

**การศึกษาการเตรียมตัวอย่างมาตรฐาน
สำหรับการวิเคราะห์หาอฟลาทอกซิน ปี 1**
**Study of In-house Reference Material Preparation
for Aflatoxin B₁ Analysis**

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บทคัดย่อ

ได้ทำการศึกษาการเตรียมตัวอย่างมาตรฐาน (In-house Reference Material) จากการเติมสารมาตรฐานอฟลาทอกซิน บี 1 ลงในตัวอย่างข้าวโพดที่ปราศจากสารดังกล่าวที่ระดับ 20 ไมโครกรัมต่อกิโลกรัม โดยวิธีแห้งและวิธีเปียก วิธีแห้งทำโดยการเติมสารมาตรฐานอฟลาทอกซินลงในตัวอย่างข้าวโพดป่นแห้งมี 2 เทคนิค คือ 1. เทคนิคการเติมแบบเลขคณิต (arithmetic dilution) 2. เทคนิคการเติมแบบเรขาคณิต (geometric dilution) วิธีเปียกทำโดยการเติมสารมาตรฐานอฟลาทอกซินลงในตัวอย่างข้าวโพดป่นที่แช่อยู่ในตัวทำละลายซึ่งมี 2 ชนิดคือ 1. เฮกเซน (hexane immersion) 2. คลอโรฟอร์ม (chloroform immersion) จากนั้นทำการสุ่มตัวอย่างจากการเตรียมทั้ง 4 วิธีมาวิเคราะห์หาปริมาณอฟลาทอกซินตามวิธีมาตรฐานของกองวิเคราะห์อาหาร กรมวิทยาศาสตร์การแพทย์ ฉบับที่ 100602002 ซึ่งดัดแปลงจากวิธีวิเคราะห์มาตรฐาน AOAC เพื่อดูความสม่ำเสมอในการกระจายตัวของสารดังกล่าวในตัวอย่างที่เตรียม

ค่าเฉลี่ยและส่วนเบี่ยงเบนมาตรฐานของปริมาณอฟลาทอกซินจากการวิเคราะห์ตัวอย่างที่เตรียมโดยวิธี arithmetic dilution, geometric dilution, hexane immersion และ chloroform immersion คือ 19.12 และ 1.45, 19.22 และ 1.23, 18.21 และ 1.80 และ 19.77 และ 0.97 ตามลำดับ ผลการวิเคราะห์แสดงให้เห็นว่าการเตรียมตัวอย่างมาตรฐานโดยวิธี chloroform immersion เป็นวิธีที่ดีที่สุด เนื่องจากวิธีดังกล่าวหาปริมาณอฟลาทอกซินจากตัวอย่างที่เตรียมได้มากที่สุด และมีการกระจายตัวของอฟลาทอกซินในตัวอย่างสม่ำเสมอ

ABSTRACT

A study of the homogeneity characteristic of aflatoxin B₁ (AFB₁) in corn as a reference material was accomplished in this project. Aflatoxin free corn sample was spiked with standard AFB₁ solution at the level of 20 parts per billion (ppb) by dry and wet method. The dry method was performed by spiking standard AFB₁ solution into dry granulated corn sample using two ways of mixing as arithmetic dilution (AD) and geometric dilution (GD). The wet method was done by spiking standard AFB₁ solution into organic solvent suspended corn sample using two kinds of solvent as hexane immersion (HI) and chloroform immersion (CI). Aliquot portions of spiked sample prepared by means of AD, GD, HI and CI were quantitatively AFB₁ analysed according to standard operating procedure No. 100602002 which was modified from AOAC official methods of analysis (1990) No. 968.22 "Aflatoxins in peanuts and peanut products".

The mean ($\bar{X}$) and standard deviation (SD) of AFB₁ content in spiked sample from AD, GD, HI and CI were 19.12 & 1.45, 19.22 & 1.23, 18.21 & 1.80 and 19.77 & 0.97, respectively. The result showed that CI was the best way to prepare an in-house reference material for AFB₁ analysis, since it gave maximum amount of AFB₁ with the smallest deviation.

Key words : Aflatoxin, Reference Material, Homogeneity .

Introduction

It has long been recognized that foods contaminated with moulds on consumption may cause serious health problems which could even end in fatalities. Mycotoxins are natural products produced by certain moulds which grow on agricultural commodities under certain conditions. The mycotoxins have been demonstrated to induce adverse effects in laboratory and farm animal. A variety of tissues and organs such as liver, kidney, nervous and gastro-intestinal system can be affected depending on mycotoxin. They may also weaken the immune system of the body. Some of them like aflatoxins have been shown to be mutagenic, carcinogenic and teratogenic. Mycotoxins have also been recorded to be responsible for imbalances in feed-live weight conversion ratio in farm animals and lowering their productivity. Although many toxic mould metabolites have been isolated

from laboratory cultures of moulds that occur on agricultural products, so far only a few of them have been found to occur naturally in foods ingredients. Aflatoxin is probably the most studied and widely known mycotoxin produced by *Aspergillus flavus* and *A. parasiticus*.

Aflatoxin is a potent carcinogenic agent classified as group I "Carcinogenic to human" from International Agency for Research on Cancer. ⁽¹⁾ The term aflatoxin is used to refer to a class of chemical compounds related structure among which aflatoxin B₁ is the most potent. The high risk commodities for aflatoxin are cereals like corn, oil seeds like groundnut. Aflatoxin became the focus of intense investigation when Turkey X disease occurred in turkey poult during the 1960. ⁽²⁾ The disease was characterized by acute hepatic necrosis with bile duct hyperplasia and acute signs of loss of appetite, lethargy, wing weakness and an

unusual attitude of the head and neck at the time of death.⁽³⁾ Aflatoxin may not be responsible for all facets of these toxic syndromes, but aflatoxin is responsible for at least the hepatic lesions and the high mortality. Aflatoxin may induce hepatocarcinomas in rat, duck, trout and possibly also in guinea pig, monkey and it would seem reasonable to assume that man is also susceptible. When pigs are rations contaminated with aflatoxin B₁, hepatic cell vacuolation in the periportal areas, enlargement of hepatic cell nuclei, and bile duct proliferation were evident.⁽⁴⁾ An LD₅₀ of 1.0–1.4 mg/kg body weight was determined for a single i.p. dose administered to guinea pigs.^(5,6) Deaths occurred in various species of monkeys at different doses of aflatoxin B₁ under different dosing regimes. A single, oral dose of aflatoxin B₁ at either 13.5 or 40.5 mg/kg body weight killed all four female macaques in each group within 6 1/2 days.⁽⁷⁾ In another study on Rhesus monkeys, extensive haemorrhagic necrosis occurred in the liver at high doses, 1 mg/kg body weight, p.o. daily for 3 weeks.⁽⁸⁾ Epidemiological studies have shown an association between dietary aflatoxin exposure and the high incidence of primary liver carcinoma in Philippines, Thailand, Indonesia, Kenya, Swaziland etc. Suspected cases of acute aflatoxicoses in humans have been reported from several Asian and African countries. The disease affected people in more than 200 villages in Western India, who consumed badly moulded corn that was contaminated with aflatoxin between 6.25 and 15.6 mg/kg of corn. It was characterized by jaundice, rapidly developing ascites,

portal hypertension, and a high mortality rate, with death usually resulting from massive haemorrhages in the gastrointestinal tract.^(9,10) A similar disease affected three children in Uganda whose diet consisted mainly of cassava, beans, fish and meat.⁽¹¹⁾ In Thailand, children were associated with acute aflatoxin consumption characterized by encephalopathy and fatty degeneration of viscera in Northeastern Thailand.^(12–14)

Regulatory control measures on mycotoxins specially aflatoxin had been initiated by several countries in order to protect the public health and to promote trade at national and international levels. These standards include limits or tolerance levels, sampling and analytical methodologies. There is a lack of uniformity in the limits fixed in various countries and international harmonization efforts are under progress. Some of the countries are fixing the limits based on economic compulsions, others on analytical capabilities to detect minimum quantities. Often criticism is levelled against such practices since they do not take into account the possible errors in sampling and analyses variabilities.

Analytical results must therefore be accompanied by an explicit quantitative statement of uncertainty. If this requirement can not be fulfilled, there are strong grounds for questioning whether analysis should be undertaken at all. Quality assurance is concerned with achieving appropriate levels in matters such as staff training and management, adequacy of laboratory environment, safety, the storage, integrity and identity of samples,

record keeping, the maintenance and calibration of instruments and the use of properly documented methods. A number of factors contribute to the production of analytical data of adequate quality. It is recognized that the quality of sampling procedure determines to a large extent the quality of the measurement produced. In nature, aflatoxin contaminated commodity is not spread all over the whole lot of sample. This study designed to establish the performance characteristics, especially the practical approaches to quality control that no inappropriate interpretation has arisen during the analytical measurement process and effected the results. In laboratory, aflatoxin may heterogenously distributed when prepared spiked sample. In order to make sure that aflatoxin is uniformly dispersed in the whole lot of the spiked sample, aflatoxin analysis has to be performed in the sample that is a truly representative of the whole lot as possible. This project studied the appropriate way to prepare aflatoxin B₁ spiked corn sample as an in-house reference material and the homogeneity characteristic of aflatoxin B₁ content in the sample.

Materials and methods

Instrument, chemical, reagent and glassware

1. balance (Shimadzu Libror ED-2000)
2. grinding machine (Wiley Mill RK₁-4525A)
3. shaking machine
4. Chromatographic tube (1.5 x 40.0 cm)

with stopcock

5. rotary evaporator (Buchi Rotavapor-R)
6. heating block with nitrogen gas supply

(Pierce Reacti-Therm)

7. hot air oven (Memmert)
8. desiccator
9. sieve No. 20 mesh (Endecotts)
10. thin layer chromatography apparatus :
TLC plate 20.0x20.0 cm Merck Art. 5721 Silica gel 60 without fluorescent indicator (activate it by drying 1 hour at 120 °C and keep in desiccator), TLC sample application (Camag Linomat IV), developing tank, UV cabinet
11. densitometer with fluorometry attachment (Shimadzu CS-9301PC)
12. ultrasonic cleaner (Branson 220)
13. automatic pipet (Gilson pipetman P20, P200, P1000)
14. Merck Art 7737 Silica gel 60 (70-230 mesh) : activate it by drying 1 hour at 105 °C. Cool and then add 1% H₂O, mix and store overnight in airtight container.
15. anhydrous sodium sulfate : dry for 6 hours at 700 °C and store in airtight container.
16. cotton defatted with hexane.
17. celite 545
18. all kinds of reagent such as hexane, chloroform, diethylether anhydrous, benzene and acetonitrile are AR grade.
19. all kinds of glassware such as Erlenmeyer flask, beaker, round bottom flask and cylinder are acid, basic and heat resistant equivalent to pyrex glass.

Preparation of sample

Freshly harvested corn (November 1994)

from control field under Field Crop Research Institute was sundrying and mechanical drying as quick as possible. Immatured and damaged corn were selected and discarded. The remainder was processed to obtain good quality grain with moisture content 10% and stored in two layer plastic bag under nitrogen atmosphere.

The grain (ca 40 kg) was grinded with grinding machine (Wiley Mill RK1-4525A) into granules. The granules were sifted with sieve No. 20 mesh (Endecotts) to get homogeneous granular material. Aliquot portions of 50 g granules were sampling from the whole lot and were analysed to make sure that the whole lot contained no aflatoxin. The homogeneous granular material was divided into small portions (ca 5 kg) and stored under nitrogen atmosphere.

Preparation of standard aflatoxin B₁

Standard AFB₁ was purchased from Makor Chemicals Ltd. Stock standard solution was prepared by dissolving standard crystal AFB₁ with benzene : acetonitrile (98:2) to get approximate concentration at 10 µg/ml. Recorded UV spectrum of the stock standard solution from 330 to 370 nm. Then it was measured absorbance at $\lambda_{\max}$ 348–349 nm and calculated accurate concentration by the following equation.

$$\text{conc } (\mu\text{g/ml}) = \frac{A \times \text{MW} \times 1000 \times \text{cf}}{\epsilon}$$

where A = absorbance of AFB₁ at $\lambda_{\max}$ 348–349 nm

MW = molecular weight of AFB₁ (312)

ϵ = molar absorptivities of AFB₁ (19800)

cf = correction factor of UV spectrophotometer (1.0107 for Shimadzu UV-160 A UV-visible recording spectrophotometer)

Stock standard solution showed absorbance 0.614 at λ_{349} , thus the accurate concentration of stock standard AFB₁ solution was calculated as 9.7786 µg/ml. Working standard solution was prepared by diluting stock standard solution (1.28 ml) with benzene : acetonitrile [98:2] (25 ml) to get 0.5 µg/ml.

Preparation of spiked sample

The homogeneous granular corn was spiked with stock standard AFB₁ solution to get 20 ppb by dry and wet method as shown in the following.

1. Dry method Aliquot portion (5 kg) of the homogeneous granular corn was added with 10.2264 ml stock standard AFB₁ solution. There were many ways of adding standard to the sample. In this research, only two ways which were general techniques in manufacturing preparation of powders were studied.

1.1 Arithmetic dilution (AD) The homogeneous granular corn was accurately divided into 10 x 500 g portions. The weighed samples were transferred from the balance to the individual plastic bags. Each bag was added with 1.0226 ml stock standard AFB₁ solution. After completely mixing, the granules from the individual

bag was combined together in the big size plastic bag enough for containing 5 kg granular corn and 3 times space of the air above the granules for mixing efficiently. The pool granules mixed thoroughly and passed through a No. 20 sieve to get spiked sample. The spiked sample was checked homogeneity by sampling 3 different parts from top, middle and bottom of the whole lot. Those samples were duplicate extraction and triplicate AFB₁ determination. The mean ($\bar{X}$), standard deviation (SD) and relative standard deviation (RSD) were calculated. Afterthat, the remainder of the spiked sample was divided into 300 g portions and put on numbers sequentially. Each portion was packed under nitrogen atmosphere in tightly closed plastic bag. The divided spiked sample was checked homogeneity as mentioned above by sampling 3 different portions of No. 1, 5 and 10.

1.2 Geometric dilution (GD) The homogeneous granular corn was divided into portions according to mathematical series. The first portion (100 g) was added with 10.2264 ml stock standard AFB₁ solution. After completely mixing, the second portion (100 g) was incorporated into the first portion by blending and combining both portions with spatula little by little until completely mixing. Then the third portion (equal weight to the combination of the first and second portion, 200 g) was incorporated into the previous mixing portion. The incorporation was proceeded by combining the same weight between the previous mixing portion and the next portion until the total mixing weight of 5 kg was obtained. The 5 kg

sample was passed through a No. 20 sieve to get spiked sample and it was checked homogeneity as mentioned in AD.

2. Wet method Aliquot portion (5 kg) of the homogeneous granular corn was suspended in the mixture of 10.2264 ml stock standard AFB₁ and 5 l organic solvent. Then the organic solvent was evaporated, and the suspended material was dried to get spiked sample. The organic solvent used in this research was hexane (AFB₁ insoluble) and chloroform (AFB₁ soluble).

2.1 Hexane immersion (HI) The mixture of standard AFB₁ and hexane was prepared in the glass container by mixing 10.2264 ml stock standard AFB₁ solution with 5l hexane. Slowly added 5 kg of the homogeneous granular corn to the mixture and stirred gently until the granular corn suspended thoroughly. Closed the container and let the corn immersion stand overnight at room temperature. Afterthat hexane supernatant was sucked through guaze filter into round bottom flask of a rotary evaporator. It was evaporated under reduced pressure at temperature 50°C continuously until there was no more hexane left in the suspended granular corn. The residual oil in the round bottom flask was AFB₁ analysed to make sure before discard that it contained no AFB₁. The damp granules were placed on trays and dried in a fume hood sufficiently to get rid of hexane vapor. Then they were dried at a temperature of 45°C in the hot air oven until they ceased to lose weight. The completely dried granules were sifted through a No. 20 sieve to get spiked sample. The

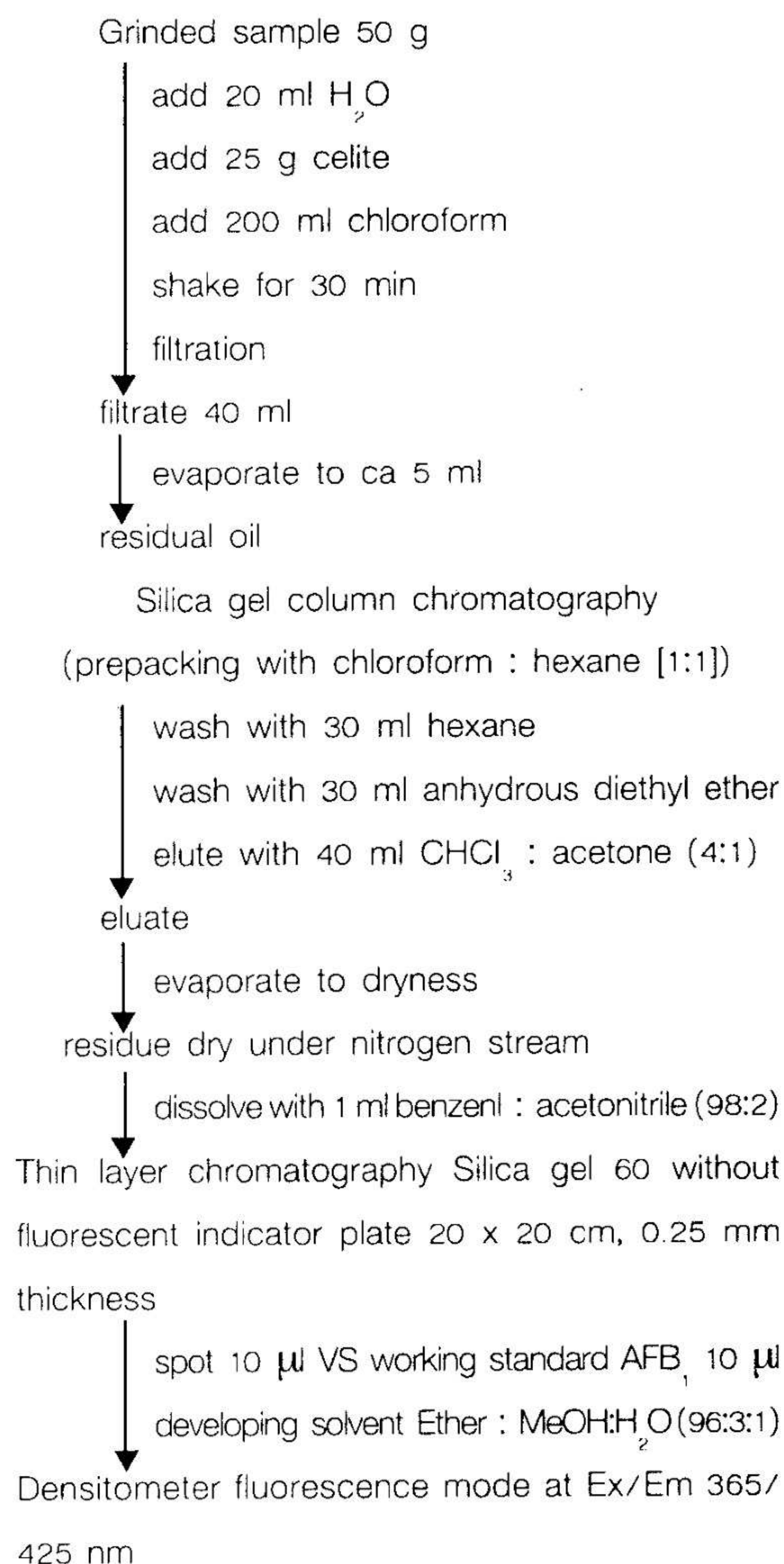
spiked sample was checked homogeneity using the same procedure as mentioned in AD.

2.2 Chloroform immersion (CI) The preparation procedure was the same as HI except using chloroform instead of hexane. The residual oil (ca 130 ml) in the round bottom flask from evaporator containing AFB₁ was transferred to separatory funnel (1 l). Added 150 ml hexane and partition extracted with 200 ml MeOH : H₂O (6 : 4) for 3 times. The hexane layer was AFB₁ analysed to make sure before discard that it contained no AFB₁. The aqueous layer was partition extracted with 200 ml chloroform for 3 times. Then the upper layer was discarded since it contained no AFB₁. The chloroform layer was sedimented in the beaker (1 l) and the supernatant was evaporated with rotary evaporator until it almost dried. The precipitate in the beaker was treated to dryness in the fume hood and hot air oven. Then the dried precipitate and the dried supernatant were mixed together. After mixing, it was incorporated with the dried suspended granules from chloroform immersion which was processed as mentioned in HI. The incorporated granules were sifted through a No. 20 sieve to get spiked sample. The spiked sample was checked homogeneity by using the same procedure as mentioned in AD.

Determination of aflatoxin B₁

The analytical method for aflatoxin was carried out under the Division of Food Analysis standard operating procedure (SOP) No. 100602002

as shown in Scheme 1. The concentration of AFB₁ was determined by comparing fluorescent intensity of sample and working standard AFB₁ from densitometer at Ex/Em 365/425 nm as shown in the following equation.



Scheme 1 Determination of aflatoxin B₁ in sample

$$AFB_1 \text{ (pph)} = \frac{A_x \times C \times D}{A_s \times S \times 10}$$

where A_x = peak area of sample

A_s = average peak area of standard
AFB₁ per 1 μ l

S = volume (μ l) of the spotted
sample giving fluorescent
intensity close to that of standard
AFB₁

C = concentration of standard AFB₁
(μ g/ml)

D = μ l dissolving solvent

10 = gram of sample applied to Silica
gel column

Where $\bar{X}$ = mean

X_i = amount of AFB₁ (ppb)

N = number of AFB₁ analyses

SD = standard deviation

RSD = relative standard deviation

Results

Small scale preparation aflatoxin B₁ (AFB₁) spiked corn samples using four different procedures were achieved in this study. All procedures were practical in the laboratory with little degree of difference. According to the analyses of samples taken before division, the highest mean value ($\bar{X}$) was detected from geometric dilution (GD) and the lowest value was seen from hexane immersion (HI). The differences of $\bar{X}$ among four procedures were as follows GD > CI > AD > HI. However, the standard deviation (SD) of GD was not the lowest. The analysis of SD of those samples showed that there were highly significant differences among procedures. The highest standard deviation (SD) was detected from hexane immersion (HI) and the lowest value was seen from chloroform immersion (CI). The differences of SD among four procedures were as follows HI > AD > GD > CI. When analysed with the relative standard deviation (RSD), CI showed the lowest value while HI showed the highest value. It meant that CI had the smallest significant differences among analyses. On the other hand, AFB₁ was uniformly dispersed in the spiked sample prepared by means of CI. The summary of $\bar{X}$, SD and RSD was shown in Table 1.

Statistic demonstration for homogeneity

The homogeneity characteristic of AFB₁ in spiked corn prepared by means of AD, GD, HI and CI was interpreted by analyses of samples taken before and after division of the whole lot using the following equation.

$$1. \quad \bar{X} = \frac{\sum_{i=1}^n x_i}{N}$$

$$2. \quad SD = \sqrt{\frac{\sum_{i=1}^n (X - \bar{X})^2}{N-1}}$$

$$3. \quad RSD = \frac{SD \times 100}{\bar{X}}$$

Table 1 Amount of aflatoxin B₁ (ppb) sampling from top (No.1), middle (No.2) and bottom (No.3) of the spiked corn prepared by AD, GD, HI and CI (before division)

No	Amount of aflatoxin B ₁ (ppb)			
	AD	GD	HI	CI
1-1-1	18.6	18.1	20.2	19.3
1-1-2	18.0	18.7	18.6	19.7
1-1-3	19.6	19.9	21.8	18.6
1-2-1	20.5	18.8	20.1	21.1
1-2-2	21.0	20.8	19.8	20.3
1-2-3	20.1	18.3	20.4	20.8
2-1-1	20.6	17.6	17.9	19.5
2-1-2	22.3	17.8	17.0	20.1
2-1-3	23.0	18.6	17.7	20.7
2-2-1	19.1	21.3	15.2	18.6
2-2-2	19.2	21.1	16.2	20.4
2-2-3	19.2	21.4	15.4	20.6
3-1-1	16.8	20.3	19.6	20.4
3-1-2	17.3	20.3	19.1	17.7
3-1-3	19.5	20.3	20.0	19.7
3-2-1	19.0	21.5	17.2	18.8
3-2-2	19.6	21.3	17.0	19.0
3-2-3	20.4	18.7	17.6	18.7
$\bar{X}$	19.66	19.71	18.38	19.67
SD	1.55	1.37	1.86	0.95
%RSD	7.88	6.95	10.12	4.83

As to comparison among four procedures of the samples taken after division, the highest mean value ($\bar{X}$) was detected from chloroform immersion (CI) and the lowest value was seen from hexane immersion (HI). The differences were as

follows CI > GD > AD > HI. While the differences of SD were as follows HI > AD > CI > GD and the lowest RSD was GD. The summary of $\bar{X}$, SD and RSD was shown in Table 2.

Table 2 Amount of aflatoxin B₁ (ppb) sampling from No. 1, No.5 and No.10 of the spiked corn prepared by AD, GD, HI and CI (After division)

No	Amount of aflatoxin B ₁ (ppb)			
	AD	GD	HI	CI
1-1-1	19.4	19.4	17.4	20.0
1-1-2	20.9	19.2	20.8	19.4
1-1-3	20.7	17.6	16.4	20.1
1-2-1	19.6	18.4	18.5	21.2
1-2-2	17.5	19.6	20.0	21.5
1-2-3	18.8	19.4	20.2	19.9
5-1-1	17.4	16.9	18.8	18.2
5-1-2	16.9	17.2	19.7	14.6*
5-1-3	18.7	18.7	18.8	19.8
5-2-1	17.7	18.5	17.7	19.2
5-2-2	18.0	19.2	17.6	19.0
5-2-3	18.8	19.4	18.8	18.1
10-1-1	19.3	19.6	14.1	19.0
10-1-2	18.8	19.4	14.7	19.9
10-1-3	17.3	19.6	16.6	20.7
10-2-1	19.4	18.0	17.8	21.1
10-2-2	17.7	18.2	18.3	19.7
10-2-3	17.7	18.8	18.4	21.1
$\bar{X}$	18.59	18.73	18.03	19.88
SD	1.15	0.86	1.77	1.01
%RSD	6.19	4.59	9.82	5.08

* error during experiment

Since CI gave the best result of the homogeneity for the samples taken before division and GD was the best for the samples taken after division. The condition revealed that care must be exercised to achieve a situation where the final samples for analyses can be as truly representative of the whole lot as possible. Thus, the total analyses of the whole samples

(combined samples together between before and after division) were considered. The analysis of $\bar{X}$ for the total 36 analyses showed that the highest $\bar{X}$ was detected from CI and the lowest value was seen from HI. The highest SD and RSD values were detected from HI and the lowest from CI. The summary of $\bar{X}$, SD and RSD was shown in Table 3.

Table 3 Summary of $\bar{X}$, SD and RSD from before division, after division and aflatoxin B₁ analyses of the spiked corn prepared by AD, GD, HI and CI

	Before division			After division			Total analyses		
	$\bar{X}$	SD	RSD	$\bar{X}$	SD	RSD	$\bar{X}$	SD	RSD
AD	19.66	1.55	7.88	18.59	1.15	6.19	19.12	1.45	7.58
GD	19.71	1.37	6.95	18.73	0.86	4.59	19.22	1.23	6.40
HI	18.38	1.86	10.12	18.03	1.77	9.82	18.21	1.80	9.88
CI	19.67	0.95	4.83	19.88	1.01	5.08	19.77	0.97	4.91

Table 3 gave interpretation that there was the smallest loss (1.15%) of AFB₁ during the preparation of spiked corn using chloroform immersion technique since the mean value analysis of AFB₁ was 19.77 ppb and the spiked amount of AFB₁ was 20 ppb. And AFB₁ was distributed uniformly in the sample since SD and RSD values were the smallest. On the other hand, CI was the best technique to prepare spiked corn sample as a reference material for aflatoxin analysis. While there was the highest loss (8.95%) of AFB₁ in hexane immersion technique, the SD and RSD values were

also the highest among the procedures. Thus, hexane immersion technique was not good at the preparation of spiked corn sample as a reference material. Because there was no homogeneity characteristic of AFB₁ content in the sample.

There were not much differences of $\bar{X}$, SD and RSD values between AD and GD. Both of them could be considered as the appropriate procedure to prepare spiked corn sample. And most of analysts acquainted with them in order to prepare small scale spiked sample for recovery analysis of the analytical method. Although they

were simple and easy technique to prepare spiked sample, they were not as good as CI for the homogeneous content of AFB₁ in the spiked sample. The amount and frequency of standard AFB₁ addition to the sample during the preparation made a little degree of differences in $\bar{X}$, SD and RSD values between AD and GD. The mean value of AD when compared to GD was a little bit lower, while SD and RSD values were a little bit higher as shown in Table 3. Since small amount of standard AFB₁ was added to aliquot portions of sample for 10 times during the spiked sample preparation by AD technique. While big amount of standard AFB₁ was added only one time during GD preparation of spiked sample. The error of standard measurement and standard addition might made AD disadvantage to GD.

Discussion

Methods of determining amount of aflatoxin in corn include TLC, HPLC, HPTLC and fluorometric iodine method.⁽¹⁵⁾ The CB method was approved for corn in official first action 1972 and final action 1988 by the AOAC and the AACC.⁽¹⁶⁾ The method used in this research was the Department of Medical Sciences, Division of Food Analysis standard operating procedure No. 100602002 which was modified from CB method mentioned above. The modification changes were as follows : amount of H₂O and organic solvents such as chloroform, hexane, anhydrous diethyl ether, size of Silica gel column as well as amount

of packing material, eluting solvent and developing solvent. This method was undergone prior to this research for appropriate validation procedures that it was reliable and accurate for aflatoxin analysis. It was applied to the granular corn spiked with AFB₁ at levels of 0, 5, 10, 20, 30 and 40 ppb for assays as follows; accuracy, precision, specificity, limit of quantitation and linearity. The accuracy and precision were done by replicate of six analyses of the spiked sample at 5 and 40 ppb. Accuracy was expressed as percent recovery of 100.00% at 5 ppb and 84.58% at 40 ppb which were acceptable for concentration below 100 ppb.⁽¹⁷⁾ Precision was expressed as the relative standard deviation of 15.94% at 5 ppb and 13.62% at 40 ppb which were also acceptable according to the equation for determining acceptable method repeatability and according to quality assurance in the analysis of foods for trace constituents by Horwitz et al typical RSD of 32% for aflatoxin at a level of 10 ppb.⁽¹⁸⁾ The equation was shown as the following

$$RSD \leq 2^{(1-0.5 \log C)} \times 0.67$$

where C = concentration of analyte as decimal fraction (i.e. 0.1% = 0.001)

(The RSD calculated at 5 ppb was 23.79% and at 40 ppb was 17.4%) Specificity was received by replicate of three analyses of blank or spiked sample at 0 ppb, the analysis condition showed no interference of AFB₁. Limit of quantitation was 5 ppb which was the lowest concentration of spiked sample that can be quantitated with an

acceptable degree of precision and accuracy in this study. Linearity (system) was performed by duplicate analyses of standard AFB₁ at concentration 0.10, 0.25, 0.50, 0.75, 1.00 and 2.00 µg/ml. The peak area and the concentration (µg/ml) were in the linear range of the detector, the correlation coefficient of the straight line graph was 0.999 and the slope was 537.83. Linearity (method) was performed by duplicate analyses of the granular corn spiked with AFB₁ at levels of 5, 10, 20, 30 and 40 ppb. The peak area and the concentration (ppb) were in the linear range of the detector, the correlation coefficient of the straight line graph was 1.000 and the slope was 5.71.

The main difficulty in sampling for aflatoxin arose from the heterogeneity of toxin distribution in the spiked corn. Sampling variability arose from the variability of toxin levels in the sampling selected in the sample. And the Ministry of Public Health Food Act (1979) Notification No 98 (revision 1986) notifies that food with contaminants shall have the standard that aflatoxin may be detected at not more than 20 micrograms per 1 kilogram. Thus, we decided to spike granular corn sample at the action level of 20 micrograms per 1 kilogram or ppb.

The expected error in estimating the amount of AFB₁ in the spiked sample is directly proportional to the number of sample analyses. Although larger samples will undoubtedly increase accuracy there will be commensurate cost increases. Therefore, author decided to use the total 36 analyses for the representative of the whole lot of preparation

instead of using 18 samples taken either before or after division for unambiguous decision of the best procedure for homogeneity characteristic of AFB₁ content in sample.

All preparation procedures proposed in this study for AFB₁ analysis were practical in the laboratory with little degree of difference. Depend on time, effort, cost, equipment, analytical result, safety, facility of the laboratory and labor necessary to carry out the procedure, one can select the appropriate procedure for in house reference material preparation.

The obvious differences between dry and wet method were observed as follows. Dry method requires less time, effort, cost and equipment than wet method. Wet method needs large quantity of organic solvents which are flammable, harmful by inhalation and irritating to skin. The distribution of AFB₁ in sample prepared by dry method may not as even as with wet method. Wet method offers well-mixed process and organic solvent is used to selectively remove lipid interfering substances from sample preparation yielding partially purified sample for analysis.

Comparison of dry method by means of arithmetic dilution (AD) and geometric dilution (GD) was concluded as the following.

AD was the simplest way of preparation since equal amount of granular corn with equal amount of standard AFB₁ were mixed together in every portion. Thus the content of AFB₁ should be homogeneous in the whole lot after mixing every portion. The result also showed the homogeneity of

AFB₁ with the relative standard deviation of 7.58% from 36 sample analyses. The standard deviation was 1.45 which was quite high due to 10 times application of small volume of standard AFB₁ to 10 portions of the granular corn.

GD was more sophisticated than AD since total volume of standard was applied only one time at the begining for mixing with the first portion of granular corn. Then it was incorporated with equal amount of granular corn from the second portion. The mixing process was continued sequentially until total amount of 5 kg was obtained. GD was less error than AD since the relative standard deviation was 6.40% and the standard deviation was 1.23.

Comparison of wet method between hexane immersion (HI) and chloroform immersion (CI) was summarized as the following. The difference between HI and CI in being used as a matrix for suspending granular corn was the solubility of aflatoxin. AFB₁ is hexane insoluble while chloroform soluble. The idea of using such organic solvent was mainly to provide homogeneous dispersion of AFB₁ in the matrix and afford perfectly contact between AFB₁ and granules. The relative standard deviation and the standard deviation of HI were 9.88% and 1.80 which were not good. This may due to uneven dispersion of AFB₁ in hexane since it was insoluble. On the contrary, CI was the best procedure in preparing spiked corn sample for aflatoxin analysis since it showed homogeneity characteristic of AFB₁ content in the sample because of the smallest relative standard deviation (4.91%) and the standard

deviation (0.97).

Further investigation is to illustrate consistency in performance of method for aflatoxin analysis by sending reference material to other laboratories as a check sample program. This may be most important as to ensure that other laboratories are analytically equivalent.

Summary

The appropriate way to prepare aflatoxin B₁ spiked corn sample as an in-house reference material is chloroform immersion technique. This paper discusses the homogeneity characteristic of aflatoxin B₁ content in the sample for a complete approach to quality in sampling. It shows that four concepts and practices used in preparation of reference material can be adapted for application to sampling.

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