection of aflatoxins in foods by RASFF from January to mid of July 2010.

Food category	No. of cases	Origin country	Range (μg/kg)
Cereal and bakery product	6	Pakistan	AFB1: 2.6 – 8.8 Total: 2.9 – 9.7
Dietetic foods, food supplements, fortified foods	1 1	Turkey Sri Lanka	AFB1: 0.819 – 7 Total: 7.8
Fruit and vegetables	15 1 1	Turkey Nigeria India	AFB1: ND – 18.1 Total: ND – 130
Herbs and spices	23 1 1 1 1 1 1	India Spain Netherlands Germany Indonesia Thailand China	AFB1: 0.0203 – 93 Total: 0.0241 – 110
Nuts, nut products and seeds	63 60 49 36 21 8 7 5 3 3 2 2 2 2 1 1 1 1 1 1 1	Argentina China Iran United States Turkey South Africa Nigeria India Egypt Brazil Vietnam Ghana Nicaragua Bolivia Togo Georgia Australia Ukraine Thailand Kyrgyzstan Indonesia	AFB1: ND – 504 Total: ND – 801

Search criteria: notified from 01/01/2010; notified until 16/07/2010; product type food; hazard category mycotoxins. ND: not detected. (RASFF, 2010)

In South-East Asia, the incidence and level of aflatoxins B1, B2, G1 amd G2 contamination in starch-based foods, commercial traditional herbal medicines, peanuts and corn products, rice and its by products as well as baby foods from Malaysia, Indonesia, Philippines and Thailand have been reported (Abdullah et al., 1998; Ali et al., 1999; Noviandi et al., 2001; Razzazi-Fazeli et al., 2004; Tassaneeyakul et al., 2004; Ali et al., 2005; Sales and Yoshizawa, 2005). Sixty-five percent of peanut products from Malaysia were found contaminated with aflatoxins at a mean level of 50 μg/kg (maximum 180 μg/kg) (Ali et al., 1999). Sulaiman et al. (2007) reported that a very high concentration of total aflatoxins (0.85 – 762.05 μg/kg) was found in 145 shelled raw peanut samples collected in Perak state, Malaysia. According to RASFF, seven cases in Malaysia which involved food products i.e. glutinous rice balls with peanut butter, candy coated peanuts, sesame snack, mixed nuts, dried roasted peanuts, peanut butter and *satay* sauce have been identified to be non-compliant to the European Commission regulations with the AFB1 that ranged from 2.7 to 26 μg/kg between 2002 to 2008 (RASFF, 2010).

2.1.5 Regulation

The number of countries regulating aflatoxins has significantly increased worldwide over the years. Table 2.2 shows the upward trend of medians, ranges and numbers of countries with legally established limits for aflatoxins from 1995 to 2003. From 1995 to 2003, the ranges and medians of limits for total aflatoxins in food for various world regions have changed dramatically, especially in Asia or Oceania and Europe (Figure 2.3). There is no observable change in Africa, Latin America and North America. In contrast, a downward trend in the limits for total aflatoxins in

Asia or Oceania and Europe is noticed. This suggests that more Asia countries have concerned about this issue.

Table 2.2 Medians, ranges and numbers of countries with legally established limits for aflatoxins in 1995 and 2003.

Aflatoxin/matrix combination	1995			2003		
	Median (µg/kg)	Range (µg/kg)	Countries	Median (µg/kg)	Range (µg/kg)	Countries
AFB1 in foodstuffs	4	0 – 30	33	5	1 – 20	61
AFB1+B2+G1+G2 in foodstuffs	8	0 – 50	48	10	0 – 35	76
AFM1 in animal milk	0.05	0 – 1	17	0.05	0.05 – 15	60
AFB1 in feedstuffs	5	5 – 50	25	5	5 – 50	39
AFB1+B2+G1+G2 in feedstuffs	20	0 – 1000	17	20	0 – 50	21

Source: FAO (2004), Table 4, pg 165

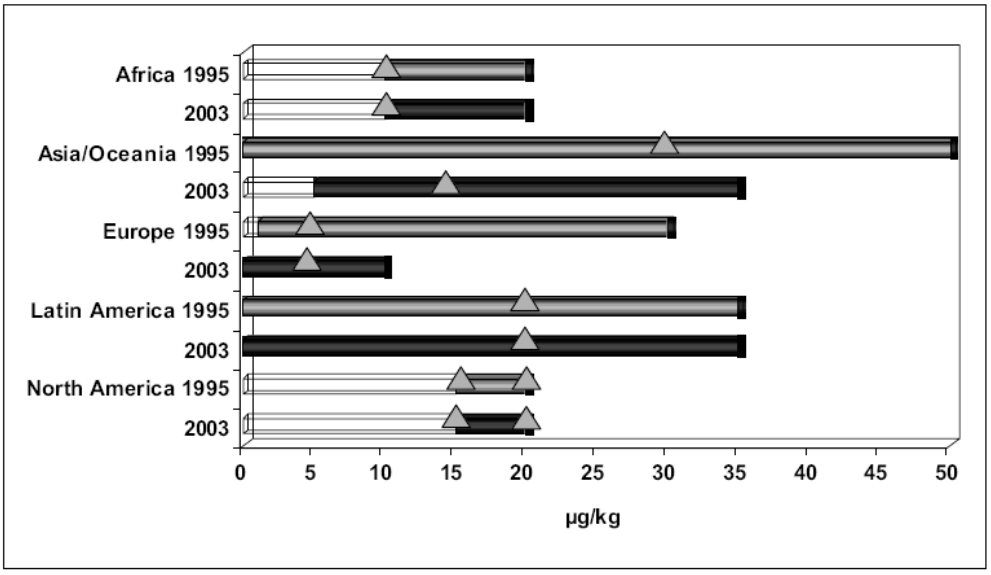

Figure 2.3 Worldwide ranges and medians of limits for total aflatoxins in foodstuffs (FAO, 2004, Figure 15, pg 20).

Aflatoxins are regulated in more than 75 countries with limits that vary from the range of 1 – 20 μg/kg and 0 – 35 μg/kg for AFB1 and total aflatoxins (AFB1 + AFB2 + AFG1 + AFG2) in food, respectively (FAO, 2004). The FDA tolerance level for aflatoxin in human food is 20 μg/kg; for breeding livestock feed 100 μg/kg; finishing beef cattle feed 300 μg/kg and other animals feed 20 μg/kg (FDA, 2011). The European Community levels are more restrictive. For nuts intended for direct human consumption or use as an ingredient in foodstuff, European Commission regulations set limits for AFB1 and total aflatoxins at 8 and 10 μg/kg for almonds, pistachios and apricot kernels; at 5 μg/kg for AFB1 and 10 μg/kg for total aflatoxins for hazelnuts and Brazil nuts; and for groundnuts (peanuts), other tree nuts and processed products thereof, are at 2 and 4 μg/kg for AFB1 and total aflatoxins, respectively (European Commission, 2010).

In Malaysian Food Act 1983 and Regulations 1985, a limit of 15 μg/kg of total aflatoxins in groundnuts for further processing has been established (Anonymous, 2006). Other South-East Asian countries such as Singapore, Indonesia, Thailand and Philippines are also having their own legislation for the aflatoxins. Singapore has the most stringent tolerance level for total aflatoxins, which is 5 μg/kg compared to other South-East Asian countries. Indonesia, Thailand and Philippines have set up the limit of 20 μg/kg for the total aflatoxins in foods (FAO, 2004).

According to van Egmond and Jonker (2004), aflatoxin regulations are becoming more diverse and detailed with newer requirements regarding official procedures for sampling and analytical methodology. Harmonisation of standards is occurring in several free trade zones (European Union, MERCOSUR, Australia and New Zealand) and participation of other countries is expected to increase (van Egmond and Jonker, 2004). Harmonization of standards is to ease the world food

trade and any disputes regarding the aflatoxins contamination that may arise between importing and exporting nations. In the newly launched 7th Framework Programme (ranging from 2007 to 2013) large collaborative projects are foreseen in which rapid methods for mycotoxins, sampling, and standardization issues will be benefited globally (van Egmond et al., 2007).

2.2 Risk Assessment of Contaminant Intake

Risk assessment has been defined as "a process of evaluating including the identification of attendant uncertainties, of the likelihood and severity of an adverse effects(s) or event(s) occurring to man or the environment following exposure under defined conditions to a risk source(s)" (European Commission, 2000). Risk assessment is one of the three components of risk analysis, which also includes risk management and risk communication (Renwick et al., 2003). These three interlocking processes of risk analysis provide a systematic, disciplined approach for making food safety decisions and promote ongoing improvements in public health. Risk analysis represents a structured decision-making process with three distinct but closely connected components as shown in Figure 2.4.

Risk assessment is considered to be the "science-based" of risk analysis while risk management is the component in which scientific information including economic, social, cultural and other factors are integrated and weighed in choosing the preferred food policy options as well as implementing the decision. Risk communication involves the interchange of information concerning risk and its perception among policy makers, industry, consumer and other related stakeholders (European Commission, 2000). Risk assessments are performed in a four-step process, namely assessing hazards (hazard identification and hazard characterization)

and intakes (exposure assessment) of specific contaminant, and integrating the hazard and exposure information thereby characterizing the probability and consequence of harm (risk characterization) (Barlow et al., 2002; Renwick et al., 2003).

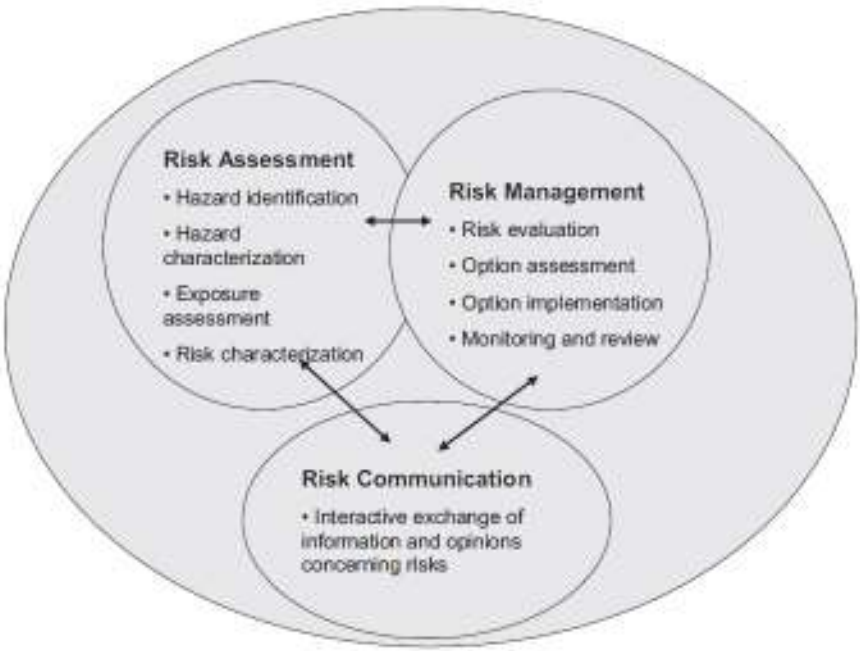

Figure 2.4 Three components of risk analysis. (IPCS, 2009, Figure 2.1, pg 2)

2.2.1 Hazard identification

Hazard identification is the first of four steps in risk assessment. It is defined as "the identification of the risk source(s) capable of causing adverse effect(s)/event(s) to human or environmental species, together with a qualitative description of the nature of these effect(s)/event(s)" (European Commission, 2000). The purpose of hazard identification is to evaluate the weight of evidence for adverse health effects, based on assessment of a variety of data, ranging from observations in humans or domestic and laboratory animals (IPCS, 2009). Primarily, it is designed to

address two questions: a) the nature of any health hazard to humans, and b) under which circumstances the identified hazard may be expressed (IPCS, 2009). From the studies and observations, the nature of any occurring adverse health effects and the affected target organs or tissues is identified. For aflatoxins, they have been identified for their adverse health effects by a vast of literature and supported by the International Agency for Research on Cancer (IARC) as the Group One human carcinogen (IARC, 1993).

2.2.2 Hazard characterization (dose-response assessment)

Hazard characterization is defined as "the quantitative or semi-quantitative evaluation of the nature of the adverse health effects to human and/or the environment following exposure to a risk source(s). This must, where possible, include a dose-response assessment" (European Commission, 2000). To define the dose-response relationships for the detected adverse effects, toxicity studies are carried out. Most of these studies are relied on animal studies while human dose-response data only available in some cases.

2.2.2.1 Dose-response assessment

Dose-response data can be analysed using two different statistical approaches for deriving a reference point (a dose without appreciable adverse health effects in the test animals under the experimental conditions). Reference point has also known as point of departure (POD). The two approaches are the no-observed-adverse-effect-level (NOAEL) and/or the lowest-observed-adverse-effect-level (LOAEL) approach and benchmark dose (BMD) approach. NOAEL/LOAEL has long been used in risk assessment of chemicals in food. The NOAEL/LOAEL forms the reference point for

deriving a health-based guidance values such as acceptable daily intakes (ADI) for food additives and pesticide residues, and tolerable daily intake (TDI) or tolerable weekly intakes (TWI) for contaminants. However, this approach does not fully utilize all the available information and critically dependent on the choice of dose intervals made, the number of subjects at the selected doses and the sensitivity of the methods to detect the adverse effect (EFSA, 2009). In order to better characterize and quantify the potential risks, the benchmark dose (BMD) approach arose as an alternative way to define a reference point in recent years.

Benchmark dose approach involves a mathematical model fitting and it uses the information in the complete dataset instead of making pair wise comparisons using subsets of the data (EFSA, 2009). Thus, the BMD approach can interpolate between applied doses, while the NOAEL approach is restricted to these doses. Key concepts in the benchmark dose (BMD) approach are shown in Figure 2.5. The BMD is a dose that corresponds to a low but measureable change in response, denoted the benchmark response (BMR). The one-sided lower 95% confidence limit of the BMD (BMDL) is normally used as the reference point due to the BMDL accounts for the greater uncertainty of a study (Muri et al., 2009). The BMD approach is applicable to all chemical in food, regardless of their category or origin, such as pesticides, additives or contaminants (EFSA, 2009). Advantages of BMD approach over NOAEL/LOAEL have been well documented (Edler et al., 2002, Jenks et al., 2004; IPCS, 2009). Both the EFSA (2005) and the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2006) have proposed the application of BMDL as the reference point for calculation of margin of exposure for substances that are both genotoxic and carcinogenic. Due to its strong points and the available of BMD

software, BMD approach is gaining more acceptances and is being used in the area of food risk assessment in these last few years.

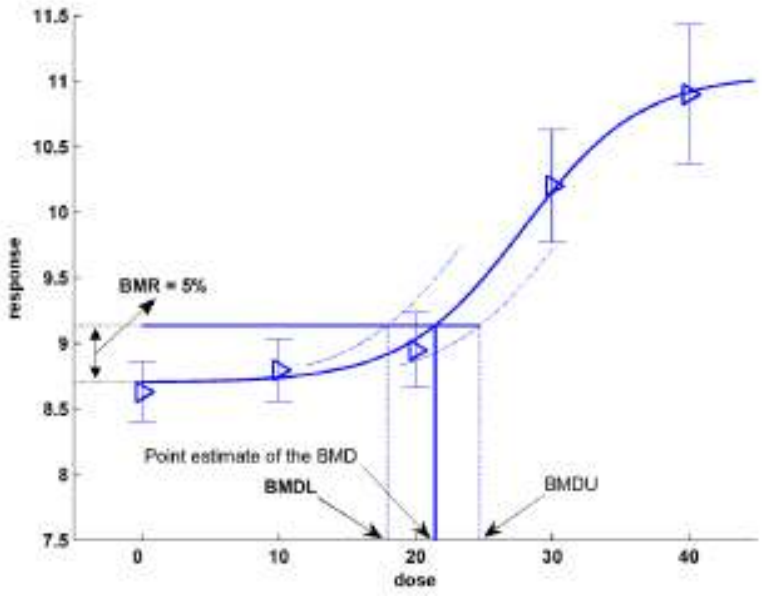

Figure 2.5 Key concepts in the benchmark dose (BMD) approach. The solid curve represents a fitted dose-response model; the triangles are observed mean responses; the dashed curves represent the one sided upper and lower 95% confidence bounds for the effect size as a function of dose. (EFSA, 2009, Figure 1, pg 10)

2.2.3 Exposure assessment

Exposure assessment is defined as "the qualitative and/or quantitative evaluation of the likely intake of biological, chemical or physical agents via food, as well as exposures from other sources if relevant" (FAO/WHO, 2006). The role of dietary exposure assessment has been widely used in performing risk assessments on chemicals (food additives, contaminants, processing aids, nutrients and residues of pesticides and veterinary drugs) in foods. Dietary exposure assessment combines food consumption data with data on the concentration of chemicals in food. Food consumption data can be estimated through food consumption surveys at an individual or household level or approximated through food production statistics or

international databases such as GEMS/Food International databases. Food consumption surveys include records/diaries, food frequency questionnaires, dietary recall and others.

2.2.3.1 Methods for food consumption data collection

The selection of which method to adopt will depend on the objectives of the assessment, costs and resources available. There are two general approaches to obtain information on the dietary habits of a population or of individuals: (a) A collection of inferred data on the movement and disappearance of foodstuffs in a region or home; and (b) A collection of direct personal data on the actual amounts of food consumed by an individual or household (WHO, 1985). In principle, there are four different types of data can be used to assess food consumption, i.e., food supply data, household consumption surveys data, dietary surveys among individuals data and the collection of duplicate diets (Kroes et al., 2002). Below are methods which have been used to generate data on the patterns of food consumption which are typical of a population group or selected individuals within a population:

A. Food diary, weighed intake

Food diary method requires the individual to write down in a diary the type and amounts of foods consumed over a period of time. Records of 24-hour duration have been undertaken in many studies but a record of intake over 3 – 7 days is preferred if a 24-hour record is felt insufficient to reflect the average consumption of foods within that population group or the sample size is not large enough to truly represent a population group (Lindsay, 1986).

The weighed food intake method is similar to the food diary approach but requires that the foods be weighed with the provided food scale prior to consumption. The higher respondent burden and cost as well as a lower response rate always limit its application to small population groups.

B. Duplicate portion

This method requires an individual to organize the purchasing and preparation of two portions of food that are usually consumed. The duplicate is weighed and stored for subsequent investigations or analysis. This study may be part of the food diary and weighed intakes studies. This technique is only suitable for small surveys and is often applied in the determination of exposure of well-defined at-risk groups (Kroes at al., 2002).

C. Dietary recall

In this method, individuals are asked to recollect the types and amounts of food they consumed at some time in the past. Due to the inaccuracy of attempting to recall food consumption, a 24-hour dietary recall has always been used. This method is also the one with the least subject burden and usually has a good response rate (WHO, 1985).

D. Food frequency

Food frequency method is used to obtain a reflection of the usual patterns of consumption for individual types of food. This approach is particularly useful in the collection of retrospective data on habitual food intakes of population-based epidemiological studies (Lindsay, 1986). Data on average serving size for certain

food is obtained on the basis of the number of times per day, week or month that each food is consumed and amount of intake expressed in standardized portion sizes (WHO, 1985). Food frequency method may also be applied to study the consumption of specific food that is known or likely to be contaminated. This method requires minimal effort from the respondent, cheap and easy to administer. It is useful to evaluate mean population intake or to categorize intake of individuals into groups of low, medium or high based on their food consumption (Kubena, 2000; Kelemen et al., 2003). Food frequency study has been used to assess national food consumption surveys by Malaysia (Jamal Khair et al., 2006) and other countries such as Singapore (Health Promotion Board, 2004), Vietnam (Khoi et al., 2002) and New Zealand (Quigley and Watts, 1997). This method also has been applied in this study because it fits the purpose of the study, cheap and has low respondent burden.

E. Food disappearance methods

Food disappearance method can be done on household or national basis. For household disappearance approach, the amount of food that disappear from a home kitchen in a given time period (usually one week) is recorded and divided by the number of people in the family to estimate the intake per person per day. More accurate estimation of food consumption data are obtained if corrections are made for food waste, food consumed away from home or guests and inedible matter (Lindsay, 1986).

In national food disappearance method, data on national food availability can be estimated from food balance sheets. National food balance is the sum of food production, food imported and food taken from stocks, subtract from food exported, added to stocks, used for sowing, used for non-edible purposes or animal feed and

food loss from harvest to kitchen (WHO, 1985). The data represents the annual average national food consumption but they do not account for seasonal variations or for the distribution of food within the population of a country (Lindsay, 1986). The application of this method is very limited and only be considered if other approaches are not feasible. Food balance sheets are available for many countries (FAOSTAT, 2010).

2.2.3.2 Estimating of dietary exposure

There are three approaches for estimating dietary exposure of a contaminant, based on the food consumption data obtained by one or more methods described in previous section (Section 2.2.3.1 A – E). The three approaches are total diet (market basket) studies, selective studies of individual foodstuffs and duplicate portion studies.

The market basket approach is based on the dietary intake of a defined population group. All food items, which are part of the average diet, are aggregated into several food groups, purchased and prepared according to standard household procedures (Kroes et al., 2002). Each food group is analysed for the constituents of interest. In the individual foodstuffs approach, a list of foods representing the products most commonly consumed is composed based on national food consumption surveys for several age-sex groups. For the duplicate portion studies, the individual daily diet as consumed is analysed (Kroes et al., 2002). Results from these studies are subsequently used to calculate an approximate dietary intake for a contaminant in composites or individual foods, by multiplying the level of contaminant for a given composite by the weight of that composite consumed. The

level of a contaminant, in this research, was the concentration of aflatoxins, is discussed in detail in Chapter 2.3.

2.2.3.3 Biomarkers of exposure

Biomarker-based methodologies may be employed to measure human exposure to food contaminants. Use of biomarkers in epidemiological study design combines the tools of standard epidemiology (such as case histories, questionnaires and monitoring of exposure) with the sensitive laboratory analysis of molecular biology (Perera, 1987). It has the advantage of being directly relevant to human risk. It also has the potential to identify the hazard, to give early warning by signalling the early effects of exposure and increased susceptibility, thus allowing opportunities to arrest disease through timely intervention (Perera, 1996). Furthermore, biomarkers can provide a useful bridge between hazard characterization and exposure assessment in cases where other components present in the food matrix affect the bioavailability and hence the systemic dose of the substance under consideration. For example a faster absorption of vitamin A when given as a supplement than its absorption from liver (Buss et al., 1994). According to Hulka et al. (1990), biomarkers can be categorized into internal dose, biologically effective dose, early biological effects and susceptibility.

In most cases the biomarker is either the food contaminant itself or a metabolite. The selected body fluid is frequently urine or blood but other options like saliva, breast milk, faeces and adipose tissue also exist. Biomarker methodology does not have universal applicability as a means of assessing human exposure to food contaminants. One of the most important determining factors is inter-individual variability in the pharmacokinetic and metabolic behaviour of the food contaminant