UNIVERSITI PUTRA MALAYSIA

SUPERCRITICAL FLUID EXTRACTION AND PURIFICATION OF ASTAXANTHIN FROM MALAYSIA TIGER SHRIMP (Penaeus monodon) WASTE

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By

SHAZANA AZFAR BINTI RADZALI

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

January 2015

DEDICATION

This thesis is dedicated to my father, Dr. Radzali Muse and my mum, Puan Hasnah Hasan, my brother, Muhammad Ariff Radzali and sister, Syairah Habrah Radzali..

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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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January 2015

Chairman: Associate Professor Badlishah Sham Baharin
Faculty: Food Science and Technology

Astaxanthin is claimed to have higher antioxidant activity than that of other carotenoids such as lutein, zeaxanthin, canthaxanthin and β-carotene; the antioxidant activity of astaxanthin is also claimed to be higher than that of α-tocopherol. *Penaeus monodon* (tiger shrimp) is the largest commercially available shrimp species and its waste is a rich source of carotenoids such as astaxanthin and its esters. The extraction of thermolabile compound like carotenoids at lower temperatures through SFE can reduce the potential isomerization and degradation of the extraction product. The main objectives of this study were to find the optimum conditions for astaxanthin extraction from Tiger shrimp waste as well as to characterize and separate the free astaxanthin and its ester from the pigment extract. The efficient and environmental friendly recovery of astaxanthin was accomplished by using supercritical fluid extraction (SFE) technique. The techniques of identification and quantification of the carotenoids employed in this study were UV spectrophotometric test and high performance liquid chromatography (HPLC) analysis. The effects of different co-solvents and their concentrations on the yield and composition of the extract were investigated in this study. The following co-solvents were studied prior to the optimization of the SFE technique: ethanol, water, methanol, 50% (v/v) ethanol-water, 50% (v/v) methanol-water, 70% (v/v) ethanol-water, and 70% (v/v) methanol-water. The ethanol extract produced the highest carotenoid yield (84.02 ± 0.8µg/g) dry weight (DW) with 97.1% recovery. The ethanol extract also produced the highest amount of the extracted astaxanthin complex (58.03 ± 0.1 µg/g DW) and the free astaxanthin content (12.25 ± 0.9 µg/g DW) in the extract. Lutein and β-carotene were the other carotenoids identified. For optimization study, a central composite design (CCD) was employed to determine the effect of three supercritical carbon dioxide (SC-CO_2) parameters namely temperature (X_1) from 40 to 80ºC, pressure (X_2) from 150 to 250 bar and extraction flow rate (X_3) from 1 to 3 ml/min on the astaxanthin yield (Y_1) and free astaxanthin content (Y_2). The nonlinear regression equations were significantly ($p<0.05$) fitted for both responses with high R^2 (>0.9261), which had no indication of lack of fit. The results indicated that a combined set of values of temperature (56.88ºC), pressure (215.68 bar) and extraction flow rate (1.89 ml/min) was predicted to provide the optimum region in terms of astaxanthin yield, (58.50 ± 2.62 µg/g) and free astaxanthin content (12.20 ± 4.16 µg/g). Later, the free astaxanthin and the isomers of astaxanthin from the extracts of the shrimp waste were successfully separated using open column chromatography (OCC). Three kinds of astaxanthin isomers; *trans*-astaxanthin (478.8 nm), 9-*cis*-astaxanthin (470.4 nm), 13-

cis-astaxanthin (468.0 nm) and their esters were separated and identified according to their retention behaviour, absorbance spectra and absorption maxima by photodiode array detection. The purified astaxanthin contained approximately 85.896% (3S, 3'S)-*trans* astaxanthin (free astaxanthin), 1.944% 9-*cis*-astaxanthin, 3.681% 13-*cis*-astaxanthin, 2.825% lutein and 4.421% impurities. These findings highlighted the potential of SFE of astaxanthin and the chromatographic analysis suitable for the recovery of astaxanthin from shrimp waste. This can reduce the problems related to waste disposal itself and solvent extraction which may post a dangerous threat to the environment.

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

PENGEKSTRAKAN BENDALIR LAMPAU GENTING DAN PENULENAN ASTAXANTHIN DARIPADA SISA UDANG HARIMAU MALAYSIA (*Penaeus monodon*)

Oleh

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Januari 2015

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Astaxanthin dikatakan mempunyai aktiviti antioksidan yang lebih tinggi daripada karotenoid lain seperti lutein, zeaxanthin, canthaxanthin, dan β-karotena; aktiviti antioksidan astaxanthin juga adalah lebih tinggi daripada α-tokoferol. *Penaeus monodon* (udang harimau) merupakan spesies udang yang terbesar yang boleh didapati secara komersial dan sisanya merupakan sumber yang kaya dengan karotenoid seperti astaxanthin dan astaxanthin ester. Pengekstrakan component 'thermolabile' seperti astaxanthin pada suhu yang lebih rendah menggunakan SFE boleh mengurangkan potensi 'isomerization' dan kehilangan bahan ekstrak. Objektif utama kajian ini adalah untuk mengkaji keadaan optimum untuk pengekstrakan astaxanthin dari sisa udang harimau dan mencirikan serta memisahkan astaxanthin dari ekstrak pigmen. Kaedah pengekstrakan yang mesra alam dan cekap telah tercapai dengan menggunakan Aplikasi Bendalir Lampau Genting (*Supercritical Fluid Extraction, SFE*). Teknik-teknik pengenalan dan kuantifikasi karotenoid yang telah digunakan dalam kajian ini adalah ujian UV spektrofotometri dan analisis Kromatografi Cecair Berprestasi Tinggi (*High-Performance Liquid Chromatography, HPLC*). Kesan aplikasi bendalir lampau genting bersama pelarut dan kepekatan yang berbeza pada hasil dan komposisi ekstrak telah dikaji dalam kajian ini. Berikut merupakan pelarut yang telah dikaji sebelum pengoptimuman teknik aplikasi bendalir lampau genting: etanol, air, metanol 50% (v/v) etanol-air, 50% (v/v) metanol-air, 70% (v/v) etanol-air, dan 70% (v/v) metanol-air. Ekstrak etanol menghasilkan karotenoid tertinggi (84.02 ± 0.8 μg/g) dengan pemulihan 97.1%. Ekstrak etanol juga menghasilkan jumlah tertinggi astaxanthin kompleks (58.03 ± 0.1 μg / g DW) dan kandungan astaxanthin bebas (12.25 ± 0.9 μg / g DW) yang diekstrak. Lutein dan β-karotena adalah karotenoid lain yang turut dikenal pasti. Untuk kajian pengoptimuman, reka bentuk komposit pusat (*Central Composite Design, CCD*) telah digunakan untuk menentukan kesan tiga parameter aplikasi bendalir lampau genting iaitu suhu (X_1) 40-80ºC, tekanan (X_2) 150-250 bar dan kadar alir pengekstrakan (X_3) 1-3 mL/min pada hasil extract astaxanthin (Y_1) dan kandungan astaxanthin bebas (Y_2). Persamaan regresi tak linear yang digunakan adalah signifikan ($p < 0.05$) untuk kedua-dua respon dengan R^2 yang tinggi (> 0.9261), yang tidak menunjukkan '*lack of fit*'. Keputusan menunjukkan bahawa set gabungan nilai-nilai suhu (56.88ºC), tekanan (215.68 bar) dan kadar alir pengekstrakan (1.89 mL/min) telah diramalkan untuk menyediakan rantau yang optimum dari segi hasil astaxanthin, (58.50 ± 2.62 μg/g) dan kandungan astaxanthin bebas (12.20 ± 4.16 μg/g). Kemudian, astaxanthin bebas dan isomer-isomer astaxanthin daripada ekstrak sisa udang telah berjaya dipisahkan menggunakan Kromatografi Turus Terbuka (*Open Column Chromatography, OCC*).

Tiga jenis isomer astaxanthin; *trans*-astaxanthin (478.8 nm), 9-*cis*-astaxanthin (470.4 nm), 13-*cis*-astaxanthin (468.0 nm), dan ester mereka dipisahkan dan dikenal pasti mengikut tingkah laku penahanan, penyerapan spektra dan penyerapan maksima oleh pengesanan susunan fotodiod. Penulinan astaxanthin mengandungi kira-kira 85.896% (3S, 3'S)-*trans* astaxanthin (astaxanthin bebas), 1.944% 9-*cis*-astaxanthin, 3.681% 13-*cis*-astaxanthin, 2.825% lutein dan 4.421% bendasing. Penemuan ini menekankan potensi pengekstrakan astaxanthin melalui aplikasi bendalir lampau genting dan analisis kromatografi yang sesuai untuk pemulihan astaxanthin dari sisa udang. Ini boleh mengurangkan masalah yang berkaitan dengan pelupusan sisa itu sendiri dan kaedah pengekstrakan menggunakan pelarut kimia.

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APPROVAL

I certify that a Thesis Examination Committee has met on 16th January 2015 to conduct the final examination of Shazana Azfar binti Radzali on her thesis entitled "Supercritical Fluid Extraction and Purification of Astaxanthin from Malaysia Tiger Shrimp (*penaeus monodon*) Waste" 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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TABLE OF CONTENTS

		Page
ABSTRACT		i
ABSTRAK		iii
ACKNOWLEDGEMENTS		v
APPROVAL		vi
DECLARATION		viii
LIST OF TABLES		xiii
LIST OF FIGURES		xvi
LIST OF NOMENCLATURE		xvii
CHAPTER		
1	**GENERAL INTRODUCTION**	
	1.1 Background of Study	1
	1.2 Problem Statement and its Significance	2
	1.3 Research Objectives	3
2	**LITERATURE REVIEW**	
	2.1 Tiger Shrimp (*Penaeus monodon*)	4
	2.1.1 Chemical Composition of *Penaeus* waste	5
	2.2 Carotenoids	5
	2.2.1 Structure of Carotenoids	5
	2.3 Astaxanthin	6
	2.3.1 Chemical Structure of Astaxanthin	6
	2.3.2 Astaxanthin Isomers and Their Biological Activity	8
	2.3.3 Polarity and Solubility of Astaxanthin	8
	2.3.4 Sources of Astaxanthin	9
	2.3.5 The role of Astaxanthin in Shrimp Pigmentation	10
	2.3.6 Astaxanthin as Antioxidant	11
	2.4 Supercritical Fluid Extraction (SFE)	12
	2.4.1 Properties of Supercritical Fluid	12
	2.4.2 Supercritical Carbon Dioxide	15
	2.4.3 Supercritical Fluid Extraction System	16
	2.4.4 Factors Influencing Yield and Selectivity	17
	2.4.5 SFE of Carotenoids	21
	2.4.6 Comparison with Other Extraction Method	24
3	**CO-SOLVENT SELECTION FOR SUPERCRITICAL FLUID EXTRACTION OF ASTAXANTHIN AND OTHER CAROTENOIDS FROM *Penaeus monodon* WASTE**	
	3.1 Introduction	25
	3.2 Materials and Methods	26
	3.2.1 Chemicals and Reagents	26

	3.2.2	Preparation of the Standard Solution and Construction of the Standard Curves	27
	3.2.3	Sample Preparation	27
	3.2.4	Solvent Extractions	27
	3.2.5	Determination of Total Carotenoid Concentration	32
	3.2.6	Determination of the Astaxanthin Yield (Astaxanthin Complex)	32
	3.2.7	Determination of Astaxanthin Content (Free Astaxanthin) and other Carotenoids Content	33
	3.2.8	HPLC Analysis	33
3.3	Result and Discussion		33
	3.3.1	Carotenoids Recovery	34
	3.3.2	Composition of the Carotenoids Extract	35
3.4	Conclusions		39
4	**OPTIMIZATION OF SUPERCRITICAL FLUID EXTRACTION FOR ASTAXANTHIN EXTRACTION FROM *Penaeus monodon* WASTE USING ETHANOL-MODIFIED CO_2**		
4.1	Introduction		40
4.2	Materials and Methods		41
	4.2.1	Chemicals and Reagents	41
	4.2.2	Sample Preparation	41
	4.2.3	Experimental Design Using Response Surface Methodology (RSM)	41
	4.2.4	Statistical Analysis	42
	4.2.5	Verification of Model	43
	4.2.6	Supercritical Fluid Extraction	43
	4.2.7	Astaxanthin Complex Yield Quantification	43
	4.2.8	Free Astaxanthin Identification and	43
4.3	Results and Discussion		43
	4.3.1	Model Fitting	43
	4.3.2	Response Analysis	
	4.3.3	Optimization Condition	54
	4.2.4	Verification of the Model	55
4.4	Conclusions		57
5	**SEPARATION AND PURIFICATION OF *TRANS*-ASTAXANTHIN FROM TIGER SHRIMP (*Penaeus monodon*) WASTE EXTRACT**		
5.1	Introduction		58

5.2 Materials and Methods 59
5.2.1 Chemicals and Reagents 59
5.2.2 Sample Preparation 59
5.2.3 Supercritical Fluid Extraction (SFE) 59
5.2.4 HPLC Analysis of Carotenoids 60
5.2.5 Hydrolysis of Astaxanthin Esters 60
5.2.6 Separation of Free Trans-astaxanthin by Open Column Chromatography (OCC) 61
5.2.7 Determination of Total Carotenoid Concentration 62
5.2.8 Determination of the Astaxanthin Yield (Astaxanthin Complex) 62
5.2.9 Determination of Astaxanthin Content (Free Astaxanthin) and other Carotenoids Content 62
5.3 Results and Discussion 63
5.3.1 Analysis of Pigments 63
5.3.2 Hydrolysis of Astaxanthin Esters 66
5.3.3 Purification of *trans*-Astaxanthin 68
5.4 Conclusions 69

6 OVERALL CONCLUSIONS AND RECOMMENDATIONS
6.1 Conclusions 70
6.2 Recommendations 71

REFERENCES 88
APPENDICES
A Component Analysis 89
A.1 Standards 89
A.2 Calibration of Standards 92
B Preliminary Studies on Solvent Extraction 95
C Calculation for Extraction Flow Rate Adjustment 96
D Experimental Design of SFE 99
E Calculation for Q-Ratio 100
BIODATA OF STUDENT 101
SUBMISSION OF ABSTRACTS AND PRESENTATIONS AT INTERNATIONAL CONFERENCES 102

LIST OF TABLES

Table		Page
2.1	Poperties of gas, liquid and supercritical fluid	14
2.2	Properties of some common supercritical fluids	15
2.3	Properties of common solvents	20
2.4	Example of supercritical fluid extraction conditions employed in carotenoid extraction	22
2.5	Quantitative comparison of carotenoid in some shrimp species extracted by different solvents and extraction methods	24
3.1	Dilution factors of five points aliquots for carotenoid standard curve construction	26
3.2	Yield and recovery of total carotenoid from *Penaeus monodon* waste for all extractions	34
3.3	Composition of yield of extract, total and individual carotenoid content (µg/g DW) in *Penaeus monodon* waste for all extractions	36
4.1	Levels of independent variables established according to the CCD	42
4.2	Matrix of the CCD	44
4.3	Regression coefficients, R^2, adjusted R^2, probability values, and lack of fit for two dependent variable [a]	45
4.4	F ratio and p value for each independent variable effects in the polynomial response surface models [a]	48
4.5	Experimental and predicted values for the response variables [a]	56
5.1	Tentative identification of some major pigments detected in the extract of *Penaeus monodon*	64
5.2	Tentative results for determination of some carotenids in *Penaeus monodon* waste	69

LIST OF FIGURES

Figure		Page
2.1	Characteristic of Tiger Shrimp (*Penaeus monodon*)	4
2.2	Chemical Structures of Some Carotenoids	6
2.3	Astaxanthin Configurational Isomers (a–c) and a Geometric *Cis* Isomer (d)	7
2.4	Temperature-pressure Phase Diagram of Carbon Dioxide	13
2.5	Influence of Pressure and Temperature on Carbon Dioxide Density	14
2.6	Simplified Schematic Diagram of SFE System	16
3.1	Sample of Tiger Shrimps (*P. monodon*)	27
3.2	Carotenoids Extract from Solvent Extraction Method	28
3.3	SFE Setup at Separation Technology laboratory in Faculty of Engineering and Built Environment UKM	30
3.4	Schematic Diagram of SFE System with Co-solvent	30
3.5	HPLC Analysis of Carotenoids Extracted Using Solvent from *Penaeus monodon* Waste, Astaxanthin Esters (AE)	38
3.6	HPLC Analysis of Carotenoids Extracted Using SFE with Ethanol as a Co-solvent, at 60 ºC and 200 bar from *Penaeus monodon* Waste (1) Free Astaxanthin, (2) Lutein, (3) *β*-carotene and Astaxanthin Esters (AE)	38
4.1	Diagnostic Plots of Astaxanthin Complex Yield	46
4.2	Diagnostic Plots of Astaxanthin Content	47
4.3	(a) Response Surfaces and (b) Contour Curves for the Extraction Yield of Astaxanthin (µg astaxanthin/g DW) as a Function of Extraction Pressure (bar) and Temperature (ºC)	50
4.4	(a) Response Surfaces and (b) Contour Curves for the Astaxanthin Content (µg Astaxanthin/g DW) as a Function of Extraction Pressure (bar) and Temperature (ºC)	51
4.5	Response Optimization of the Effects of 215.68 bar, 56.88ºC and 1.89 mL/min on Astaxanthin Complex Yield (µg astaxanthin/g DW) and Astaxanthin Content (µg astaxanthin/g DW)	53
5.1	Saponified Astaxanthin Extract	61
5.2	Separation of Astaxanthin on OCC	62
5.3	HPLC Chromatogram of the Main Carotenoids Detected in *Penaeus monodon* Waste Using SFE with Ethanol as a Co-solvent, at 57ºC and 215.68 bar (1) (3S, 3'S)-9-*cis*-astaxanthin (470.4 nm), (2) (3S, 3'S)-13-*cis*-astaxanthin (371.8 and 468 nm), (3) (3R, 3'R)-*trans*-astaxanthin (477.4 nm), (4) (3S, 3'S)-*trans*-astaxanthin (478.8 nm), (5) Lutein (422, 445, 473), Un-hydrolyzed Astaxanthin Esters (AE), and (6) *β*-carotene (425, 450, 477)	63
5.4	Absorption spectra of (a) *β*-carotene, (b) Lutein, (c) (3S, 3'S)-9-*cis*-astaxanthin, (d) (3S, 3'S)- *trans*-astaxanthin, (e) (3R, 3'R)-*trans*-astaxanthin, (f) (3S, 3'S)-13-*cis*-astaxanthin in the Mobile Phase Containing Acetonitrile:water (9:1 v/v) and Ethyl Acetate	65
5.5	HPLC Chromatograms of the Pigment Extracts During Saponification for (a) 3 hours, (b) 6 hours, (c) 9 hours, and (d) 12 hours. Peaks: 1, (3S, 3'S)-9-*cis*-astaxanthin; 2, (3S, 3'S)-13-*cis*-astaxanthin; 3, (3R, 3'R)-*trans*-astaxanthin; 4, (3S, 3'S)- *trans*- astaxanthin; 5, Lutein, Un-hydrolyzed Astaxanthin Esters (AE) and 6, *β*-carotene	67

5.6 HPLC Chromatogram of the Saponified and Purified *trans* Astaxanthin. (1) (3S, 3'S)-9-*cis*-astaxanthin (470.4 nm), (2) (3S, 3'S)-13-*cis*-astaxanthin (371.8 and 468 nm), (3) (3R, 3'R)-*trans*- astaxanthin (477.4 nm), (4) (3S, 3'S)-*trans*-astaxanthin (478.8 nm) and (5) Lutein (422, 445, 473) 68

LIST OF ABBREVIATIONS

AE	Astaxanthin Esters
ANOVA	Analysis of Variance
ASES	Aerosol Solvent Extraction System
BPR	Back Pressure Regulator
CAC	Codes Alimentarius Commission
CCD	Central Composite Design
cm	Centimeter
CO_2	Carbon dioxide
DW	Dry Weight
DWB	Dry Weight Basis
e.g.	For example
et al.	(et alia): and others
FAO	Food and Agriculture Organization
g	Gram
GAS	Gas Anti-Solvent
GRAS	Generally Recognized As a Safe
HPLC	High Performance Liquid Chromatography
LC-MS	Liquid Chromatography–Mass Spectrometry
M	Morality
Mg	Milligram
mL	Millilitre
mm	Millimetre
MW	Molecular Weight
ND	Not Detected
nm	Nanometre
OCC	Open Column Chromatography
P	Probability
PDA	Photodiode array detector
RESS	Rapid Expansion of Supercritical Solutions
RSM	Response Surface Methodology
RT	Retention Time
SAS	Supercritical Anti-Solvent
SC-CO_2	Supercritical Carbon Dioxide
SCF	Supercritical fluid
SD	Standard Deviation
SFC	Supercritical Fluid Chromatography
SFE	Supercritical Fluid Extraction
TC	Total Carotenoids
TLC	Thin Layer Chromatography
UV	Ultra Violet
v	Volume
w	Weight
WWB	Wet Weight Basis
µg	Microgram
µm	Micrometre
µL	Microlitre

LIST OF NOMENCLATURES

A_{468}	Absorbance at 468 nm	-
λ	Absorbance maxima	-
R^2_{adj}	Adjusted R-square	-
Y_1	Astaxanthin yield	µg/g
°C	Celsius	-
Δu^{evap}	Cohesive energy density	J.mol/mL
C_b	Concentration at 648 nm	µg/g
C_a	Concentration at 666 nm	µg/g
P_c	Critical pressure	bar
T_c	Critical temperature	ºC
D_m	Diffusivity	cm^2/s
Y_o	Experimental value	µg/g
X_3	Flow rate	mL/min
ρliq	Fluid density at liquefied state	g/cm^3
ρ	Fluid density at supercritical state	g/cm^3
Y_2	Free astaxanthin content	µg/g
$>$	greater than	-
δ	Hildebrand solubility parameter	$J^{1/2}/m^{3/2}$
b_{ij}	Interaction effects	-
b_0	Intercept term	-
$<$	less than	-
b_i	Main effects for each variable	-
Y_i	Predicted value	µg/g
X_2	Pressure	bar
p	Probability	-
Q-ratio	Ratio of the height of the maximum absorbance peak to the *cis* peak	-
R^2	Regression coefficient	-
Y_o-Y_i	Residue	µg/g
Y_i	Response variable	µg/g
$\pm$	standard deviation	-
γ	Surface tension	cal/mol. $Å^2$
T	Temperature	ºC
X_1	Temperature	ºC
C_{x+c}	Total carotenoids concentration at 480 nm	µg/g
v	Viscosity	g/cm.s
$V_{extract}$	Volume of extract	mL
W_{sample}	Weight of sample	g

CHAPTER I

INTRODUCTION

1.1 Background of Study

Astaxanthin (3,3-dihydroxy-β,β-carotene-4,4-dione) is the most valuable ketocarotenoid, both from a biotechnological and commercial point of view. It has been found in most crustaceans like shrimps, crabs and lobsters (Guerin *et al.* 2003). This carotenoid pigment is also found in birds like flamingo, and in insects, microorganisms, and micro-green alga (*Haematococcus pluvialis*) (Guerin *et al.* 2003). It exhibits a vibrant red color and higher antioxidant activity compared to other carotenoids such as α-carotene, β-carotene, lutein, lycopene, canthaxanthin, and vitamin E (Kurashige *et al.* 1990; Shimidzu *et al.* 1996).

Astaxanthin has been applied as a food colorant and in pharmaceutical products (Johnson and An 1991; Lorenz and Cysewski, 2000). It is also an important source of pigmentation in aquaculture industries (Hussein *et al.* 2006; Lorenz and Cysewski, 2000). Recently, some studies proved that astaxanthin inhibits the invasion of cancer cells inflammation, *Helicobacter pylori* infection, aging and age-related macular degeneration and play key roles in enhancement of the immune response, liver function, heart, eye, joint and prostate health (Guerin *et al.* 2003).

Nowadays, a diverse range of global industries has used large scale of organic solvents. Unfortunately, this can cause potential danger to the environment. As an eco-friendly process, SFE is an alternative to the conventional solvent extraction method. The growth of SFE technologies has been stimulated by the rise of stricter environmental regulations related to the use of industrial solvents that can harm human health. The applications of supercritical fluids are more beneficial owing to less deterioration of thermolabile compounds and simplicity (Bruno *et al.* 1993). Compared to the conventional extraction, less time is required for SFE due to greater rate of mass transfer in supercritical fluids.

1.2 Problem Statement and its Significance

In recent years, the global production of shrimp has been growing gradually and this trend is predicted to continue (FAO, 2005). In the last 20 years, shrimp has contributed to about 20% of the total value of exported fishery products (CAC, 2002). According to Lowther (2005), in 2003, the global production of shrimps has been estimated at 1,804,932 tonnes per year (Lowther, 2005). Major shrimp-culturing countries are Thailand, Indonesia, Malaysia, China, India, Bangladesh, Vietnam, the Philippines, Myanmar and Australia (FAO, 2005). Other than that, shrimp processing plants produce a large volume of waste products. The shrimp body parts that are processed for human consumption comprises only 70% of the overall shrimp landing (Sachindra *et al.* 2007). Hence, a remarkable tonnage of shrimp waste is generated, from which astaxanthin, one of the major carotenoids, can be obtained.

Penaeus monodon is also endemic and found in the waters of Malaysia (Mazuki, 2008). Astaxanthin is the major carotenoid in *Penaeus monodon*'s waste and it exists mostly as astaxanthin esters (Boonyaratpalin *et al.* 2001; Okada *et al.* 1994). This pigment is a potential source of carotenoids for the aquaculture and poultry industries, which need enormous supplies of this carotenoid.The increasing demand for natural food has encouraged research on the extraction of astaxanthin from natural sources. Normally, organic solvents such as dichloromethane and acetone are used for the extraction of astaxanthin; however, the usage of the aforementioned solvents may cause safety issues. The poor yields (almost 50% of the pigments are lost) and considerable environmental concern of the traditional extraction methods have motivated further studies on SFE as an effective alternative to the traditional methods (Delgado-Vargas and Paredes-Lopez, 2003).

SFE has been used for the extraction of bioactive compounds from foods (Mendes *et al.* 2003; Sun and Temelli, 2006). This method is more significant when thermo-labile compounds are present. In addition, the use of toxic solvents can be avoided, since carbon dioxide (CO_2) is inexpensive and generally recognized as a safe (GRAS) solvent, which is easy to separate from the extract (Sahena *et al.* 2009; Mercadante, 2008; Reverchon and De Marco, 2006). Supercritical fluids have outstanding extractive properties such as liquid-like density, high compressibility, high diffusivity, and low viscosity (Lim *et al.* 2002).

However, one shortcoming of supercritical carbon dioxide (SC-CO_2) is that it is often incapable of extracting slightly polar analytes due to its poor interaction with the matrices and weak solvating power of polar compounds (Pawliszyn, 1993). For extraction of astaxanthin, polar entrainers used can increase the extraction efficiency of CO_2 by improving the solubility of astaxanthin in the mixture and help to reduce the interaction between analytes and the matrix (Charest *et al.* 2001; Machmudah *et al.* 2006; Lim *et al.* 2002). Other than that, numerous studies have been conducted on the SC-CO_2 extraction of carotenoids from crustacean and marine animal waste (Charest *et al.* 2001; Yamaguchi *et al.* 1986; Hardardottir and Kinsella, 1988). However, in those studies, the recovery of astaxanthin was relatively low compared to that of conventional methods, and the selectivity of the compounds extracted from the shrimp waste remained uncertain.

Penaeus monodon or Tiger shrimp is one of the most significant commercial species found in Malaysian waters, and in terms of production, it has continued to be the most important species for the past 5 years at a value of USD 160, 186 (FAO, 2008). Astaxanthin is the main carotenoid extracted from the *P. monodon* species (Katayama *et al.* 1972; Katayama *et al.* 1971). Despite Malaysia being one of the most important producers of this species, the shrimp processing waste generated in Malaysia is not commercially exploited for the recovery of astaxanthins (FAO, 2005). To the best of our knowledge, there is no report on the determination of carotenoid contents from the wild Malaysian *P. monodon* waste. Thus, this study will offer new insights into the determination of carotenoids in the *P. monodon* waste from Malaysia. From a green technology point of view, the health food industry will benefit tremendously if this precious pigment could be extracted from an inexpensive raw material instead of it being chemically synthesized. Therefore, this study is designed to extract astaxanthins from *P. monodon* waste owing to the abundance of this species in Malaysia.

1.3 Research Objectives

This study embarked on the following objectives:

1) To extract astaxanthin from Malaysian Tiger shrimp waste (*P. monodon*) using Supercritical Fluid Extraction (SFE) and to optimize the SFE extraction conditions

2) To separate and purify the free *trans*-astaxanthin from the astaxanthin complex mixture of the Tiger Shrimp (*P. monodon*) waste

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