



UNIVERSITI PUTRA MALAYSIA

**ISOLATION, IDENTIFICATION AND ANTIOXIDATIVE ACTIVITY OF
PHENOLICS IN PALM OIL MILL EFFLUENT**

NORMALA BT HALIMON.

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ACTIVITY OF PHENOLICS IN PALM OIL MILL EFFLUENT**

By

NORMALA BT HALIMON

**Thesis Submitted to the School of Graduate Studies, University Putra Malaysia, in
Fulfilment of the Requirements for the Degree of Doctor of Philosophy**

January 2006



DEDICATION

Bismillahirrahmannirrahim,

Alhamdulillah.

Specially dedicated to my beloved father, Halimoon Bin Hamid and my mother, Rahamah Bt Mohd, my loving husband, Khairul Husni Bin Kamaruddin, my cute daughter, Nurhusna Aina Bt Khairul Husni, and my handsome son, Muhammad Zafran Bin Khairul Husni, all my siblings and my friends.

Thanks for your loving support during the difficult time.

Amin.

**Abstract of dissertation submitted to the Senate of Universiti Putra Malaysia
in fulfilment of the requirements for the degree of Doctor of Philosophy**

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Chairman: Associate Professor Azizah Binti Abdul Hamid, PhD

Faculty: Food Science and Technology

The objectives of the study are to isolate, and identify the phenolic compounds present in the palm oil mill effluent (POME) and evaluate their antioxidative activity. Combinations of column and partition chromatography were used in the isolation of the phenolic compounds. Antioxidative activities of the POME fractions were evaluated for free radical scavenging activity, lipid thiocyanate, β -carotene-linoleate and reducing assays. Identification of the phenolic compounds in POME filtrate was carried out using high performance liquid chromatography (HPLC), followed by confirmation of the compounds using gas chromatography mass spectrometry (GC/MS) and liquid chromatography mass spectrometry (LC/MS). The study revealed the presence of cinnamic acid derivatives, which were measured at 320 nm. These included chlorogenic, *p*-coumaric, caffeic, ferulic acids and rutin hydrate. Benzoic acid derivatives, which were identified as protocatechuic, gentisic, *p*-hydroxybenzoic and vanillic acids were also present when measured at 260 nm. Hydrolysis of the sample did not affect the

chromatographic pattern obtained indicating the stability of the phenolic compounds in the POME filtrate. Flavan-3-ol groups (catechins) were identified from ytterbium precipitation method. Four fractions have been isolated from POME filtrate based on flash chromatography coupled with uv/vis detection. The antioxidative activity of each fraction was then evaluated. All the POME fractions demonstrated excellent antioxidative activity in all the assays used. This is especially true for both Fractions 3 and 4. Fraction 1 exhibited highest antioxidative activity based on reducing assay followed by Fraction 3 indicating the excellent metal chelating ability of the phenolics in these fractions. The POME fractions were found to contain different levels of total phenolics, which did not necessarily correspond to the strength of antioxidative activity, measured for each fraction. Fraction 3 showed strong free radical scavenger and phenolic content was also high. Fraction 2 showed low antioxidant activity, while total phenolic content was low. However, Fraction 4 showed high antioxidant activity with low total phenolic content. This indicates the presence of different phenolic compounds in the different fractions. Results showed that Fraction 1 contained an unknown polar compound and protocatechuic acid with strong metal chelating activity, Fraction 2 was rich in *p*-hydroxybenzoic acid, Fraction 3 contained three unknown cinnamic acid derivatives and Fraction 4 contained known cinnamic acid derivatives compounds including *p*-coumaric acid, rutin hydrate and ferulic acid with lower polarity. The cinnamics in Fraction 3 consistently exhibited higher antioxidative properties compared with benzoics in Fraction 2. Combination study of the fractions revealed that mixture of Fraction 3 and ascorbic acid had better free radical scavenging activity. However, the synergistic effect was observed only at low concentration of 2.5 and 5 mg/l of Fraction 3 and ascorbic acid. The

individual compounds of Fraction 3 (cinnamic acid derivatives) including Compounds 14, 15 and 16 exhibited the highest free radical scavenging activity. Highest antioxidative activity was also measured when Compounds 15 and 16 were combined (double combined) while for triple combinations, the highest activity was seen with Compounds 13, 14, and 15 at 15 mg/l of total phenolic contents. The results showed that there was a synergistic effect between the compounds in particular those in Fraction 3. GC/MS and LC/MS were employed to confirm identify of unknown compounds in Fraction 3. Three compounds in Fraction 3 were observed to exhibit similar absorbance spectra using HPLC with diode array detection. LC/MS revealed presence of dimer with maximum molecular weight of 335 m/z. GC/MS revealed the presence of compounds with a general cinnamic acid structure and derivatives with silylate group. Antioxidative activity of the compounds was in the following order; Compound 14 > Compound 16 > Compound 15. Compounds 14, 15 and 16 were tentatively identified as 2,4-dihydroxycinnamic acid, 2,5-dihydroxycinnamic acid and 2,3-dihydroxycinnamic acid.

**Abstrak disertasi yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi syarat untuk Ijazah Doktor Falsafah**

**PEMENCILAN, PENGENALPASTIAN DAN AKTIVITI ANTIOKSIDAN
FENOLIK DALAM AIR SISA KELAPA SAWIT**

Oleh

NORMALA BT HALIMOON

January 2006

Pengerusi : Profesor Madya Azizah Binti Abdul Hamid, PhD

Fakulti : Sains dan Teknologi Makanan

Objektif kajian ini merangkumi pemencilan, dan mengenalpastian sebatian fenolik yang wujud dalam air sisa buangan minyak kelapa sawit (POME) dan menilai aktiviti antioksidannya. Gabungan turus dan titikan kromatografi telah digunakan untuk memenculkan sebatian fenolik. Aktiviti antioksidan fraksi POME telah dinilai dengan menggunakan asai aktiviti memerangkap radikal bebas, asai β -karoten-linoleat, asai tiosianat dan aktiviti penurunan. Sebatian fenolik dikenal pasti di dalam tapisan POME menggunakan kromatografi cecair berkeupayaan tinggi (HPLC), diikuti dengan proses pemastian menggunakan kromatografi gas spektrometri jisim (GC/MS) dan kromatografi cecair spektrometri jisim (LC/MS). Kajian ini telah menunjukkan kehadiran terbitan asid cinnamik terdapat di dalam tapisan POME pada 320 nm termasuklah asid klorogenik, asid *p*-koumarik, asid kaffeik, asid ferulik dan rutin hidrat. Terbitan asid benzoik iaitu asid protokatekuik, asid gentisik, asid *p*-hidroksibenzoik dan asid vanillik juga hadir pada 260 nm. Sampel yang dihidrolisis didapati tiada perubahan kromatografi menunjukkan

tapisan POME hanya mengandungi sebatian fenolik yang stabil. Kumpulan Flavan-3-ol (catekin) dikenalpasti melalui kaedah pemendakan ytterbium. Empat fraksi telah dipencarkan dari tapisan POME berdasarkan imbasan kromatografi bersama dengan penentuan uv/vis. Aktiviti antioksidan setiap fraksi telah dinilai. Semua fraksi POME yang diuji menunjukkan aktiviti antioksidan yang tinggi dalam semua asai yang digunakan. Keadaan ini adalah jelas bagi kedua-dua Fraksi 3 dan 4. Aktiviti antioksidan yang tinggi bagi Fraksi 1 berdasarkan asai penurunan diikuti dengan Fraksi 3, ini menunjukkan bahawa fenolik berkebolehan mengikat logam dalam fraksi tersebut. Fraksi POME didapati mengandungi sebatian fenolik yang berbeza dan aktiviti antioksidan yang ditentukan tidak perlu bergantungan di antara satu sama lain. Fraksi 3 menunjukkan perangkap radikal bebas yang kuat dan kandungan fenoliknya juga tinggi. Fraksi 2 menunjukkan aktiviti antioksidan yang rendah sementara kandungan jumlah fenoliknya juga rendah. Walau bagaimanapun, Fraksi 4 menunjukkan aktiviti antioksidan yang tinggi dengan kandungan fenolik yang rendah. Ini menunjukkan kehadiran sebatian fenolik yang berbeza di dalam fraksi yang berbeza. Keputusan juga menunjukkan bahawa Fraksi 1 mengandungi sebatian polar yang tidak diketahui termasuk asid protokatekuik dengan aktiviti pengikatan logam yang kuat, Fraksi 2 kaya dengan asid *p*-hidroksibenzoik, Fraksi 3 mengandungi tiga terbitan asid cinnamik yang tidak diketahui dan Fraksi 4 mengandungi terbitan asid cinnamik termasuklah asid *p*-koumarik, rutin hidrat dan asid ferulik iaitu sebatian yang tidak polar. Terbitan asid cinnamik dalam Fraksi 3 menunjukkan sifat antioksidan yang tinggi secara seragam berbanding dengan terbitan asid benzoic dalam Fraksi 2. Kajian gabungan sebatian fraksi POME mendapat campuran Fraksi 3 dengan asid askorbik menunjukkan aktiviti perangkap radikal bebas

yang lebih baik. Bagaimanapun, kesan sinergistik hanya pada kepekatan yang rendah iaitu 2.5 dan 5 mg/l bagi Fraksi 3 dan asid askorbik. Sebatian tunggal Fraksi 3 (terbitan asid cinnamik) termasuklah sebatian 14, 15, dan 16 dalam menunjukkan aktiviti perangkap radikal bebas yang tertinggi. Aktiviti antioksidan yang tinggi juga ditentukan bagi gabungan dua sebatian 15 dan 16 serta gabungan tiga sebatian iaitu sebatian 13, 14 dan 15 memberikan aktiviti yang tinggi pada jumlah fenolik 15 mg/l. Ini menunjukkan kesan sinergistik berlaku diantara sebatian yang wujud dalam Fraksi 3. GC/MS dan LC/MS telah digunakan untuk memastikan sebatian fenolik yang tidak dikenalpasti. Keputusan HPLC dengan penentuan diod arrai menunjukkan bahawa tiga sebatian di dalam Fraksi 3 didapati menunjukkan absorban spektra yang sama. LC/MS menunjukkan kehadiran dimer dengan berat molekul yang maksima pada 335 m/z. GC/MS menunjukkan kehadiran struktur umum asid cinnamik dan terbitannya dengan sililate. Aktiviti antioksidan adalah seperti berikut; Sebatian 14 > Sebatian 16 > Sebatian 15. Sebatian 14, 15 dan 16 dikenali sebagai asid 2,4-dihidroksicinnamik, asid 2,5-dihidroksicinnamik dan asid 2,3-dihidroksicinnamik.

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LIST OF ABBREVIATIONS

AOA	antioxidant activity
APCI	atmospheric pressure chemical ionization
BOD	biological oxygen demand
BSTFA	bis(trimethylsilyl)trifluoro-acetamide
C	carbon
COD	chemical oxygen demand
Cu	copper
DCM	dichloromethane
DNA	deoxyribonucleic acid
°C	degree Celsius
DOE	Department of Environment
DPPH	1, 1 – diphenyl – 2 - picrylhydrozyl
ELSD	evaporative light scattering detector
ESI	electrospray ionization
Fe	ferric
Fe³⁺	ferrous
FES	filtrate extract supernatant
FC	Folin-Ciocalteu
FPLC	flash pack liquid chromatography
FTC	ferric thiocyanate
GAE	gallic acid equivalent
GC/MS	gas chromatography/mass spectrometry
g	gravity
g	gram
HCl	hydrochloric acid
HPLC	high performance liquid chromatography
hr	hour
H₂O₂	hydrogen peroxide

LC/MS	Liquid chromatography/mass spectrometry
LDL	low-density lipoprotein
Nm	nanometer
MPOB	Malaysia Palm Oil Board
mg	milligram
mg/g	milligrams per gram
min	minute
μm	micrometer
μl	microlitre
mg/l	milligram per litre
ml	millilitre
mm	millimeter
M	molar
OD	optical density
OFE	original filtrate extract
O&G	oil & grease
OH	hydroxyl radicals
OMWW	olive mill wastewater
O₂	superoxide anions
PDA	photodiode array detector
CPO	crude palm oil
POME	palm oil mill effluent
ppm	parts per million
%	percentage
RFE	recovered filtrate extract
ROS	radical oxygen species
ROOH	lipid peroxy radicals
TCA	trichloroacetic acid
TFA	trifluoroacetic acid
TLC	thin layer chromatography
TS	total solids

t/t	tonne per tonne
SS	suspended solids
v/v	volume per volume
Yb-ppt	ytterbium-precipitated
Yb³⁺	ytterbium ion