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

LEILI AFSAH HEJRI

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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
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.
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

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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
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$_2$O/MeOH (8:54:38), temperature of 24°C 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.
Production of AFB\textsubscript{1} and OTA by the isolates in culture media was analyzed using the optimized HPLC methods. Three-day-old purified green \textit{Aspergillus} isolates grown at 30\textdegree C 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\textsubscript{1} and OTA production of isolates. A total of 14 aflatoxigenic, 5 ochratoxigenic and 4 non-aflatoxigenic/non-ochratoxigenic \textit{Aspergillus} were isolated from raw peanuts marketed in Malaysia.
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 AFLATOKSN DAN OKRATOKSN A DALAM KACANG TANAH

oleh

LEILI AFSAH HEJRI

Januari 2012

Pengerusi: Profesor Jinap Selamat, PhD

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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 ceair 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
menggunakan tindak balas rantai polimer (PCR). Kesan keadaan HPLC iaitu komposisi fasa gerak, suhu dan kadar aliran terhadap luas puncak empat AF sasaran (aflatoksin B₁ (AFB₁), aflatoksin B₂ (AFB₂), aflatoksin G₁ (AFG₁) dan aflatoksin G₂ (AFG₂)) 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/H₂O/MeOH: 8/54/38, suhu 24 ºC dan kadar aliran 0.4 mL/min. Nilai had pengesan (LOD) adalah 0.03, 0.01, 0.09 dan 0.06 ng / mL bagi AFB₁, AFB₂, AFG₁ dan AFG₂, masing-masing. Nilai dapatan semula bagi kaedah untuk aflatoksin AFB₁, AFB₂, AFG₁ dan AFG₂, 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 AFB₁ 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 ºC 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
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 AFB₁ 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 AFB₁ 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.
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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.

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Date:
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
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BIODATA OF STUDENT

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