UNIVERSITI PUTRA MALAYSIA

OPTIMIZATION OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND PCR FOR DETERMINATION OF AFLATOXIN AND OCHRATOXIN A IN PEANUTS

LEILI AFSAH HEJRI

FSTM 2012 1

OPTIMIZATION OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND PCR FOR DETERMINATION OF AFLATOXIN AND OCHRATOXIN A IN PEANUTS

By

LEILI AFSAH HEJRI

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Doctor of Philosophy

January 2012

© COPYRIGHT UPM

DEDICATION

This thesis is dedicated to my husband and son, who were the greatest supporters throughout my doctoral program. They remind me every day just how precious the time is that we have together, and they make my heart full of love and joy. I love you from depth of my heart.

© COPYRIGHT UPM

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Doctor of Philosophy

OPTIMIZATION OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND PCR FOR THE DETERMINATION OF AFLATOXINS AND OCHRATOXIN A IN PEANUTS

By

LEILI AFSAH HEJRI

January 2012

Chairman : Professor Jinap Selamat, PhD

Faculty : Food Science and Technology

Aflatoxins (AFs) are carcinogenic, mutagenic, and hepatotoxic compounds, and ochratoxin A (OTA) is a possible carcinogen to humans. Malaysia is a tropical country favoring the growth of aflatoxin and ochratoxin producer strains. To reduce the health risk from the consumption of aflatoxin and ochratoxin-contaminated food, accurate and effective detection methods are highly needed. This research was conducted to optimize a reversed phase high performance liquid chromatography fluorescence detection method for the determination of AFs and OTA and to isolate potential aflatoxin and ochratoxin producer strains from raw peanuts marketed in Malaysia. Moreover, aflatoxin and ochratoxin-producer strains were also detected using polymerase chain reaction analysis. The effect of high performance liquid chromatography (HPLC) conditions, including mobile phase composition, temperature and flow rate, on the peak areas of four target AFs (aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1) and

© COPYRIGHT UPM

aflatoxin G_2 (AFG_2)) from spiked peanuts was investigated using response surface methodology (RSM). The highest quantification value for target AFs was obtained with the following HPLC conditions: mobile phase composition of ACN/H_2O/MeOH (8:54:38), temperature of 24ºC and flow rate of 0.4 mL/min. The limits of detection (LOD) values were 0.03, 0.01, 0.09 and 0.06 ng/mL for AFB_1, AFB_2, AFG_1 and AFG_2, respectively. The recovery values for AFB_1, AFB_2, AFG_1 and AFG_2, were 91.57, 89.37, 89.42 and 76.14 %, respectively. The recommended optimal HPLC conditions provided peak areas for all target AFs by 1 to 2.5-fold higher than two other conditions that are generally used for aflatoxin detection. The level of AFB_1 contamination in six brands of raw peanuts ranged from 0.45 to 977.16 ng/g. A significant nonlinear response surface model was fitted to evaluate the effect of HPLC parameters (pH, temperature and flow rate) on the quantification level of OTA. All the HPLC variables had a significant ($p <$ 0.05) effect on the quantification level of OTA. The highest quantification value for OTA was obtained with the following HPLC conditions: mobile phase consisting of 5 mM sodium acetate (pH 2.36)/ACN/MeOH (40:30:30), excitation of 333 nm, emission of 467 nm, temperature of 24°C, and flow rate of 0.4 mL/min. The recommended optimal HPLC conditions provided a 2.2-fold to 4.7-fold higher peak area for OTA when compared to two other routinely used HPLC conditions. The recovery and LOD values of OTA under the recommended optimal conditions were 95.4% and 0.05 ng/g, respectively. The OTA contents of the analyzed peanut samples ranged from 2.82 to 7.41 ng/g. Sixty raw peanut samples were used for fungal isolation. Based on the results of the morphological studies, *Aspergillus* species were isolated from the peanut samples. A total of 23 *Aspergillus* (black and green aspergilli) isolates were obtained from the samples.

© COPYRIGHT UPM

Production of AFB_1 and OTA by the isolates in culture media was analyzed using the optimized HPLC methods. Three-day-old purified green *Aspergillus* isolates grown at 30ºC on potato dextrose agar (PDA) plates were screened for their aflatoxin-producing ability using ammonia vapor. The pink/red color of colony reverse represented aflatoxin-producing strains. The results of the screening test were in agreement with the HPLC results of toxin production in media. To detect aflatoxigenic and ochratoxigenic isolates, specific primers targeting the genes responsible for aflatoxin and ochratoxin production were used. Three primer sets were used for the detection of aflatoxigenic isolates, and three primer sets were used for the detection of ochratoxigenic isolates. The results of the PCR amplification were in agreement with the HPLC chromatogram of AFB_1 and OTA production of isolates. A total of 14 aflatoxigenic, 5 ochratoxigenic and 4 non-aflatoxigenic/non-ochratoxigenic *Aspergillus* were isolated from raw peanuts marketed in Malaysia.

© COPYRIGHT UPM

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENGOPTIMUMAN KROMATOGRAFI CECAIR PRESTASI TINGGI DAN PCR UNTUK PENENTUAN AFLATOKSIN DAN OKRATOKSIN A DALAM KACANG TANAH

oleh

LEILI AFSAH HEJRI

Januari 2012

Pengerusi: Profesor Jinap Selamat, PhD

Fakulti: Sains dan Teknologi Makanan

Aflatoksin (AFs) merupakan sebatian karsinogenik, mutagenik dan hepatotoksik manakala okratoksin A (OTA) berpotensi karsinogen terhadap manusia. Malaysia adalah negara tropika yang mempunyai potensi pertumbuhan jenis pengeluar aflatoksin dan okratoksin. Untuk mengurangkan risiko kesihatan daripada pengambilan makanan yang terkontaminasi dengan aflatoksin dan okratoksin, kaedah pengesanan yang tepat dan efektif sangatlah diperlukan. Kajian ini dijalankan untuk mengoptimumkan kaedah pengesanan menggunakan kromatografi cecair berprestasi tinggi fasa terbalik bagi penentuan AFs dan OTA, dan untuk mengasingkan kemungkinan pengeluar AFs dan OTA daripada kacang tanah mentah yang dipasarkan di Malaysia dan mengesannya dengan

© COPYRIGHT UPM

menggunakan tindak balas rantai polimer (PCR). Kesan keadaan HPLC iaitu komposisi fasa gerak, suhu dan kadar aliran terhadap luas puncak empat AF sasaran (aflatoksin B_1 (AFB_1), aflatoksin B_2 (AFB_2), aflatoksin G_1 (AFG_1) dan aflatoksin G_2 (AFG_2)) daripada kacang tanah yang dipakukan telah dikaji dengan menggunakan kaedah permukaan gerakbalas (RSM). Nilai penaksiran yang tertinggi bagi AFs sasaran telah didapati dalam keadaan HPLC seperti berikut: komposisi fasa gerak ACN/H_2O/MeOH: 8/54/38, suhu 24 ºC dan kadar aliran 0.4 mL/min. Nilai had pengesanan (LOD) adalah 0.03, 0.01, 0.09 dan 0.06 ng / mL bagi AFB_1, AFB_2, AFG_1 dan AFG_2, masing-masing. Nilai dapatan semula bagi kaedah untuk aflatoksin AFB_1, AFB_2, AFG_1 dan AFG_2, adalah 91.57, 89.37, 89.42 dan 76.14%, masing-masing. Keadaan HPLC optimum yang dicadangkan menghasilkan luas puncak yang lebih tinggi bagi semua AFs sasaran sebanyak 1 hingga 2.5 kali berbanding dengan dua kaedah lain yang biasa digunakan dalam pengesanan AFs. Tahap kontaminasi AFB_1 dalam enam jenis kacang tanah mentah adalah dalam lingkungan 0.45 hingga 977.16 ng/g. Suatu model permukaan gerak balas tidak linear yang signifikan telah disesuaikan untuk menilai kesan parameter HPLC (iaitu pH, suhu dan kadar aliran) terhadap tahap penaksiran OTA. Semua parameter HPLC menunjukkan kesan yang signifikan ($p < 0.05$) terhadap tahap penaksiran OTA. Nilai penaksiran tertinggi bagi OTA telah didapati dengan menggunakan kaedah HPLC seperti berikut: fasa gerak yang terdiri daripada 5 mM sodium asetat (pH 2.36) / ACN / MeOH (40:30:30); pengujaan pada 333 nm; pemancaran pada 467 nm; suhu 24 °C dan kadar aliran 0.4 mL / min. Keadaan HPLC optimum yang dicadangkan menghasilkan luas puncak 2.2 dan 4.7 kali lebih tinggi bagi okratoksin A apabila dibandingkan dengan dua kaedah HPLC lain yang biasa digunakan. Dapatan semula dan nilai

© COPYRIGHT UPM

LOD bagi OTA pada keadaan optimum yang dicadangkan adalah 95.4% dan 0.05 ng / g, masing-masing. Kandungan OTA dalam sampel kacang tanah adalah dalam lingkungan 2.82 hingga 7.41 ng / g. Enam puluh sampel kacang mentah digunakan untuk pengasingan fungal. Berdasarkan hasil kajian morfologi spesis *Aspergillus* dapat diasingkan. Sejumlah 23 *Aspergillus* (aspergilli hitam dan hijau) didapati daripada sampel. Penghasilan AFB_1 dan OTA dalam media kultur dianalisa dengan menggunakan kaedah HPLC yang dioptimumkan. Aspergilli hijau tulen yang tumbuh selama 3 hari pada 30 ºC pada plat potato dextrose agar (PDA) telah disaring untuk melihat kebolehan penghasilan aflatoksin dengan menggunakan wap ammonia. Koloni terbalik berwarna merah jambu mewakili pengeluar bagi aflatoksin. Hasil ujian saringan wap adalah selari dengan hasil HPLC bagi penghasilan toksin dalam media. Untuk mengesan ceraian aflatoksigenik dan okratoksigenik, primer khusus menyasarkan gen bertanggungjawab dalam perghasilan aflatoksin dan okratoksin A telah digunakan. Tiga set primer telah digunakan untuk pengesanan aflatoksigenik dan tiga pasang untuk ceraian okratoksigenik. Hasil amplifikasi PCR ceraian tersebut adalah selari dengan kromatogram HPLC bagi pengasilan AFB_1 dan OTA dalam ceraian tersebut. Keseluruhannya sebanyak 14 aflatoksigenik, 5 okratoksigenik dan 4 *Aspergillus* bukan aflatoksigenik/okratoksigenik yang diasingkan daripada kacang tanah mentah yang dipasarkan di Malaysia.

© COPYRIGHT UPM

ACKNOWLEDGEMENTS

First and foremost, I would like to sincerely thank my supervisor, Prof. Dr. Jinap Selamat, for her guidance, constant encouragement and valuable advices during my time in this program. I appreciate all her contributions of time, ideas, and funding to make my Ph.D. experience productive and stimulating.

I also wish to thank my supervisory committee, Prof. Dr. Son Radu and Dr. Farinazleen Mhd Ghazali, for their guidance, helpful suggestions and supports.

I gratefully acknowledge the funding sources that made my Ph.D. work possible. It was funded by Universiti Putra Malaysia (UPM), under Research University grants (RUGs) Fund project No. 02/01/07/0024RU.

None of this would have been possible without the laboratory technical assistance of Mrs Norliza (Food safety and Molecular Lab), Mrs Sorya and Mr Hamezan (Chromatography Lab) , Mr Azman (Biochemistry Lab) and Mr Zulkefli, Ms Fatihah and Mrs Noratina (Microbiology Lab).

In particular, I wish to thank Dr. Hamed Mirhosseini for his helpful suggestions in statistical design and analysis.

© COPYRIGHT UPM

Certainly, I wish to acknowledge many friends of other labs whom I have known over the past years, and their assistance and suggestions made it possible to accomplish some difficult parts of my work and fine-tune the whole process; Dr. Farid Azizi, Nahid Babaei and Mahmodreza Shabani Mofrad.

It is a pleasure to thank my good friends Dr. Parvaneh Hajeb, Dr. Maryam Jalili, Dr. Anosheh Rahmani, Dr. Farhang Soleimany, Sahar Arzandeh and Diana Jafar, who have helped and encouraged me during my research work.

In particular, I would like to thank Gisia Daniali and Morvarid Akhavan Rezaei for being my good friends. Their friendship during these years really helped to smooth out some rough spots that I experienced.

I would also like to thank all of my lab mates: Dr. Afsaneh Farhadian, Dr. Elham Moazami, Farnaz Faridnia, Wendy Lim, Maimunah Sunny, Hapizah, Diyana Syamim, Babak Hatami, Wael Mousa and Jahurul Akanda in Center of Excellence for Food Safety Research (CEFSR) for their friendship and creating a wonderful environment for learning and living.

Last but certainly not least, my eternal gratitude goes to my parents, Atarod Kalantari Afsah Hejri and Sayed Abolfazl Afsah Hejri, for their unconditional love and never-ending support. They have always been a tremendous help in my life and have encouraged me to succeed academically while showing me by example that faith in God and love of family should always come first. You have

© COPYRIGHT UPM

always been a perfect role model for me to follow. Words cannot express how grateful I am to you.

My sisters (Maryam and Nahid) and brother (Sayed Ali) have always been there when I needed someone to talk to. Over the years, we have continued to grow closer and enjoy life together. Although we are living in different parts of the world now, I look forward to many happy moments yet to come. Thanks for your support and encouragement.

Finally, I am indebted to my husband Kazem for being supportive. His understanding is a firm foundation from which I attain my goals. Thank you for your love and for the way you show your love in all that you do. I am blessed to be able to share my life with you. Your understanding, sacrifices and endless love and constant support have sustained me during times of hardship. For all of this, I shall be always grateful.

My deepest gratitude is to the dearest person in my life, my little angel Parsa. For these years, we have been alone here most of the time, and he has always been with me in my difficult times. Sweetheart, I will never forget those days you stayed with mom in the lab to finish an experiment. I know that the time I have spent pursuing my doctorate was sometimes difficult, but we have persevered. Thanks for your understanding and being so helpful to mom. I love you more than words can express.

© COPYRIGHT UPM

APPROVAL

I certify that an Examination Committee has met on 17/01 /2012 to conduct the final examination of Leili Afsah Hejri on her thesis entitled " **Optimization of High Performance Liquid Chromatography and PCR for determination of aflatoxin and ochratoxin A in peanuts** " 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 PhD of Food Safety.

Members of the Thesis Examination Committee were as follows:

Alfi Khatib, PhD
Doctor
Faculty of Food Science and Technology
Universiti Putra Malaysia
(Chairman)

Nazamid Saari, PhD
Professor
Faculty of Food Science and Technology
Universiti Putra Malaysia
(Internal Examiner)

Abdulkarim Sabo Mohammed, PhD
Associate Professor
Faculty of Food Science and Technology
Universiti Putra Malaysia
(Internal Examiner)

Russell M. Paterson, PhD
Professor
IBB Institute of Biotechnology and Bioengineering
University of Minho
Portugal
(External Examiner)

_____

SEOW HENG FONG, PhD
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

© COPYRIGHT UPM

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

Jinap Selamat, PhD
Professor
Faculty of Food science and Technology
University Putra Malaysia
(Chairman)

Son Radu, PhD
Professor
Faculty of Food science and Technology
University Putra Malaysia
(Member)

Farinazleen Mohamad Ghazali, PhD
Associate professor
Faculty of Food science and Technology
University Putra Malaysia
(Member)

BUJANG BIN KIM HUAT, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

© COPYRIGHT UPM

DECLARATION

I declare that the thesis is my original work except for quotations and citations, which have been duly acknowledged. I also declare that it has not been previously and is not concurrently submitted for any other degree at UPM or at any other institutions.

LEILI AFSAH HEJRI

Date: 17 January 2012

© COPYRIGHT UPM

TABLE OF CONTENTS

	Page
DEDICATION	ii
ABSTRACT	iii
ABSTRAK	vi
ACKNOWLEDGEMENTS	ix
APPROVAL	xii
DECLARATION	xiv
LIST OF TABLES	xix
LIST OF FIGURES	xxi
LIST OF ABBREVIATIONS	xxvii
CHAPTER	
INTRODUCTION	**1**
Background of study	1
Importance of study	2
Objectives	4
LITRATURE REVIEW	**5**
Peanuts (*Arachis hypogaea L.*)	5
Aspergillus species	7
Aspergillus Section *Flavi*	10
Pathogenicity	11
Clustering of aflatoxin genes	12
Genetic and regulation of aflatoxin biosynthesis	14
Aspergillus section *Nigri*	15
Pathogenicity	17
Genetics and regulation of OTA biosynthesis	17
Aflatoxins (AFs)	18
Chemical characteristics of AFs	18
Physical properties of AFs	21
Occurrence of AFs	24
Adverse effects of AFs	28
Toxicity Kinetics of AFs	31
International regulations for AFs	34
Ochratoxin A (OTA)	35
Chemical and Physical Properties of OTA	37
Occurrence of OTA	38
Adverse effects of OTA	40
Toxicity Kinetics of OTA	42
International regulations for OTA	44

© COPYRIGHT UPM

Detection methods for AFs, OTA and producer strains 46
Culture techniques 46
Bioassay or Immunochemical techniques 48
Chromatographic techniques 49
Principles for the quantification of AFs and OTA by HPLC 50
Molecular techniques 55
Principles of conventional PCR 57
Procedures involved in the detection of aflatoxigenic and ochratoxigenic strains by PCR 60

OPTIMIZATION OF HPLC CONDITIONS FOR QUANTITATIVE ANALYSIS OF AFLATOXINS IN CONTAMINATED PEANUTS 62

Introduction 62
Materials and methods 66
Materials 66
Sample preparation 66
Mobile phase preparation 67
Instruments 68
Experimental design and data analysis 68
Optimization and validation procedures 70
Conditions and data analysis used for comparison study 72
Moisture content measurement 72
Results and discussion 73
Optimization procedure for predicting the highest detection level of target AFs 80
Verification of the final reduced models 83
Comparison study 85
Aflatoxin content of peanut samples 90
Conclusions 93

OPTIMIZATION OF HPLC CONDITIONS FOR QUANTITATIVE ANALYSIS OF OCHRATOXIN A IN CONTAMINATED PEANUTS 95

Introduction 95
Materials and Methods 99
Materials 99
Sample preparation 100
Mobile phase preparation 101
Instruments 103
Experimental design and data analysis 104
Optimization and validation procedures 107
Conditions and data analysis used for comparison study 108
Results and Discussion 109
Preliminary test for mobile phase composition 109
Optimization study 115
Optimization procedure for predicting the highest detection level of OTA 122
Verification of the final reduced model 124
Method efficiency 125

© COPYRIGHT UPM

OTA content of peanut samples 128
Conclusions 131

ISOLATION OF ASPERGILLUS FROM PEANUTS AND SCREENING OF AFLATOXIGENIC STRAINS 133

Introduction 133
Material and methods 138
Fungal strains 138
Peanut samples 138
Isolation of fungal flora from peanuts 139
Screening for aflatoxigenic *Aspergillus* 140
AFB_1 and OTA producing ability of the isolates 140
AFs and OTA standards 140
Extraction of AFs from media 141
Extraction of OTA from media 142
Quantification of AFB_1 using HPLC 142
Quantification of OTA using HPLC 143
Statistical analysis 143
Results and discussion 144
Conclusion 158

DETECTION OF AFLATOXIGENIC AND OCHRATOXIGENIC ASPERGILLUS USING POLYMERASE CHAIN REACTION 160

Introduction 160
Materials and Methods 165
Materials 165
Instruments 166
Fungal isolates 166
DNA extraction 166
PCR primers 167
PCR mixture 168
PCR protocol and PCR product 170
Confirmation: HPLC analysis of AFB_1 and OTA 170
Results and discussion 171
Optimization of PCR program 174
Application of PCR programs to samples 179
Conclusion: 187

SUMMARY, GENERAL CONCLUSION AND RECOMMENDATION FOR FUTURE RESEARCH 188

Summary 188
Conclusion and recommendation 191

REFERENCES 194

APPENDICES 218

Appendix A: Standard calibration curve of AFs 218

© COPYRIGHT UPM

Appendix B: Standard calibration curve of OTA 221

BIODATA OF STUDENT **222**

LIST OF PUBLICATIONS **223**

© COPYRIGHT UPM