UNIVERSITI PUTRA MALAYSIA

DEVELOPMENT, OPTIMIZATION AND VALIDATION OF RP-HPLC-FL METHOD FOR SIMULTANEOUS DETERMINATION OF AFLATOXINS, OCHRATOXIN A AND ZEARALENONE IN CEREALS

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By

ANOSHEH RAHMANI

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

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Dedicated to my beloved husband

Farhang

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

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October 2010

Chairman : Professor Jinap Selamat, PhD

Faculty : Food Science and Technology

The heightened awareness of consumer-health risk from cumulative mycotoxin exposure has resulted in the establishment of regulatory limits that can protect human risk from the consumption of food products containing mycotoxin. Consequently, cost-effective, time saving, trace level simultaneous detection methods for controlling main mycotoxins in foods became highly desired. This research has been conducted to develop a reverse phase high performance liquid chromatography fluorescence detection (RP-HPLC-FLD) method for simultaneous determination of six mycotoxins (aflatoxin B1, B2, G1 and G2, ochratoxin A and zearalenone) in cereals. It has been found that injecting 100µL of mycotoxin standard to a symmetry C18 column in appropriate combination of methanol: acetonitrile: phosphoric acid (1%) (30:10:60 (v/v) for 0-14 min and 10:45:45 (v/v) for 12-35min in suitable excitation and emission wavelengths of fluorescent detector can grant separation of six mycotoxins in 35 minutes. Then, the developed method was optimized using an experimental design including fractional factorial design (FFD)

followed by response surface methodology (RSM). In this study, the effect of seven variables including temperature (20-40^{o}C), flow rate (0.8-1.2 ml/min), acid concentration in aqueous phase (0-2%), organic solvent percentage at start (40-50%) and end (50-60%) of the gradient mobile phase, ratio of methanol/acetonitrile at start (1-4) and end (0-1) of gradient mobile phase, have been evaluated on HPLC responses (separation and resolutions of peaks and HPLC run time). The statistical optimized HPLC condition for response variables (separation and resolutions of peaks and HPLC run time) was validated statistically and experimentally. The optimal conditions was 41% and 60% organic solvent percentage at start and end of gradient mobile phase, 1.93 and 0.2 methanol/acetonitrile ratio at start and end of gradient respectively, 0.1% acid concentration in aqueous phase, at 1 ml/min flow rate and in 40^{o}C column oven temperature. After that, the efficiency of three extraction solvents including methanol: water (80:20 v/v), acetonitrile: water (80:20 v/v) and acetonitrile: water (60:40 v/v) and three clean-up procedures including multi-functional AOZ immunoaffinity column (IAC), OASIS HLB solid phase extraction column and multi- functional MycoSep column were compared using spiked rice sample. The highest recovery of mycotoxins (93-104%) was obtained by using methanol: water (80:20 v/v) as extraction solvent and IAC as clean-up method. Application of optimized simultaneous determination method on spiked cereal samples (rice, barley, oat, wheat and corn) showed 74-104 % recovery for all six mycotoxins and acceptable precision in all cereals. The optimized HPLC- FLD method was validated through determination of selectivity, sensitivity, linearity and precision. Limit of detection (LOD) and quantification (LOQ) of these mycotoxins ranged 0.004 - 0.5ng/g and 0.015 -2 ng/g, respectively. To confirm the performance of HPLC method for simultaneous determination of mycotoxins on real

cereal samples a total of 61 samples of cereals (including rice, oat, barley, wheat and maize) were randomly collected from food stores in Selangor State, Malaysia and were analyzed for mycotoxins using the HPLC simultaneous determination method. The results showed low occurrence of these mycotoxins in commercial cereals marketed in Malaysia. In conclusion, the newly developed, optimized and validated HPLC-FLD method is an efficient and accurate method for simultaneous determination of these mycotoxins and could be used for routine analysis in the mycotoxin laboratories.

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

PEMBANGUNAN, PENGOPTIMUMAN DAN PENGESAHAN KAEDAH RP-HPLC-FL UNTUK PENENTUAN SERENTAK AFLATOXINS, OCHRATOXIN A DAN ZEARALENON DALAM BIJIRIN

Oleh

ANOSHEH RAHMANI

Oktober 2010

Pengerusi : Profesor Jinap Selamat, PhD

Fakulti : Sains dan teknologi makanan

Kesedaran yang tinggi terhadap risiko kesihatan pengguna daripada pendedahan mikotoksin telah menghasilkan pembangunan had pengawalan yang boleh melindungi pengguna daripada risiko pengambilan produk makanan yang mengandungi mikotoksin. Akibatnya, kaedah pengesanan pada tahap surih yang mempunyai kos berkesan serta penjimatan masa untuk mengawal mikotoksin utama dalam makanan adalah amat diperlukan. Penyelidikan ini telah dijalankan untuk membangun kan kaedah pengesanan pendarfluor kromatografi cecair prestasi tinggi fasa terbalik (RP-HPLC-FLD) untuk penentuan enam mikotoksin (aflatoksin B1, B2, G1 dan G2, okratoksin A dan zearalenon) dalam bijirin. Didapati bahawa dengan menyuntik 100µL larutan piawai mikotoksin ke dalam turus C18 simetri menggunakan kombinasi methanol:, asetonitril : asid fosforik (1%) (30:10:60 (v/v) yang sesuai untuk 0-14 minit dan 10: 45: 45 (v/v) untuk 12-35 minit dalam pengujaan serta jarak gelombang pemancaran pengesan pendarfluor yang sesuai

boleh membenarkan pemisahan enam mikotoksin dalam masa 35 minit. Kemudian, kaedah yang telah dibangunkan dioptimumkan menggunakan satu rekabentuk ujikaji termasuk rekabentuk faktor pecahan (FFD) diikuti dengan kaedah permukaan gerak balas (RSM). Dalam kajian ini, kesan tujuh parameter termasuk suhu (20-40 °C), kadar aliran (0.8-1.2 ml/minit), kepekatan asid dalam fasa akueus (0-2%), peratusan pelarut organik pada permulaan (40-50%) dan pengakhiran (50-60%) bagi fasa gerak kecerunan, nisbah bagi metanol/asetonitril pada permulaan (1-4) dan pengakhiran (0-1) bagi fasa gerak kecerunan, telah dinilai pada gerak balas HPLC (pemisahan dan resolusi puncak, dan masa analisis HPLC). Keadaan optimum ialah 41% dan 60% peratusan pelarut organik pada permulaan dan pengakhiran fasa gerak kecerunan, 1.93 dan 0.2 nisbah metanol dan asetonitril pada permulaan dan pengakhiran kecerunan, 0.1% kepekatan asid dalam fasa akueus, kadar aliran pada 1 ml/min dan suhu dalam ketuhar 40°C. Selepas itu, kecekapan tiga pelarut pengekstrakan termasuk metanol:air (80:20 v/v), asetonitril:air (80:20 v/v) dan asetonitril:air (60:40 v/v) dan tiga prosedur pembersihan termasuk turus immunoafiniti AOZ pelbagai fungsi (IAC), turus pengekstrakan fasa pepejal OASIS HLB dan turus MycoSep pelbagai fungsi telah dibandingkan menggunakan sampel beras yang ditambah dengan larutan piawai. Dapatan semula mikotoksin yang tertinggi (93-104%) telah diperolehi dengan menggunakan metanol : air (80:20 v/v) sebagai pelarut pengekstrakan dan IAC sebagai kaedah pembersihan. Penggunaan kaedah penentuan yang telah dioptimumkan terhadap sampel bijirin (beras, barli, oat, gandum dan jagung) yang ditambah dengan larutan piawai menunjukkan dapatan semula sebanyak 74-104 % bagi semua enam mikotoksin dan ketepatan yang boleh diterima dalam semua bijirin.

HPLC- FLD yang telah dioptimumkan telah disahihkan melalui penentuan pemilihan, kepekaan, kelinearan dan ketepatan. Julat had pengesanan (LOD) dan had penaksiran (LOQ) untuk mikotoksin ialah 0.004 - 0.5 ng /g dan 0.015 – 2 ng/g masing-masing. Untuk mengesahkan prestasi kaedah HPLC bagi penentuan mikotoksin pada sampel sebenar bijirin sejumlah 61 sampel bijirin (termasuk beras, oat, barli, gandum dan jagung) telah dikutip secara rawak dari kedai makanan di negeri Selangor, Malaysia dan telah dianalisis untuk mikotoksin menggunakan kaedah penentuan HPLC yang telah dibangunkan. Keputusan juga menunjukkan kedapatan mikotoksin yang rendah dalam bijirin komersial di pasaran Malaysia. Sebagai kesimpulan, kaedah HPLC-FLD yang baru dibangunkan, dioptimumkan dan disahihkan merupakan kaedah yang cekap dan tepat untuk penentuan serentak mikotoksin dan boleh digunakan untuk analisis rutin di makmal mikotoksin.

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In the name of the most compassionate. One day I asked God for instructions on how to live on this earth? God wispered in my ear, Be like flowers, loving the sun, but faithful to your roots. Be like the firefly, although small, it casts its on light. Be like the water, good and transparent. Be like the river, always moving forward and above all things, be like the heavens, ahome for God. Thanks God who is speaking to me. Thanks God. He is always here beside me, lovingly accompanying me every step of the way on my lifes journey. Lord, don't let me to remain where I am; Help me reach where you want me to be.

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I certify that an Examination Committee met on 18/10 /2010 to conduct the final examination of Anosheh Rahmani on her PhD degree of Food Science thesis entitled "Development, Optimization and Validation of RF-HPLC-FL Method for Simultaneous Determination of Aflatoxins, Ochratoxin A and Zearalenone in Cereals" in accordance with Universiti Pertanian Malaysia (higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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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 Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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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.

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ANOSHEH RAHMANI

Date:

TABLE OF CONTENTS