METHOD DEVELOPMENT FOR DETERMINATION OF MULTI-MYCOTOXIN IN VEGETABLE OIL USING QuEChERS TECHNIQUE AND LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY

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FSTM 2015 32

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By

SHARMILI D/O KUPPAN

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

December 2015

Dedicated to my beloved parents, husband, sons

and

rest of my family members

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

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In recent years, vegetable oils were reported to be contaminated by mycotoxins. At the same time, demand for vegetable oils has been increasing due to its health benefit and also for its direct use or as ingredients in food. The objectives of this study were to develop and validate the QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) extraction and clean-up procedures for mycotoxins in vegetable oil by liquid chromatography tandem mass spectrometry (LC-MS/MS) and to verify the established method in vegetable oils in Malaysian market. In this study, a new, fast, cost efficient, sensitive and accurate assay method was developed, optimized and validated for the determination of aflatoxins (AFB_1, AFB_2, AFG_1 and AFG_2), ochratoxin A (OTA), zearalenone (ZEA) and deoxynivalenol (DON) in vegetable oil by a modified QuEChERS approach, which was conventionally developed for pesticides analysis. A quick procedure of mycotoxins extraction and clean-up was achieved in two steps. Firstly, target analytes were extracted into the optimized solvent acetonitrile and followed by dispersive solid phase extraction (d-SPE) clean up with the optimized sorbents to remove the co-extracts. Three types of d-SPE sorbents; C18, primary secondary amine (PSA) and graphitized carbon black (GCB) and four combination ratios of two selected d-SPEs were evaluated to achieve an optimum and acceptable result. The maximum co-extracts were removed when d-SPE C18 and GCB were used at ratio 3:1 with $MgSO_4$ anhydrous followed by LC-MS/MS analysis, using an electro spray-ionization interface (ESI). Methanol as an organic phase and formic acid (0.1%) with 5 mM ammonium acetate as a polar phase, with total run of 16 minutes and flow rate 300 $\mu Lmin^{-1}$ were applied. The validation study indicated the seven mycotoxins could be detected in the concentration range of 0.01 ngg^{-1} to650.17ngg^{-1} with correlation coefficient > 0.98. The limit of quantitation (LOQ) ranges from 0.04 ngg^{-1} to 2000 ngg^{-1}. The recoveries were in the range of 87.9% to 106.6%. The repeatability and reproducibility of the analysis were within the acceptable level (0.5 – 1.3) for precision using HORRAT ratio. Significant matrix effect was compensated using

the matrix matched calibration curves. After validation, the method was successfully applied to 25 vegetable oil samples including corn oil, palm oil and sunflower oil. ZEA, AFG_2, AFG_1 and AFB_1 were among the detected mycotoxins. A new fast, cost efficient, sensitive and accurate assay method was successfully developed, validated and applied for the determination of seven mycotoxins in vegetable oils.

Abstark tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PEMBANGUNAN KAEDAH BAGI PENENTUAN MULTI-MIKOTOKSIN DALAM MINYAK SAYURAN MENGGUNAKAN TEKNIK QuEChERS DAN KROMATOGRAFI CECAIR SPEKTROMETER JISIM (LC-MS/MS)

Oleh

SHARMILI D/O KUPPAN

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Sejak kebelakangan ini, minyak sayuran dilaporkan dicemari oleh mikotoksin. Pada masa yang sama, permintaan bagi minyak sayuran telah meningkat disebabkan oleh manfaat kesihatan dan sebagai bahan dalam makanan. Objektif kajian ini adalah untuk membangunkan dan mengesahkan kaedah pengekstrakan teknik QuEChERS (*Quick, Easy, Cheap, Effective, Rugged and Safe*) dan prosedur pembersihan sampel untuk mengekstrak mikotoksin dari minyak sayuran dan menganalisis menggunakan kromatografi cecair spektrometri jisim (LC-MS/MS) serta untuk mengesahkan kaedah yang dikembangkan melalui penentuan pencemaran mikotoksin dalam minyak sayuran di pasaran. Dalam kajian ini, satu kaedah yang baru, cepat, cekap, sensitif, murah dan tepat telah dibangunkan, dioptimumkan dan disahkan bagi menentukan aflatoksin (AFB_1, AFB_2, AFG_1, AFG_2), ochratoxin A (OTA), zearalenone (ZEA) dan deoxynivalenol (DON) dalam minyak sayur-sayuran oleh pendekatan teknik QuEChERS yang diubahsuai dari analisis racun perosak. Prosedur yang cepat bagi pengekstrakan mikotoksin dan pembersihan sampel telah dicapai dalam dua langkah. Pertama, analit sasaran diekstak ke dalam pelarut asetonitril dan diikuti oleh pengekstrakan dari serakan fasa pepejal (d-SPE) untuk mendapatkan nilai kejituan mikotosin dan nilai sisa ekstrak yang optimum. Tiga jenis penjerap d-SPE; C18, *primary secondary amine* (PSA) dan *graphitized carbon black* (GCB) dan empat nisbah gabungan dua penjerap telah dinilai untuk mencapai hasil yang optimum dan boleh diterima.
Keputusan yang maksimum iaitu nilai kejituan bagi mikotoksin yang tinggi serta nilai sisa ekstrak yang minimum dicapai apabila penjerap C18 dan GCB telah digunakan pada nisbah 3: 1 dengan $MgSO_4$ kontang diikuti oleh analisis LC-MS/MS, menggunakan antara muka elektro semburan-pengionan (ESI). Metanol sebagai fasa organik dan asid formik (0.1%) dengan 5 mM ammonium asetat sebagai fasa cecair, dengan jumlah jangka masa 16 minit dan kadar aliran 300 $\mu L min^{-1}$ telah digunakan. Kajian pengesahan menunjukkan tujuh mikotoksin dapat dikesan dalam julat kepekatan terendah (*LOD*) antara 0.01 ngg^{-1} hingga 650.17 ngg^{-1} dengan pekali korelasi > 0.98. Had kuantifikasi (*LOQ*) adalah di antara 0.04

ngg^{-1} hingga 2000 ngg^{-1}. Nilai kejituan adalah dalam lingkungan 87.9% kepada 106.6%. Kebolehulangananalisis adalah dalam tahap yang boleh diterima iaitu di antara 0.5-1.3 untuk ketepatan menggunakan nisbah *HORRAT*. Kesan matriks yang ketara telah diatasi menggunakan matriks sepadan dengan keluk penentukuran. Selepas pengesahan, kaedah yang telah berjaya digunakan untuk menganalisa 25 sampel minyak sayur-sayuran termasuk minyak jagung, minyak sawit dan minyak bunga matahari. ZEA, AFG_2, AFG_1 dan AFB_1 adalah antara mikotoksin yang dikesan. Satu kaedah baru yang cepat, efektif kos, sensitif dan tepat telah dibangunkan, divalidasi dan digunapakai dengan jayanya untuk menentukan kandungan tujuh mikotoksin dalam minyak sayuran.

ACKNOWLEDGEMENTS

My very first gratitude goes to Lord Shiva for His utmost blessings in giving the opportunity and allowing me to complete my MSc thesis. Thanks for being there all the time to guide and support this humble soul.

I would like to express my deep and sincere appreciation to Prof. Dr. Jinap Selamat for her guidance, encouragement and support throughout completion of this work. Special thanks to Dr. Rashidah for her helpful suggestions and invaluable guidance.

My warmest thanks and gratitude also goes to my beloved parents and husband Gunaselan. I cannot express how much I would like to say thank you to them for their love and supporting me from day one. Not forgetting my two sons whom tolerated my absence while completing this work. Thanks for being my entertainer during my late night thesis writing. Mummy always love you my boys!

Special gratitude goes to Ministry of Health for awarding scholarship to support my studies and fulfil my ambitions. My deepest thank to Food Safety and Quality Division and National Public Health Laboratory for their supports. Also, many thanks to my friends especially Yasmin, Tan Cheng Keng, Leela and Ghanthimathi for their moral support, encouragement, motivation and love. Not forgetting my dear colleagues Faizah Puad, Rafidah Ismail, Zuliana, Rosshanida, Zurainah, Nor Azlina Shari and Junizah for their contribution and help during my research work. Special thanks to my beloved brother Jayasuthan for his contribution during the final thesis preparation. I wish to extend my heartfelt appreciations to all those soul who were involved in this project directly or indirectly. Thanks dear all.

I certify that a Thesis Examination Committee has met on 8 December 2015 to conduct the final examination of Sharmili a/p Kuppan on her thesis entitled "Method Development for Determination of Multi-Mycotoxin in Vegetable Oil Using QuEChERS Technique and Liquid Chromatography Tandem Mass Spectrometry" 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 fulfilment of the requirement for the degree of 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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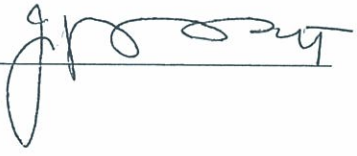
Signature: 
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LIST OF ABBREVIATIONS

AA	Acetic acid
ADI	Acceptable daily intake
AFB_1	Aflatoxin B_1
AFB_2	Aflatoxin B_2
AFG_1	Aflatoxin G_1
AFG_2	Aflatoxin G_2
AFs	Aflatoxins
AOAC	Association Officials of Analytical Chemistry
ANOVA	Analysis of variance
APCI	Atmospheric pressure chemical ionization
APPI	Atmospheric pressure photo ionization
CIT	Citrinin
CV	Coefficient of variation
C18	Octadecyl
DON	Deoxynivalenol
DSPE	Dispersive solid phase extraction
EC	European Commission
EFSA	European Food Safety Authorities
ESI	Electrospray ionization
EU	European Union
FA	Formic acid
FAO	Food and Agriculture Organization
FB	Fumonisin
FDA	Food and Drug Administration
FSA	Food Standard Agency
GC	Gas chromatography
HPLC	High peformance liquid chromatography
IAC	Immunoaffinity column
IARC	International Agency for Research on Cancer

ISO	International Organization for Standardisation
IUPAC	International Union of Pure and Applied Chemistry
LC	Liquid chromatography
LC-MS/MS	Liquid chromatography tandem mass
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantitation
MeCN	Acetonitrile
MeOH	Methanol
$MgSO_4$	Magnesium sulphate
min	Minute
MPOB	Malaysian Palm Oil Board
MRL	Maximum residue limit
MS	Mass spectrometry
MSMS	Tandem mass spectrometry
MSPD	Matrix solid phase dispersion
MUFA	Monounsaturated fatty acid
OLS	Ordinary least square
OTB	Ochratoxin B
pH	Negative logarithmic value of the hydrogen ion (H^+)
PKO	Palm kernel oil
PO	Palm olein
PSA	Primary secondary amine
PUFA	Polyunsaturated fatty acid
QuEChERS	Quick, easy, cheap, effective, rugged, safe
RSD	Relative standard deviation
R^2	Correlation
S/N	Signal/noise
SD	Standard deviation
SIM	Selection ion monitoring
SFA	Saturated fatty acid

SPE	Solid phase extraction
SRM	Selected reaction monitoring
TIC	Total ion chromatography
UPLC	Ultra performance liquid chromatography
ZEA	Zearalenone
et.al.	and other authors
g	Gram
$gmol^{-1}$	Gram per mole
kg	Kilogram
L	Liter
mg	Miligram
mgL^{-1}	Miligram per liter
mL	Mililiter
$mLmin^{-1}$	Mililiter per minute
m/z	Mass per electrical charge ratio
ngg^{-1}	Nanogram per gram
µL	Microliter
$µgL^{-1}$	Microgram per liter
%	Percent
Σ	Sum
C	Celcius

CHAPTER 1

INTRODUCTION

1.1 Background of study

Mycotoxins are toxic chemical products produced as secondary metabolites by certain filamentous fungal that can contaminate many agricultural commodities and processed food, either in the field or during storage wherever humidity and temperature are adequate especially in tropic areas (Cavaliere et al., 2010). These mycotoxins have wide range of toxic effects due to their largely diversified chemistry and toxicology properties (Ibanez-vea et al., 2011). Among hundreds of mycotoxins, aflatoxins (AFs), ochratoxin A (OTA), zearalenone (ZEA), deoxynivalenol (DON), fumonisins (FBs), T-2 and HT-2 toxins are the major health concern (Soleimany et al., 2012).

Human exposure to mycotoxins is inevitable and despite many years of study, applying good manufacturing practices during food production, storage and distribution, mycotoxins continue to contaminate a wide range of food and feed. Commodities and products frequently contaminated with mycotoxins include wide range of vegetable commodities such as cereals like wheat, maize, rye, rice, oat, and barley, nuts, peanuts, fruits, oilseeds, dried fruits, cocoa, spices, beer, herbal teas and coffee (Anukul & Vangnai, 2013). Recently, occurrence of some mycotoxins in vegetable oil has been reported worldwide and has received a great deal of attention by the regulatory bodies (Marin et al., 2013).

1.2 Statement of problems

Corn germ and sunflower seeds are very vulnerable to mycotoxins contamination and studies have showed number of occurrence of mycotoxins in corn and sunflower oils (Qian et al., 2015; Schollenberger et al., 2008). However, there is no data been collected to identify the level of mycotoxins contamination in palm oil produced by palm fruit which is at high risk of mycotoxins contamination due to its cultivation in tropical country such as Indonesia and Malaysia. This is important since Malaysia is known as the second largest producer of palm oil in the world after Indonesia.

With increasing findings on mycotoxins contamination in vegetable oil and its health hazard to human, total AFs in oilseeds before human consumption and intended for direct consumption have been regulated. In addition, ZEA level also been regulated particularly in refined maize oil by European Commission (EU) Regulatory body (European Commission Regulation, 2007) due to its highest occurrence in this commodity but in Malaysia, regulation yet been implemented under Food Act 1983 and Food Regulation 1985 for mycotoxins in vegetable oil. It is important to assess the presence and amount of mycotoxins to ensure safety and quality of vegetable oil produced and consumed in Malaysia thus reliable, fast and cost effective analytical method is important for an effective monitoring.

Numerous methods have been developed for the determination of single or multi-mycotoxin in vegetable oil employing different type of extraction and quantification techniques such as liquid-liquid extraction (Elzupir et al., 2010), immunoaffinity column (IAC) (Bao et al., 2010), matrix solid phase dispersion (MSPD) (Cavaliere et al., 2007), dispersive liquid-liquid microextraction (DLLME) (Afzali et al., 2012), acid base extraction (Majerus et al., 2009) and gel permeation chromatography (GPC) (Qian et al., 2015). Most of the researchers quantified using chromatographic methods including high performance liquid chromatography (HPLC) coupled with fluorescence detectors (FLD) (Majerus et al., 2009) or mass spectrometry (MS) (Cavaliere et al., 2007), and gas chromatography (GC) coupled with MS detectors (Qian et al., 2015). Though the methods are reliable and reproducible, the procedure are limited to single mycotoxin analysis or time consuming, require large volume of solvents and costly. In addition, oil which is a lipid poses a challenging task in extraction of mycotoxins which have a wide range of different physicochemical properties in term of pH, stability, solubility, diversity of chemical structure and molecular weight.

1.3 Importance of study

Recently many methods have been developed using QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) technique in mycotoxin analysis in matrixes like cereal, spices, egg, wine, dairy product, beer and rice (Jia et al., 2014), yet there is no method been developed to analyse mycotoxins in vegetable oil. It is known for its simplicity, quickness in extraction and cheaper compared to solid phase extraction (SPE) and immunoaffinity column (IAC) and does not require supporting equipment such as vacuum manifold. For the very first time, QuEChERS technique will be employed to extract multi-mycotoxin from vegetable oil and this will open opportunity to conduct preliminary study on the mycotoxins in vegetable oil; particularly in corn oil, sunflower oil and palm olein which are highly consumed by Malaysian and also in other countries. Since the H[WUDFWLRQ ZLOO EH HPSOR\LQJ QHZ 4X(& optimizing various types of dispersive solid phase extraction (d-SPE) sorbents and extraction solvents, the optimized method will be tested for its performance by conducting detailed method validation including comparison with IAC extraction technique.

This study involves three major components namely method development for seven mycotoxins, followed by method validation of the developed method of the specific compounds and next, determination of multi-mycotoxin in three vegetable oils.

1.4 Objectives

The objectives of this study are as follows:

i. To develop and validate a reliable, fast and cost effective QuEChERS based extraction and clean-up procedures for multi-mycotoxin in vegetable oil
ii. To determine the contamination of multi-mycotoxin in three different vegetable oils using the developed method

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