UNIVERSITI PUTRA MALAYSIA

SIMULTANEOUS DETERMINATION OF AFLATOXINS AND OCHRATOXIN A IN SELECTED SPICES USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTOR

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By

WAN AINIZA BINTI WAN MUSTAPHA

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirement for the Degree of Master of Science

October 2015

DEDICATION

This work is especially dedicated to my loving husband (Jailani Talib), who always given me the encouragement, support, patience, understanding and love throughout my research period.

Lots of love to my beautiful daughters (Syakirah Jaida, allahyarhamah Adriana Jaida and Karlissa Jaida). They are my guiding lights.

Also never forget my parents, a bundle of love and hugs to my mother, Raimah Baheran, also with respect and love to my late father, allahyarham Wan Mustapha Wan Taib.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia
in fulfillment of the requirement for the degree of Master of Science

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Chairman : Professor Jinap binti Selamat, PhD
Faculty : Food Science and Technology

Mycotoxins are found in a wide range of food making it possible for aflatoxins (AFs) and ochratoxin A (OTA) to occur simultaneously in spices and other commodities. Previously, immunoaffinity columns were designed to be used for single mycotoxin clean up and separate extractions was required if many mycotoxins were to be determined in a single food sample. Now, due to advances in technology, multi-mycotoxins immunoaffinity column were developed, making simultaneous determination possible. The objectives of this study were to develop a simultaneous determination method of AFs and OTA in selected spices, to validate the extraction procedure and to verify the method performance using curry mixture samples which were collected from retail markets. The simultaneous determination developed was a modification of a published method using multi-toxin immunoaffinity column and reverse-phased HPLC-FLD with photochemical derivatization system. Recoveries from spiked samples for AFB_1, AFB_2, AFG_1 and AFG_2 were ranged from 77.2% to 105.7% for coriander, chilli, cumin, curry mixture and soup mixtures; and from 63.6% to 90.1% for fennel, turmeric and kurma mixture. Recoveries for OTA ranged from 70.0% to 109.3% in coriander, chilli, cumin, fennel, turmeric and all spice mixtures. Acceptable recoveries are ranged from 70% to 110%. Relative standard deviation (RSD) for AFB_1, AFB_2, AFG_1, AFG_2 and OTA in all spices ranged from 3.18% to 10.81%. The method was validated using curry powder samples for selectivity, limit of detection (LOD), limit of quantification (LOQ), linearity and working range, accuracy (taken as recovery) and precision. The LOQ was found to be at 0.4µg/kg for AFB_1/AFG_1, 0.2µg/kg for AFB_2/AFG_2 and 0.5µg/kg for OTA respectively. The correlation coefficients of >0.99 have been obtained for all AFs and OTA. The accuracy and precision for this method were acceptable. The recoveries were between 77.7% to 88.1% for all analytes, meanwhile the precision were < 7.3% for repeatability and < 8.7% for reproducibility. This method was verified when 30 retails samples were analysed and 63.3% were found to contain AFs >LOQ, and 70.0% contained OTA >LOQ. The current results indicated potential of the extraction procedure

to be used as a simultaneous determination for official food safety program and also for research purposes.

Abstrak tesis yang dikemukan kepada Senat Univeristi Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Master Sains

PENENTUAN SERENTAK AFLATOKSIN DAN OKRATOKSIN A DALAM REMPAH TERPILIH MENGGUNAKAN KROMATOGRAFI CECAIR BERPRESTASI TINGGI DENGAN PENGESAN PENDARFLUOR

Oleh

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Mikotoksin boleh didapati dalam pelbagai jenis makanan, ini membolehkan aflatoksin (AFs) dan okratoksin A (OTA) untuk hadir serentak dalam rempah ratus dan komoditi-komoditi lain. Sebelum ini, turus *immunoaffinity* telah dibangunkan untuk pembersihan mikotoksin secara tunggal dan pengekstrakan berasingan perlu dilakukan sekiranya penentuan pelbagai jenis mikotoksin hendak dilakukan dalam satu jenis sampel makanan. Pada masa terkini, dengan kemajuan dalam teknologi, lajur *immunoaffinity* untuk pembersihan pelbagai mikotoksin telah dibangunkan, oleh itu penentuan serentak boleh dijalankan. Objektif kajian ini adalah untuk membangunkan satu kaedah penentuan serentak AFs dan OTA dalam rempah terpilih, untuk mengesahkan prosedur pengekstrakan dan untuk mengesahkan prestasi kaedah tersebut ke atas sampel campuran serbuk kari yang dikumpulkan dari pasaran runcit. Penentuan serentak yang telah dibangunkan adalah suatu pengubahsuaian dari kaedah yang diterbitkan menggunakan lajur immunoaffinity pelbagai toksin dan HPLC-FLD secara fasa terbalik, dengan sistem penerbitan secara fotokimia. Perolehan semula ke atas sampel yang telah ditambah AFB_1, AFB_2, AFG_1 dan AFG_2 adalah dari 77.2% hingga 105.7% untuk ketumbar, cili, jintan putih, campuran kari dan campuran sup; dan dari 63.6% hingga 90.1% untuk jintan manis, kunyit dan campuran kurma. Perolehan semula untuk OTA adalah dari 70.0% hingga 109.3% dalam ketumbar, cili, jintan putih, jintan manis, kunyit dan semua campuran rempah. Perolehan semula yang diterima adalah antara 70% hingga 110%. Sisihan piawai relative (RSD) untuk AFB_1, AFB_2, AFG_1, AFG_2 dan OTA dalam semua rempah telah adalah antara 3.18% hingga 10.81%. Kaedah ini telah divalidasi menggunakan sampel serbuk kari untuk kepilihan, had pengesanan (LOD), had kuantifikasi (LOQ), kelinearan, ketepatan (diambil sebagai perolehan semula) dan kejituan. Nilai LOQ diperolehi pada 0.4 µg/kg untuk AFB_1/AFG_1, 0.2 µg/kg untuk AFB_2/AFG_2 dan 0.5 µg/kg untuk OTA masing-masing. Pekali korelasi >0.990 telah diperolehi untuk semua AFs and OTA. Ketepatan dan kejituan bagi kaedah ini adalah diterima. Perolehan semula adalah antara 77.7% hingga 88.1% untuk semua

analit, manakala kejituan adalah < 7.3% for keterulangan dan < 8.7% untuk kebolehulangan semula. Kaedah ini telah diverifikasi dengan menganalisa 30 sampel pasaran 63.3% dijumpai mengandungi AFs >LOQ, dan 70.0% mengandungi OTA >LOQ. Keputusan kajian telah menunjukkan bahawa kaedah pengekstrakan ini mempunyai potensi untuk digunakan sebagai penentuan serentak untuk program rasmi keselamatan makanan dan juga untuk tujuan-tujuan penyelidikan.

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I certify that a Thesis Examination Committee has met on 6 October 2015 to conduct the final examination of Wan Ainiza binti Wan Mustapha on her thesis entitled "Simultaneous Determination of Aflatoxins and Ochratoxin a in Selected Spices using High Performance Liquid Chromatography with Fluorescence Detector" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of the Master of Science. The members of the Supervisory Committee were as follows:

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This is to confirm that:

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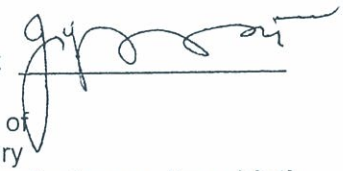
Signature: 
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LIST OF ABBREVIATIONS

µg	microgram
AFs	Aflatoxin
AFB_1	Aflatoxin B_1
AFB_2	Aflatoxin B_2
AFG_1	Aflatoxin G_1
AFG_2	Aflatoxin G_2
EC	European Commission
EU	European Union
FAO	Food and Agricultural Organisation
FLD	Fluorescent detector
FTIR	Fourier transforms infrared spectroscopy
GC	Gas Chromatography
HPLC	High Performance Liquid Chromatography
IAC	Immunoaffinity column
IARC	International Agency of Research on Cancer
ISO	International Standard Organisation
kg	kilogram
L	litre
LC MS/MS	Liquid Chromatography Tandem Mass Spectrometry
LLC	Liquid-liquid extraction
LOD	Limit of Detection
LOQ	Limit of Quantification
OTA	Ochratoxin A
mL	mililiter

MFC	Multifunctional column
MPP	Maximum permitted proportion
MS-FID	MS-flame ionization detector
ng	nanogram
PVDF	Polyvinylidene fluoride
SFE	Supercritical fluid extraction
SPE	Solid phase extraction
TFA	Trifluoroacetic acid
QuEChERS	Quick, Easy, Cheap, Effective, Rugged and Safe
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

Mycotoxins are found in a wide range of food, therefore aflatoxins (AFs) and ochratoxin A (OTA) could occurs simultaneously in spices or any other commodities. Spices are widely used in our daily cooking making it possible for the mycotoxin to accumulate in the body if the spices are contaminated. As mycotoxins are highly stable compounds, cooking at normal temperature will not cause any or cause only little degradation. AFs and OTA are classified as Group 1 and Group 2 carcinogen respectively by International Agency of Research on Cancer (IARC). AFs are known to be potent toxic, carcinogenic, mutagenic, immunosuppressive agents, produced as secondary metabolites by the fungus *Aspergillus flavus* and *Aspergillus parasiticus* on variety of food products. Major members of AFs are aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1) and aflatoxin G_2 (AFB_2). Food products contaminated with AFs include cereal (maize, sorghum, pearl millet, rice, wheat), oilseeds (groundnut, soybean, sunflower, cotton), spices (chillies, black pepper, coriander, turmeric, ginger), tree nuts (almonds, pistachio, walnuts, coconut) and milk. OTA is regarded as carcinogenic and nephrotoxic. It is mainly produced by *Aspergillus ochraceus* and *Penicillium verracusom*. Food that is susceptible to OTA contamination are cereals, spices, coffee beans, cocoa beans, wine and beer.

Spices are commonly used in Malaysia to give flavours into food and are also well known for their medicinal value. Spices are mostly produced in tropical countries and often dried up in open air that could lead to fungus growth and cause mycotoxin contaminations. Currently there is no complete baseline data on mycotoxin contamination in such commodities in Malaysia. There are reports on the occurrence of AFs and OTA in European countries and in the Middle East. Spices that will be studied are in single form (individual spice) and also in mixed form (mixture of spices). These spices are chosen because they are the most commonly used in making curry, kurma and soup mixture.

Currently, there are very limited research conducted on the simultaneous determination of AFs and OTA in spices in Malaysia and also in other countries. Most of the references obtained provide studies on single mycotoxin analysis, either on AFs or OTA only. There are some studies on simultaneous occurrence of AFs and OTA but the determination or extraction were done separately on the same samples. Most of the researches conducted in the recent years very much concentrated on black pepper, red paprika, chilli powder or ginger. There are many more types of spices to be analysed because these spices are susceptible to mycotoxin contamination. Most mycotoxin extraction methods are matrix dependant meaning that one method may only suit one type of spices. Therefore, there will be a lot of extraction procedures to conduct and it will incur time and cost.

The complexity of spices due to high pigmentation, high lipid, essential oil compounds and its inhibitory effect on mycotoxin formation will interfere during the extractions of mycotoxin. In the past, immunoaffinity columns are designed for single mycotoxin clean up and this will require separate extractions, clean-up and quantification if many mycotoxin are to be analysed in a single sample. However due to advances in technology, multi-mycotoxin immunoaffinity column are developed making simultaneous determination possible.

Due to these limitations, there is a need to develop a simultaneous determination of AFs and OTA in spices using immunoaffinity column clean-up and quantification using high performance liquid chromatography. The Malaysian Food Regulation 1985 specified that the maximum level of AFs in processed groundnuts and other food shall not be greater than 10 ug/kg, while groundnut for further processing not greater than 15 ug/kg. Currently there is no regulatory limit set for OTA in spice in Malaysia. Legal limits should therefore be set to as low as reasonably achievable as both aflatoxins and ochratoxins A are carcinogen.

The main objective of this study is to select and validate the most suitable method of extraction that could simultaneously determine AFs and OTA in a variety of spices using one single extraction procedure. To accomplish this, the study will address the following specific objectives:

i) To select the extraction methods for the simultaneous determination of AFs and OTA in spices.
ii) To validate the extraction method for the simultaneous determination of AFs and OTA in curry powder mixture, and then to verify the method performance on real samples from the retail markets.

Hypothesis or the expected outcomes are as below:

i) At least one of the extraction methods tested is able to simultaneously extract both AFs and OTA from variety of spices mixture.
ii) To be able to use this extraction method as the standard method for simultaneous determination of AFs and OTA in spices.

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