



UNIVERSITI PUTRA MALAYSIA

***DETERMINATION AND VALIDATION OF DISPERSIVE SOLID PHASE
EXTRACTION AND ITS APPLICATION IN DETERMINATION OF
ANTHELMINTICS AVERMECTINS IN FISH***

RAFIDAH ISMAIL

FSTM 2014 40



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By

RAFIDAH ISMAIL

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

November 2014

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DEDICATION

Naskah ini ditujukan kepada suami (Biswadi) yang tercinta, anak-anak yang tersayang Haziq, Hafiz dan Haziqah, mak (Azizah) dan ayah (Ismail) yang dikasihi di atas dorongan, sokongan dan kesabaran yang diberikan dalam menjayakan nukilan ilmu ini. Tiada bahasa dapat menggambarkan cinta dan sayang kalian di hati ini.



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

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RAFIDAH ISMAIL

November 2014

Chair : Nor Ainy Mahyudin, PhD

Faculty : Food Science and Technology

Anthelmintics avermectins are veterinary drugs used as anti-parasites in food producing animals. An analytical method which will yield fast and reliable results for the determination of avermectins in aquaculture fish is an essential to ensure compliance with food safety monitoring programmes. The following objectives are set in order to achieve the above goal: to investigate the effectiveness of octadecyl (C18), primary secondary amine (PSA) and alumina clean-up sorbents used in the Dispersive Solid Phase Extraction (d-SPE) procedure in determination of avermectins in barramundi (*Lates calcarifer*); to select the clean-up sorbent(s) which will give the highest recovery rate in the detection of avermectins; to validate the use of the selected clean-up sorbent(s) as well estimate the measurement uncertainties and to verify the effectiveness of the d-SPE method for the determination of avermectins in five aquaculture fish species.

A total of five avermectin compounds is evaluated in this study, namely, emamectin, abamectin, doramectin, moxidectin and ivermectin. Homogenised barramundi (*L. calcarifer*) samples are extracted with acetonitrile and purified using the d-SPE procedure. The presence of avermectins in the samples were quantified using LC-MS/MS. It is found that the use of combined PSA and C18 sorbents at a ratio of 1:1 results in high recovery rates for all avermectins with a range of 94 – 103% and relative standard deviations (RSDs) less than 11%,

compared to the use of individual sorbents. Hence, the combined PSA and C18 sorbents are selected for further analysis using the d-SPE method.

Method validation has been carried out and the LOD within the range of 0.3 – 0.4 µg/kg, whereas the LOQ is 1 µg/kg. The linearity of the method is 1 – 15 µg/kg for all avermectins. The precision of the method is less than 20%, while the trueness of the method is within the range of 87 - 102% for low, medium and high concentration levels of 1, 2 and 5 µg/kg. The measurement uncertainty has been estimated.

The use of the combined PSA and C18 sorbents with the d-SPE method has been verified to determine the presence of avermectins in five aquaculture fish species with dissimilar fatty acid content, namely, barramundi (*L. calcarifer*), tilapia (*Oreochromis* sp.), river catfish (*Pangasius* sp.), grouper (*Epinephelus* sp.) and snapper (*Lutjanus* sp.). A total of 34 fish samples are used for this purpose and the applicability of the d-SPE method has been demonstrated by the detection of emamectin in two fish samples, i.e. grouper (*Epinephelus* sp.) and snapper (*Lutjanus* sp.), which corresponds to 5.9%. Confirmatory analysis has been carried out and confirm that the emamectin residues are detected above the LOD but below the LOQ (1 µg/kg) for both samples. Based on the findings of this study, it is recommended that the d-SPE method is implemented as part of the veterinary residue monitoring plan in Malaysia in order to establish a comprehensive database on the use of avermectins in aquaculture fish.

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

**PENENTUAN DAN PENGESAHAN PENGEKSTRAKAN SERAKAN FASA
PEPEJAL DAN APLIKASINYA DALAM PENENTUAN AVERMEKTIN
ANTELMINTIK DALAM IKAN**

Oleh

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Avermektin antelmintik merupakan ubatan veterinar yang digunakan sebagai agen anti-parasit dalam industri penternakan haiwan sebagai sumber makanan. Pembangunan satu kaedah yang dapat memberikan keputusan yang cepat dan boleh dipercayai bagi menentukan kehadiran sisa avermektin dalam ikan akuakultur sangat diperlukan demi menyokong program pemantauan keselamatan makanan. Demi mencapai matlamat tersebut, objektif-objektif kajian ini telah ditetapkan seperti berikut: untuk mengkaji keberkesanan penjerap penulenan oktadekil (C18), amina primer sekunder (PSA) dan alumina dalam kaedah pengekstrakan serakan fasa pepejal (d-SPE) bagi menentukan sisa avermektin dalam ikan siakap (*Lates calcarifer*); untuk memilih penjerap penulenan yang memberikan kadar perolehan tertinggi dalam pengesanan sisa avermektin; untuk mengesahkan kaedah d-SPE dengan penggunaan penjerap penulenan terpilih serta menganggarkan ketidakpastian pengukuran berkaitan; dan untuk menentusahkan kaedah d-SPE bagi menentukan kehadiran sisa avermektin dalam lima spesis ikan akuakultur.

Sejumlah lima sebatian avermektin telah dinilai dalam kajian ini, iaitu emamektin, abamektin, doramektin, moksidektin dan ivermektin. Sampel ikan siakap (*L. calcarifer*) dihomogenkan terlebih dahulu dan kemudiannya diekstrak dengan menggunakan asetonitril dan ditulenkan dengan menggunakan kaedah

d-SPE. Kehadiran sisa avermektin dalam sampel ikan ditentukan dengan menggunakan Kromatografi Cecair Spektrometri Jisim Tandem (LC-MS/MS). Hasil kajian menunjukkan bahawa penggunaan penjerap gabungan PSA dan C18 pada nisbah 1:1 menghasilkan kadar perolehan yang tinggi untuk kesemua avermektin berbanding penggunaan penjerap penulenan secara berasingan. Hasil kajian menunjukkan bahawa julat kadar perolehan adalah antara 94 – 103% sementara sisihan piawai relatif adalah kurang daripada 11%. Oleh yang demikian, penjerap gabungan PSA dan C18 telah dipilih untuk analisis seterusnya dengan menggunakan kaedah d-SPE.

Pengesahsahihan kaedah d-SPE telah dilaksanakan dan menunjukkan LOD berada dalam lingkungan 0.3 – 0.4 µg/kg manakala nilai LOQ adalah 1 µg/kg. Kelinearan kaedah d-SPE untuk kesemua avermektin didapati berada dalam lingkungan 1 – 15 µg/kg. Hasil kajian juga menunjukkan bahawa kepersisan kaedah d-SPE adalah kurang daripada 20%, manakala kejituan pula berada dalam lingkungan 87-102% untuk tahap kepekatan rendah, sederhana dan tinggi iaitu 1, 2 and 5 µg/kg. Analisis ketakpastian juga dilaksanakan.

Penentusahan telah dijalankan ke atas keadah d-SPE dengan penggunaan penjerap gabungan PSA dan C18 bagi menentukan kehadiran sisa avermektin dalam lima spesis ikan akuakultur. Spesis ikan akuakultur yang dikaji dalam kajian ini mempunyai kandungan asid lemak berbeza, iaitu siakap (*L. calcarifer*), tilapia (*Oreochromis* sp.), patin (*Pangasius* sp.), kerapu (*Epinephelus* sp.) dan merah (*Lutjanus* sp.), dan sejumlah 34 sampel ikan telah digunakan untuk tujuan ini. Kebolehgunaan kaedah d-SPE telah terbukti berjaya dengan pengesanan sisa emamektin dalam dua sampel ikan iaitu kerapu (*Epinephelus* sp.) dan merah (*Lutjanus* sp.), dengan peratusan sebanyak 5.9%. Analisis pemastian telah dilaksanakan dan sisa emamektin untuk kedua-dua sampel didapati lebih daripada LOD dan kurang daripada LOQ (1 µg/kg) berdasarkan hasil keputusan analisis pemastian. Berdasarkan hasil kajian, adalah diperakukan bahawa kaedah d-SPE boleh dilaksanakan sebagai sebahagian daripada pelan pemantauan sisa ubatan veterinar bagi mewujudkan satu pangkalan data yang komprehensif berkenaan penggunaan avermektin dalam ikan akuakultur di Malaysia.

ACKNOWLEDGEMENTS

In the name of Allah, Most gracious, Most merciful, Alhamdulillah, with his blessing, I have completed the preparation of this manuscript.

I would like to express my deepest gratitude and respect to my kind supervisor, Associate Professor Dr. Nor Ainy Mahyuddin, for her guidance, encouragement, and support throughout my study. I also would like to extend my appreciation and gratitude to the members of the advisory committee Professor Fatimah Abu Bakar for their invaluable contributions and support.

I will be always indebted to the Food Safety and Quality Division, Ministry of Health Malaysia for the scholarship and funding of my study. Paramount gratitude is also extended to the staff of National Public Health Laboratory, Ministry of Health Malaysia, especially Veterinary Drug Residue Unit who contributes one way or another towards the completion of my study.

Last but not least, I would like to express my sincere gratitude to my beloved husband and family for their support, advice and encouragement and also to my friends, especially Ghanthimathi, Tan Cheng Keng, Wan Ainiza, Norazlina, Jamilah, Zawiyah, Tosiah, Nur Ain, Rogayah, Haslina, Nora Azila and Zamidah for their kindness, friendship and corporations to complete my study.

This thesis 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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LIST OF ABBREVIATIONS

µm	micrometer
ANOVA	Analysis of variance
APCI	Atmospheric pressure chemical ionization
C18	Octadecyl
d-SPE	Dispersive Solid Phase Extraction
ESI	Electrospray ionization source
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
ISO	International Organisation for Standardisation
LC MS	Liquid Chromatography Mass Spectrometry
LC MS/MS	Liquid Chromatography Tandem Mass Spectrometry
LOD	Limit of Detection
LOQ	Limit of Quantification
m/z	mass per charge
mL	millilitre
MRL	Maximum residue limit
ng	nanogram
PSA	Primary and Secondary Amine
PVDF	Polyvinylidene fluoride
QuEChERS	Quick, Easy, Cheap, Effective, Rugged and Safe
RSD	Relative standard deviation
SPE	Solid Phase Extraction
US FDA	United States Food and Drug Administration
USA	United States of America
v/v	Volume per volume
µg/kg	microgram per kilogram
µg/mL	microgram per milliliter
µL	microliter

CHAPTER 1

INTRODUCTION

Malaysia is one of the developing countries which aim to increase exports of fishery products to the countries listed under the European Union (EU). The export value of fishery commodities from Malaysia increased from RM 1,155 million in 1999 to RM 2,826 million in 2011 (Department of Statistics Malaysia, 2012). However, there was a significant loss in export value between year 2008 and 2009 (with a value of 16%) due to the economic downturn as well as suspension of fishery products by the EU authorities. The Member States of the EU approved the launch of negotiations with Malaysia for a Free Trade Agreement in September 2010 (Trade Countries and Regions Malaysia, 2014). It is therefore compulsory for the Government of Malaysia to provide comprehensive residue monitoring plans and accompanying data to the Commission of the European Communities for evaluation and further clarification in order to fulfil the requirements stipulated by the EU for aquaculture products (European Council Directive No 96/23/EC, 2007).

One of the demands set by the European Commission is that it is obligatory for the Government of Malaysia to draft and implement monitoring programs for all veterinary drug residues listed in the European Union Council Regulation No. 2377/90 and the monitoring plans must be accredited according to the ISO/IEC 17025 standard procedure for fishery products. At present, the residue monitoring plans in Malaysia are accredited to test the presence of a limited range of veterinary drugs (specifically chloramphenicol, nitrofurans, tetracycline, antibacterial substances and anthelmintic benzimidazole) in fishery products and it is therefore crucial for the Government of Malaysia to expand the scope of the accreditation to include other veterinary drugs such as anthelmintics and avermectins in order to preserve and prolong trade on fishery products with the Member States of the EU. The Food and Veterinary Office under the European Commission is authorized to evaluate the effects of aquaculture and fishery products on public health, specifically those which are produced from raw materials intended for export to the EU, and thus it is vital for the Government of Malaysia to ensure that all fishery products comply with the requirements set by the European Commission (DG (SANCO), 2012).

Anthelmintics are categorized as Group B substances and they are registered veterinary drugs which need to be monitored on an ongoing basis (European

Council Directive 96/23/EC, 1996). At present, the National Public Health Laboratory under the Food Safety and Quality Division, Ministry of Health Malaysia, is accredited to test the presence of anthelmintic benzimidazole and hence there is a critical need to develop an analytical method to test the presence of other anthelmintics (specifically avermectins) in order to support food safety monitoring programs and fulfil the requirements stipulated by the European Commission. The findings of this study will be beneficial to the Ministry of Health Malaysia in updating the Malaysia Food Act 1983 & Regulations 1985 and serves as the groundwork to assess risks before changes are implemented.

Owing to the importance of supporting food safety monitoring programmes and ensuring compliance with the requirements set by the European Commission, it is essential to devise an analytical method which will yield fast, reliable and accurate test results. Hence, a highly selective sample preparation technique needs to be established for this purpose, which includes an extraction and clean-up procedure as well as highly sensitive instrumentation. The Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) multi-residue technique developed by Anastassiades and Lehotay (2003) is particularly attractive for this purpose, and was originally developed for the analysis of pesticide residues in fruits and vegetables. The QuEChERS multi-residue technique involves the use of Dispersive Solid Phase Extraction (d-SPE) clean-up procedure, and has been implemented successfully to analyse the presence of veterinary drug residues in a number of studies, with modifications on the clean-up sorbents used for purification. It is thus necessary to investigate and validate the effectiveness of the d-SPE technique in determining the analytes of interest, particularly when the technique has been adjusted or expanded for application in specific matrices.

Since this study is deemed beneficial to Malaysia in terms of economical and technical contribution, there is a need to devise an analytical method which will yield fast and reliable results, specifically for the determination of avermectins in aquaculture fish. The main aim of this study is to implement the d-SPE method for extracting and purifying sample extracts in order to determine the presence of five avermectins (emamectin, abamectin, doramectin, moxidectin and ivermectin) in aquaculture fish. The following objectives are set in order to achieve this goal:

1. To investigate the effectiveness of clean up sorbents alumina, primary secondary amine (PSA) and C18 sorbents used and to select the clean-up sorbent(s) with the highest recovery rate in the determination of avermectins in barramundi (*Lates calcarifer*) by the d-SPE method
2. To validate and estimate the measurement of uncertainty associated with the use of the selected clean-up sorbent(s) with the d-SPE method for the determination of avermectins in barramundi (*Lates calcarifer*).
3. To verify the effectiveness of the d-SPE method of confirmatory analysis using LC-MS/MS for the determination of avermectins in five aquaculture fish species with dissimilar fatty acid content i.e. barramundi (*Lates calcarifer*), tilapia (*Oreochromis* sp.), river catfish (*Pangasius* sp.), grouper (*Epinephelus* sp.) and snapper (*Lutjanus* sp.).

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