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**Pertanika Journal of Tropical Agricultural Science**
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## Contents

# Foreword

Welcome to the **Second Issue 2013** of the Journal of Tropical Agricultural Science (JTAS)!

JTAS is an open access journal for the Tropical Agricultural Science published by Universiti Putra Malaysia Press. It is independently owned and managed by the university and run on a non-profit basis for the benefit of the world-wide science community.

In this issue, there are **8 articles** published; out of which **2 articles** are short communication and **6 articles** are regular research papers. The authors of these articles vary in country of origin (***Malaysia, Nigeria, and Iran***).

The short communications in this issue report an update made by the group of researchers in Malaysia. One of the groups has successfully isolate the mitochondrial control region of White-nest Swiftlets (*Aerodramus fuciphagus*) by using the Primer Walking techniques (*Goh, W. L., Lim C. K. and Rahman, M. A.*) while the other research group has successfully identified sexes of Colugo (*Galeopterus variegatus*) by using the gliding motion photography technique (*Dzulhelmi M. N. and Suriyanti S. N. P.*).

The regular articles cover a wide range of topics. Group of researchers from University of Agriculture, Abeokuta, Nigeria share their findings that the different particle size of pelletized chicken's feed will affect the carcass characteristics and intestinal morphology of Broiler chicken (*Aderibigbe, O. B., Sogunle, O. M., Egbeyale, L. T., Abiola, S. S., Ladokun, O. A. and Ajayi, O. L.*).

Researchers from Universiti Putra Malaysia reported that the use of different extraction methods towards the marine microalgae *Nannochloropsis oculata* and *Chaetoceros gracilis* will yield a different composition of fatty acid and carotenoid yield (*Loh, S. P. and Lee, S. P.)*, soil factors and soil types will influence a heavy metals concentration in medicinal plants (*Dayang S. N. and I. Che Fauziah*) and the appropriate desorption isotherm model for a Malaysian rough rice variety (MR219) was derived by comparing five most commonly used desorption models (*M. Nordin Ibrahim, K. Tajaddodi Talab, S. Spotar, Kharidah, M. and Rosnita, A. T.*).

Besides Universiti Putra Malaysia, researchers from Universiti Malaysia Sabah have identified a potential bacterial that play a role in bacteria-algae association of toxin-producing *Pyrodinium bahamense* var. *compressum* (Böhm) that contribute to paralytic shellfish poisoning (*Chin G. J. W. L., Teoh P. L., Kumar S. V. and Anton A.*). I conclude with the researchers from Universiti Teknologi MARA, Malaysia in which they successfully evaluated the protein hydrolysate produced from Mentarang (*Pholas orientalis*) (*Normah, I. and Nurfazlika Nashrah, M. P.*).

I anticipate that you will find the evidence presented in this issue to be intriguing, thought provoking, and hopefully useful in setting up new milestones. Please recommend the journal to your colleagues and students to make this endeavour meaningful.

I would also like to express my gratitude to all the contributors; namely the authors, reviewers and editors for their professional contributions who have made this issue feasible. Last but not the least the editorial assistance of the journal division staff is fully appreciated.

JTAS is currently accepting manuscripts for upcoming issues based on original qualitative or quantitative research that opens new areas of inquiry and investigation.

**Chief Executive Editor**
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*Short Communication*

# Isolation of Mitochondrial Control Region for White-nest Swiftlets (*Aerodramus fuciphagus*) Using Primer Walking Techniques

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## ABSTRACT

This paper reports on a novel DNA sequence located at the mitochondrial control region (D-loop) of the white-nest swiftlet (*Aerodramus fuciphagus*). This hypervariable control region sequence is potentially useful for studying genetic relationships among the white-nest swiftlet populations. The isolation of the control region involves a primer walking technique, which is simple, fast and cost-effective. In this study, the variability of the control region was assessed and discussed.

*Keywords: Aerodramus fuciphagus*, control region, Mitochondrial DNA, primer walking

## INTRODUCTION

The most commonly used DNA markers in the molecular studies of swiftlets are cytochrome *b* of mitochondrial DNA (mtDNA; Lee *et al*., 1996; Thomassen *et al*., 2003; Price *et al*., 2004; Thomessen *et al*., 2005; Aowphol *et al*., 2008) and NADH dehydrogenase sub-unit 2 of mtDNA (NADH-2; Price *et al*., 2004; Thomassen *et al*., 2005; Aowphol *et al*., 2008). In particular, nuclear 12S and beta-fibrinogen intron regions were sequenced by Thomassen *et al*. (2005), whereas a microsatellite genotyping method was established by Aowphol *et al*. (2008). Notably, most of these markers were not specially developed for resolving the relationships of the swiftlets at lower taxonomic-levels. A non-coding region

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in the mtDNA is, therefore, expected to provide more informative characters in examining the phylogenetic relationships among the swiftlet populations.

One of the most variable regions in the mtDNA genome is the control region, also known as D-loop (Rahman *et al*., 2010). The control region of avian mtDNA contains three domains based on the distribution of the variable nucleotide positions and the differential nucleotide frequencies of parts of the control region (Quinn & Wilson, 1993). It was reported that Domains I and III were more variable compared to Domain II, as the average substitution rates for Domain I, Domain II and Domain III were 16%, 2.7%, 18.6%, respectively (Delport *et al*., 2002). There could also be a big difference in the substitution rate between the first half and second half of Domain I, for example, 2% and 20% were reported for the first and second half of Domain I (Randi & Lucchini, 1998). Sbisà *et al*. (1997) and Randi and Lucchini (1998) suggested the adoption of the following nomenclature for the three D-loop domains: the extended termination-associated sequence (ETAS) of Domain I, the central conserved domain of Domain II, and the conserved sequence blocks (CSB) of Domain III.

As there has been no mtDNA control region sequence reported for the white-nest swiftlet or its related species, this study aims to acquire the DNA sequence of this region using the white-nest swiftlets. This study also intends to develop a primer walking strategy for sequencing a DNA region with no prior information. Primer walking is a rapid and simple strategy developed for obtaining the sequences of large DNA fragments using the DNA cloning method (Strauss *et al*., 1986). This strategy is then widely used with several modifications customised for different circumstances (Kieleczawa *et al*., 1992; Kotler *et al*., 1994; Lodhi & McCombie, 1996; Gromek & Kaczorowski, 2005; Cairns *et al*., 2009).

## MATERIALS AND METHODS

### *Primer Walking*

The total DNA of the white-nest swiftlet embryo was extracted using the Promega DNA Extraction Kit following the manufacturer's instructions. The avian universal primers for the mtDNA region spanning the NADH6 to the control region, Thr (L) and H1251 (Desjardins & Morais, 1990) were used in the first step of primer walking. The sequence of the light strand of the 2 kb polymerase chain reaction (PCR) product was determined up to 500 bp from the 5' end. From this sequence, the second forward primer (L453) was designed. A primer pair of L453 and H1251 was used to amplify the DNA sample to give a 1.5 kb PCR product. This process of primer design, PCR and sequencing was continued until the whole control region was sequenced. Primers L12 and H12 were designed to amplify the range of 'partial ND6–$tRNA^{Glu}$–partial control region' for the phylogenetic analysis. All the primers, represented by 1 – 7 (Table 1) and their position in the mitochondrial genome are shown in Fig.1. Polymerase chain reaction (PCR) was run using a Perkin

TABLE 1
The primers used to design the primers of control region in this study.

| No. | Primer name | Primer sequence (5' – 3') | Forward / Reverse |
|---|---|---|---|
| 1. | Thr(L) | TTG TAA CAA GGA CAT TTG GTT TCT | Forward |
| 2. | H1251 | TCT TGG CAT CTT CAG TGC CRT GC | Reverse |
| 3. | L453 | CAA CGA CAC AAA GGA GAG GC | Forward |
| 4. | L103 | CAT AAG AGT TTC CAC TTG GC | Forward |
| 5. | H238 | AAA TGC CGC GAT TAC GGG TG | Reverse |
| 6. | L12 | AAC CAA CCA CCC CAT AGT AA | Forward |
| 7. | H12 | GAG ATA GCG GCA TAC CTA GC | Reverse |

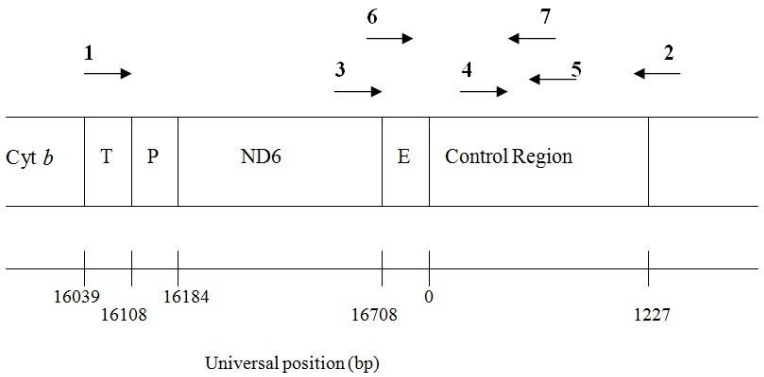


Fig.1: The universal positions of the primers. The numbers indicate the primer listed in Table 2.3. The arrows indicate the direction of the primers. T refers to $tRNA^{Thr}$, P refers to $tRNA^{Pro}$, E refers to $tRNA^{Glu}$

Elmer GeneAmp 9600 Thermocycler with the programme set at 2 min at 95.0°C; 30 cycles of 30 a at 94.0°C, 45 s at annealing temperature, 1 min at 72.0°C; 5 min at 72.0 °C; hold at 4.0°C. Annealing temperatures ranged from 55.0°C to 62.0°C. The PCR reaction mixture contained 1.5 mM $MgCl_2$, 0.5 µM forward and reverse primers each, 0.2 mM of dNTPs, 1× PCR buffer and ~10 ng of DNA samples. PCR products were purified using the Promega PCR Clean-up System kits following the instructions by the manufacturer. The purified PCR products were sent to the commercial laboratories [FirstBase Laboratories Sdn. Bhd. and Century Science Equipment (Sarawak) Sdn. Bhd.] for direct sequencing. All the DNA sequences obtained were deposited into Genbank (Accession number: JF269187–JF269235).

The partial control region sequences of 35 individuals were then obtained using Primers L12 and H12 (Table 1). These individuals were collected from the swiftlet houses of five localities: 10 from Endau-Rompin (02° 40−48' N; 103° 29−36' E), nine from Kuantan (03° 49' N; 103° 19' E), seven from the West Coast of Peninsular Malaysia (Perak and Selangor, i.e., 03° 46' − 04° 13' N; 100° 41−59' E), six from Sumatra

(03° 35' N; 98° 40' E) and three from Sibu (02° 18' N; 111° 49' E).

*Data Analysis*

The DNA sequences were trimmed to readable bases on both ends of the strands. In most cases, the scoring of the bases started by the light-strand complementing the light-strand towards the centre. The mitochondrial control region sequence of the closest related species thus far reported was that of the *Apus apus* (Apodiformes, the swift family) (GenBank accession no.: NC008540; Slack *et al*., 2009). This sequence was aligned with one of the DNA sequences obtained for the white-nest swiftlets in the present study.

To assess the variability of the control region, the sequences of all individuals were aligned using the Clustal X version 1.81 (Thompson *et al*., 1997) and adjusted manually using Bioedit (Hall, 1999) whenever necessary. Indels were coded following the Simple Indel Coding method (Simmons & Ochoterena, 2000) using the FastGap1.1 programme (Borchsenius, 2009). Maximum parsimonious (MP) analysis was conducted using PAUP4.0 (Swofford, 2002) and the phylogenetic tree was rooted with *Apus apus* (GenBank accession no. as mentioned above), *Alectura lathami* (GenBank accession no.: NC007227; Slack *et al*., 2005) and *Anser albifrons* (GenBank accession no.: AF363031; Slack *et al*., 2003). Bootstrap analysis was run for 1000 replicates.

## RESULTS AND DISCUSSION

In the present study, the primer walking technique (Strauss *et al*., 1986) was modified to design the species-specific PCR primers for the mtDNA region without prior knowledge. This method is rapid and simple as it involves only repeated steps of PCR, direct sequencing and primer design. It is especially suitable for the organellar genomes, for instance, mtDNA, because organellar genomes are circular and relatively smaller in size compared to the nuclear genome.

The DNA sequences obtained using Primers L12 and H12 consisted of the 3' end of the NADH region (~40 bp), tRNA-Glu (~73 bp) and a partial control region (~346 bp). The typical strings of Cs at the beginning of the avian mitochondrial control region were also observed in the white-nest swiftlets (represented by the individual KT152; Fig.2). Unlike *Apus apus*, the white-nest swiftlets have three C-strings (Fig.2). The starting point of the control region falls at position 113 following the mtDNA characterisation of the *Apus apus* (Slack *et al*., 2009). The sequence upstream to the starting point is tRNA-Glu and NADH-6. The control region sequence obtained in this study was located in Domain I assuming that the white-nest swiftlet mtDNA did not differ much from the typical avian mtDNA gene arrangement and sizes (Quinn & Wilson, 1993; Quinn. 1997). The control region of the white-nest swiftlet affirms the findings of Randi and Lucchini (1998), that the second half of Domain I had a greater degree of variation (20%) than the

first half of Domain I (2%). The variations occurred in abundance after position 302 (data matrix not shown).

The aligned DNA matrix of the 35 white-nest swiftlet individuals (i.e. without the outgroups) was 350 characters in length, including 341 bases and nine indels. Among the 50 variable characters, 18 were parsimony-informative, that was 5.14%. A comparison with the cytochrome-*b* data obtained in Goh (2007) suggests that the control region of the white-nest swiftlets has a higher variability compared to the cytochrome-*b* (Table 2). Among the white-nest swiftlets sampled in this study, 15 individuals formed a well-supported clade (bootstrap value=84%; Fig.3), indicating that there are at least two distinct lineages among the house-farmed swiftlet populations.

TABLE 2
Comparison of the DNA data variability of control region and cytochrome-*b* sequence among the house-farmed white-nest swiftlets.

| | DNA characters | Indel characters | Total characters | Variable characters (%) | Parsimony-informative characters (%) |
|---|---|---|---|---|---|
| Cytochrome-*b* (Goh, 2007) | 558 | 0 | 558 | 17 (3.05) | 6 (1.08) |
| Control region (present study) | 341 | 9 | 350 | 50 (14.29) | 18 (5.14) |

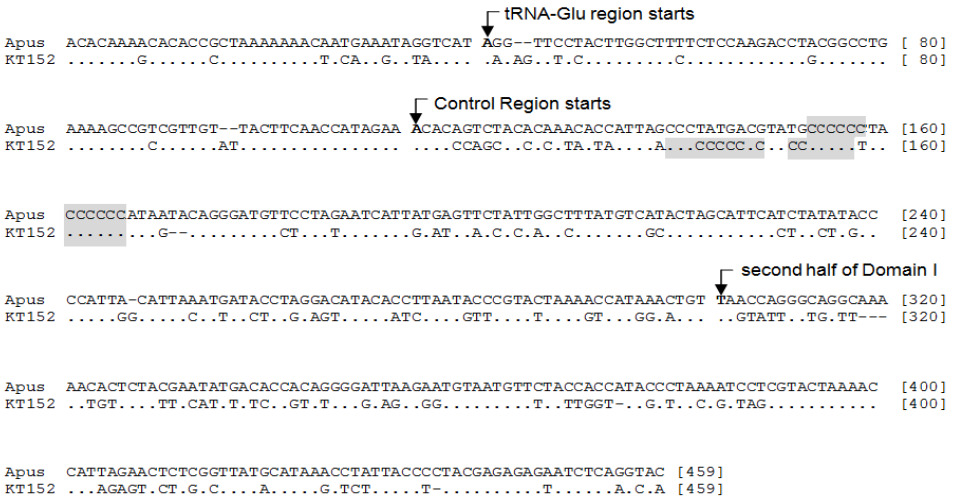


Fig.2: Characterisation of the mtDNA sequence obtained in this study. *Aerodramus fuciphagus* was represented by the individual 'KT152' and the sequence was aligned with the mtDNA sequence of *Apus apus* (NC008540.1; Slack *et al*., 2009). Dots indicate characters identical with *A. apus* sequence. Letters designate base substitutions. ' – ' indicates gap.

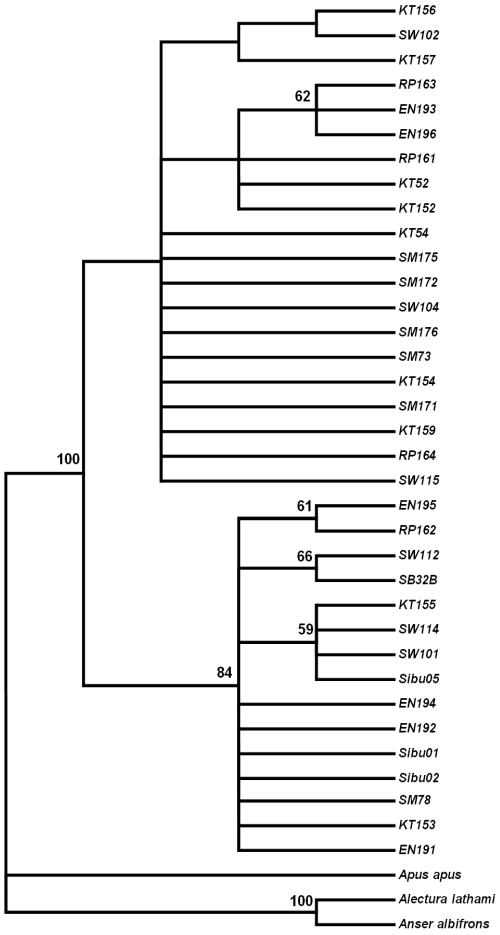


Fig.3: Strict consensus of the 72 most parsimonious trees based on the mitochondrial control region of the white-nest swiftlets. Bootstrap values of >50 % were shown next to the nodes. The prefix in the sample ID indicates the sampling localities (KT=Kuantan, RP=Rompin, EN=Endau, SM=Sumatra, SW=Perak, SB=Selangor, Sibu=Sibu).

## CONCLUSION

This study suggests that the control region is a promising DNA marker for resolving the lower-level phylogenetic relationships among the closely related lineages of the swiftlets as well as to understand the genetic structure of the white-nest swiftlet populations. This study does not recommend if the control region is more advantageous over other mtDNA regions (such as cytochrome-*b*), but it provides one more choice of DNA markers which could be incorporated in future studies on the white-nest swiftlets. Primers L12 and H12 were proven to be specific to the white-nest swiftlets. Alternatively, Primers L12 and H1251 could be used if one were to sequence the full length of the control region. However, an additional step (e.g. DNA cloning) may have to be taken because H1251 is less species-specific. A similar technique can be used for developing other mtDNA regions of the white-nest swiftlets or the mtDNA control region primers for other avian groups.

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*Short Communication*

# Determining the Colugo Sexes by Gliding Motion Photographs

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## ABSTRACT

The Colugo is a nocturnal arboreal mammal that inhabits the tropical rainforest in South East Asia. Photographs of the Colugo in gliding motion were taken using Digital SLR camera with the aid of speed light and flashlight to determine the Colugo sexes. These photographs technique could be used to determine the Colugo sexes without the need to capture the animal. It may also be a useful technique to determine the sexes of other gliding mammals and help in assisting the conservation effort of the mammal species.

*Keywords:* Colugo, Dermoptera, *Galeopterus variegatus*, gliding motion, photograph method, sexes

## INTRODUCTION

Colugo (*Galeopterus variegatus*) is a nocturnal arboreal mammal from the Order Dermoptera (Stafford, 2005). *G. variegatus* is widely distributed in the tropical rainforest in South East Asia within various habitats, while *Cynocephalus volans* is strictly found in the southern parts of the Philippines (Stafford, 2005). Previous studies revealed that the Colugo does not retreat when detected by human unless if it is directly disturbed (Dzulhelmi, 2011). Capturing the Colugo, using the capturing techniques described by Wischusen and Richmond (1989) have been used for field studies (Wischusen, 1990; Byrnes *et al*., 2011) and can directly determine the Colugo sexes. However, capturing the animal alive and unharmed is rather challenging (Wischusen, 1990; Byrnes *et al*., 2011).

Meanwhile, field research had also been conducted without capturing the Colugo. This includes the study on the population estimation (Lim, 2004; Agoramoorthy *et*

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*al*., 2006; Lim & Ng, 2010), diet preferences (Lim, 2004; Agoramoorthy *et al*., 2006; Dzulhelmi & Abdullah, 2009b), activity patterns (Dzulhelmi & Abdullah, 2009a; Byrnes *et al*., 2011) and roosting sites (Dzulhelmi, 2011). This research constrains have posed some degree of difficulties, especially in sex determination, which may affect the outcomes and the analyses of the study. For example, although Agoramoorthy *et al*. (2006) determined the sexes of the Colugo through his field survey, they did not mention any specific method (e.g. fur colouration) used to determine the male and female Colugo. Previously, Chasen and Kloss (1929) determined the Colugo sexes by fur colouration. Dzulhelmi and Abdullah (2009a) also distinguished the Colugo individuals based on fur colouration and carried infant (if any).

However, Lim (2004) stated that fur colouration might not be reliable to determine the Colugo sexes. Besides determining the Colugo sexes by fur colouration, there is no other available technique to determine the Colugo sexes without capturing it. Due to this constraint, Lim (2004) issued a need for a reliable tool to investigate the sexes of the Colugo without the need for capturing these animals. The present paper presents the use of Digital SLR camera to determine the Colugo sexes by photographing the Colugos ventral view while in gliding motion.

## MATERIALS AND METHOD

The photographs of the Colugos in its free ranging habitat were captured at Bako National Park (Sarawak) in June 2011 and Pulau Langkawi (Kedah) in October 2011. All the Colugo photographs were taken during the Colugos active period (Dzulhelmi & Abdullah, 2009). The photographs were taken using Nikon D90 aided with a zoom lens (AF Nikkor 70-300mm), a speed light (Nissin Di866) and a flashlight. The camera mode was set to exposure control, with shutter-priority auto, shooting mode: continuous high shooting mode, image size (pixel): 4288x2848, ISO: 200 and shuttle speed: 1/4000 seconds. The speed light was set to multi-flash mode. The Colugo was manually focused on using the zoom lens with the aid of the low intense flashlight.

Once the Colugo jumped off the tree and expanding its patagium to glide, multiple photographs of the Colugos ventral view were captured in a gliding motion. The sexes of the Colugo were examined by identifying the presence and absence of the testicles and any carried infant using a Picasa photo viewer (Fig.1 to Fig.3).

## RESULTS AND DISCUSSION

This technique successfully captured the Colugo in a gliding motion and both were from the Bako National Park (<10 photographs) and Pulau Langkawi, respectively (<20 photographs). The photo evidence identified that the Colugos comprised of a male, female, and a female carrying an infant (Figures 1-3). The Colugo glides at an average of 4-29 times per night which is less than 1% of its total activity (Byrnes *et al*., 2011). The habitat structure and the Colugo population were the crucial factors for obtaining the photographs of the

Fig.1: The presence of testicles can be visibly observed and identified on a male Colugo

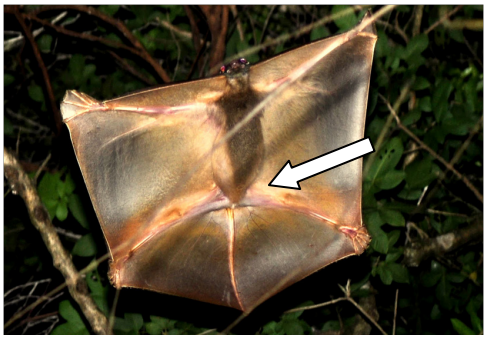

Fig.2: The absence of the testicles can be observed and this is identified as a female Colugo

Fig.3: An infant can be evidently noticed on the female Colugo and this differentiated between the two female Colugo individuals (Fig.2)

Fig.1-3: The male (Fig.1), female (Fig.2) and female with an infant (Fig.3) Colugos in a gliding motion.

Colugo in gliding motion. Others such as the photographic techniques, assistance, time, locations and weather should also be taken into consideration.

To date, the population and the ratio of the male to female Colugos are still unknown. As the ratio of the male and female Colugo in a particular area is one male to four or five females (1:4) (personal observation), prediction on the Colugo population could be made. Thus, for field survey (e.g. line transects survey), identifying the male Colugo would be a priority.

A combination of the field observations with the aid of Digital SLR camera would facilitate in the future research for the Dermopteran. The images of the Colugo ventral view during gliding could be a very useful and reliable tool to determine the Colugo sexes, while the presence of the carried infant would also assist in individual identification without the need to capture and mark the Colugo. The photographs may also enhance the understanding on the existence of the territorial behaviour portrayed between the male Colugos and also to verify the postulation that the male

Colugo takes over the maternal nature of carrying infant. This photograph technique could enhance the study of other gliding mammals such as the Colugo and the Flying Squirrel in other part of the tropical rainforest and therefore could assist the wildlife management in the conservation effort on these species.

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# Pelletized Feed of Different Particle Sizes: Effects on Performance, Carcass Characteristics and Intestinal Morphology of Two Strains of Broiler Chicken

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## ABSTRACT

This study was conducted to determine the effects of feeding pelletized feeds of different particle sizes on the performance, carcass characteristics and intestinal morphology of two strains of broiler chickens. A total of one hundred and eighty (180) birds consisting of 90 birds each of Marshal MY and Hubbard strains of broiler chicken were used in the experiment. They were brooded for 14 days and thereafter divided into two treatment groups, namely, fed with feed of different particle sizes of 1 and 2mm. These were further divided into 3 replicates of 15 chicks and the experimental period was 42 days. The performance of the chicks was monitored weekly. At week eight, 3 birds per replicate were randomly selected, weighed, slaughtered via neck slit, defeathered, singed and eviscerated for carcass evaluation. The intestinal morphometry and histomorphometry of the birds were analysed. Data collected were arranged in a 2 x 2 factorial layout and subjected to 2-way Analysis of Variance. Significant ($P<0.05$) differences were observed in the final weight, weight gain and protein efficiency ratio with Hubbard strain having better values than Marshal. Mortality ($P<0.05$) was lower in the birds fed with feed particle size of 2 mm. In addition, significantly ($P<0.05$) higher values were obtained in the head, drum stick, shank and keel of birds fed with feed with particle size of 2mm. Meanwhile, the highest ($P<0.05$) villus height was recorded

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for Hubbard fed 2mm feed particle size while Marshal had the least value. Hence, pelletized feed of 2mm particle size was recommended for broiler production.

*Keywords:* Particle size, pelletized feed, performance, carcass characteristic, intestinal morphology, Hubbard strain, Marshal strain

## INTRODUCTION

In poultry production, whole grain feeding is associated with increased gut development leading to a more muscular gizzard and less recurrence of proventricular dilation (Jones & Taylor, 2001). However, reducing grain particle size has been shown to increase hammer mill energy and production rate. A review of the past literature (Reece *et al.,* 1986a; Lott *et al.,* 1992) revealed inconsistencies in the recommended grain particle size for optimal poultry performance. However, the authors reported improved broiler performance when corn particle size decreased from 1289 to 987µm and from 1173 to 710µm, respectively. Further decrease from 900 to 300µm has also been reported by Healy (1992) to improve performance in the feed efficiency.

Particle size encompasses both the size of the various feed ingredients used in poultry diets as well as the consistency of the particle size. It is noteworthy that ingredient texture impacts on two areas of the poultry industry. Firstly, it impacts directly on the bird itself and the manner in which it utilizes the nutrients in its diet. The issue is further complicated by the fact that the manner in which materials are digested differs between ingredients. Secondly, it impacts on the manner in which ingredients are handled and processed in the feed mill.

Particle size is established by the geometric mean diameter (GMD). However, the complete information on particle size must include a measure of dispersion. This measure is the geometric standard deviation (GSD), which establishes the range of variation among the different particle sizes (Nir *et al*., 1994). Both these measures are described by ASEA (1983), but sadly they are seldom reported in literature as they independently affect broiler growth and performance. The average particle size of the sample is then determined by standard formula and given as geometric mean diameter (GMD), expressed as microns (µ). Particle size uniformity is described by geometric standard deviation (GSD), a small GSD representing higher uniformity.

Relatively, a study (Morel & Cottam, 2007) was conducted on the effects of feed form (pellet or mash) on the intestinal morphology in broilers' overall performance. The improved villus height and villus crypt depth for various segments of birds fed pelletized diets were in agreement with enhanced growth performance and increased metabolizability of nutrients. Extension of the villus enlarges total luminal villus absorptive area and subsequently results in adequate digestive enzyme action and higher transport of nutrients at the villus surface. In addition, the higher villus crypt depth in the broilers fed pellet diets is an indication for a decreased turnover rate of the intestinal mucosa. Meanwhile, a slower turnover rate

of the intestinal epithelium results in a lower maintenance requirement which leads to a higher growth rate of the animal. Thus, changes in intestinal morphology influence nutrient metabolizability and performance. Feed millers in Nigeria produce mash feeds which have ≤ 0.5 mm feed particle size (Personal Communication). However, Nir (1994) reported that feeding large particle corn may produce beneficial effects similar to whole grain feeding though the digestion and utilization of the feed particle sizes by birds would vary due to the texture of the feed and this will affect the birds' performance. The author further stated that feed particle size and the form in which it is presented are of great importance in assessing the overall productivity of broiler. For broiler to be highly productive, a good level of feeding is needed. The efficiency of feed utilization is a function of the feed particle size and the form of presentation which is directly related to the performance of broiler. The paucity of information on the effects of feed particle size of pelletized feeds on the performance of broiler chickens necessitates this research.

## MATERIALS AND METHODS

*Experimental Site*

The experiment was carried out at the poultry unit of the Teaching and Research Farm, University of Agriculture, Abeokuta (UNAAB), Ogun State, Nigeria. The area is situated in the south-western part of Nigeria which is a derived savannah zone with an annual mean temperature of 34.70$^0$C and a relative humidity of 82%. It is in the region 70 m above the sea level of latitude 7$^0$5' to 7$^0$8'N and longitude 3$^0$11.2'E (University of Agriculture, Abeokuta, Meteorological Station).

*Experimental Birds and Management*

A total of 180 (90 each of Marshal MY and Hubbard strains) chicks were used in the experiment. The 180 chicks were brooded for two weeks (14 days). Thereafter, they were divided into two treatment groups of 1 and 2mm pelletized feed particle sizes consisting of 45 chicks each which were further divided into 3 replicates of 15 chicks each and maintained for 42 days. The chicks were fed the dietary mix shown in Table 1, with pelletized feed particle sizes of 1 and 2mm *ad libitum*. Fresh water was also given to the chicks *ad libitum*.

*Experimental Diet Mix*

The macro feed ingredients (maize, soybean, wheat offal and ground nut cake) were milled and mixed together. The macro feed ingredients were sieved using 1 mm mesh and the particles that passed through the mesh were considered as the feed particle size ≤ 1 mm, whereas the feed that remained on the mesh were then sieved through 2mm mesh to get ≤ 2 mm particle size feed. The micro feed ingredients (ground bone meal, fish meal, ground oyster shell, vitamin and mineral premix, salt, lysine and methionine) were then equally divided into the two treatment groups and then mixed thoroughly with the already sieved macro feed ingredients of 1 and 2 mm feed particle sizes, respectively. The feeds

were then pelletized. Pelletizing was done using water as a binding agent in a pellet mill, where the feeds were conditioned and thermally treated in the fitted conditioners of a pellet mill.

*Performance Characteristics*

Data were taken weekly on the performance of the chicks: feed intake and weight gain. The data on feed which included gain, protein intake, protein efficiency ratio and mortality were also calculated. The protein intake was calculated by multiplying the percentage protein content of the feed by the actual intake while the protein efficiency ratio was the ratio of the weight gain to the protein intake.

*Carcass Characteristics Determination*

At the 8$^{th}$ week, 3 birds whose weights were close to the average replicate weight were selected per replicate, weighed, slaughtered, defeathered, singed and eviscerated. The dressed weights were determined. Cut-up parts such as head, neck, shank, thigh, drumstick, back and breast were weighed. The organs such as liver, gizzard and heart were also removed and weighed. These parts

TABLE 1
Composition of the experimental diet (g/kg)

| Ingredient | Composition | |
|---|---|---|
| Maize | 450.0 | |
| Soybean meal | 150.0 | |
| Wheat offal | 215.0 | |
| Groundnut cake | 125.0 | |
| Fish meal | 10.0 | |
| Bone meal | 25.0 | |
| Oyster shell | 15.0 | |
| *Vitamin and mineral premix | 2.5 | |
| Salt | 2.5 | |
| Lysine | 2.5 | |
| Methionine | 2.5 | |
| **Total** | **1000.0** | |
| **Determined Analysis (g/kg)** | 1mm pellet size | 2mm pellet size |
| Dry matter | 918.7 | 925.6 |
| Crude protein | 242.6 | 238.7 |
| Crude fibre | 35.9 | 37.4 |
| Ether extract | 34.8 | 35.1 |
| Ash | 450.6 | 461.7 |
| Nitrogen-free extract | 236.1 | 227.0 |
| Gross energy (KJ/kg) | 11.92 | 11.92 |

*Premix contained the following: (Univit. 15 Roche) 1500I.U., Vit. A; 1500I.U., Vit. D; 3000I.U., Vit. E; 3.0g, Vit. K; 2.5g, Vit. $B_2$; 0.3g, Vit. $B_6$; 8.0mg, Vit. $B_{12}$; 8.0g, Nicotinic acid; 3.0g, Ca-Pantothenate; 5.0mg, Fe; 10.0g, Al; 0.2g, Cu; 3.5mg, Zn; 0.15mg, I; 0.02g, Co; 0.01g Se.

were expressed as the percentage of the live weight. In addition, the weight of the empty gizzards, and length small intestines were also taken using a top-loading scale and a measuring tape, respectively.

*Small Intestine Sampling and Specimen Preparation for Light Microscope*

At the end of the experiment, 2 chicken from each replicate were randomly selected and killed by decapitation under light diethyl ether anaesthesia. Thereafter, the entire intestines were removed and placed immediately into a mixture of 3% glutaraldehyde and 4% paraformaldehyde fixative solution in 0.1M cacodylate buffer (pH7.4). The midpoint of the bile duct and Meckel's diverticulum (jejunum) and the midpoint between Meckel's diverticulum and the ileo-caecal junction (ileum) were cut and prepared for light and scanning electron microscopy, as enunciated by Shamoto and Yamauchi (2000). The segments (2 to 3 cm in length) were washed with 0.1M phosphate buffered saline (pH7.4), fixed in Bouin's solution for 6 hours and dehydrated in a graded ethanol series. Each segment was embedded in paraffin wax using a standard technique. The values of the villus area were calculated from the villus height, basal width and apical width according to the method described by Iji *et al.* (2001). The average villus area from the two birds was expressed as a mean villus area for one treatment group.

*Intestinal Histomorphometry*

The slides were examined under the microscope and using a calibrated eye piece graticule (Graticule Ltd. Tonbridge Kent, England). The following measurements were taken: Villus Height (VH), lamina propria depth (LPD), Apical Width (AW) and Basal Width of the villi (BW). Only those villi attached to the lamina propria and with defined tips were measured. The lamina propria measurement extended from the base of the villus to the muscular mucosa. A total of six measurements were taken for each of the parameters via each sample of the intestinal segment. The mean of the measurements for each parameter was later statistically evaluated.

*Proximate Analysis*

The proximate analysis of the two diets of 1 and 2mm particle sizes were determined according to the methods of AOAC (1995). The moisture content was determined by oven-drying 2 grams of each diet for 26 hours at $60^0$C to constant weight. The gross energy of the feeds was determined using Adiabatic Bomb® calorimetric method.

*Statistical Design and Analysis*

The data collected were subjected to 2-way analysis of variance using SAS (1999) in a 2 x 2 factorial arrangement. Significant ($p < 0.05$) means among the variables were separated using Duncan's Multiple Range Test as contained in the SAS (1999) package. The model used was:

$$Y_{ijk} = \mu + A_i + B_j + (AB)_{ij} + \varepsilon_{ijk}$$

Where,
$Y_{ijk}$ = individual observation
$\mu$ = overall mean
$A_i$ = effect of Factor A
(Particle size: i = 1mm, 2mm)
$B_j$ = effect of Factor B
(strain: j = Marshal, Hubbard)
$(AB)_{ij}$ = effect of interaction AB
(Particle size*strain)
$\varepsilon_{ijk}$ = Experimental error

## RESULTS

In the main effects of strain and feed particle sizes on the performance and carcass characteristics of broiler chickens (Table 2), significant ($P<0.05$) differences were observed in the final weight, weight gain and protein efficiency ratio. The Hubbard strain had a higher final weight, weight gain and a better protein efficiency ratio (1.56 ) than that of Marshal. In the feed particle size, significant ($P< 0.05$) difference was observed only in the mortality with the feed particle size of 2mm having a lower mortality of 1.67% as compared to 4.00% which was recorded for the birds on 1mm feed particle size. Significant ($P<0.05$) differences were also observed in the head, neck, wing, shank and large intestine. The Hubbard strain had higher head (2.84 %), neck (5.84 %) wing (9.99 %) and shank (5.27 %) than the Marshal strain of broiler chicken. Similarly, the Marshal strain recorded a better large intestine (14.90 cm) than the Hubbard strain. In the feed particle size, significant ($P<0.05$) differences were observed in the head, drum stick, and large intestine with feed particle size of 2 mm recording a higher value of 2.70 %, 11.29 %, and 14.47 cm for the head, drumstick, and large intestine, respectively.

The effects of the interaction between strain and particle size on the performance and carcass characteristics of the broiler chicken are shown in Table 3. Significant ($P<0.05$) differences were recorded in the protein efficiency ratio and mortality. The Hubbard strain on the 2mm feed particle size had the best protein efficiency ratio of 1.56 while the least value (1.38) was recorded in the Marshal strain on the feed particle size of 1mm. The highest percentage mortality of 4.67 was recorded in the Marshal strain on the 1mm feed particle size, while the Hubbard strain on the 2mm feed particle size had the least value of 1.33. Significant ($P<0.05$) results were found in the dressed percentage, head, neck, thigh, drumstick, shank, keel, large intestine and caeca. The Hubbard strain on 2mm feed particle size recorded the highest value of dressing percentage (89.77%), head (3.03%), neck (5.73%), thigh (11.20%), drumstick (12.18%), shank (5.87%), keel (20.61%), large intestine (13.47cm) and ceaca (16.33cm).

The main effects of the strain and feed particle size on the morphology of ileum and jejunum in broiler chicken are shown in Table 4. Significant ($P<0.05$) difference was observed in the lamina propria depth with Marshal strain recording a higher value of 249.17µm compared to the Hubbard strain. However, feed particle size had no significant ($P>0.05$) effect on the parameters considered, except for the

TABLE 2
The main effect of strain (±SE) and feed particle size (±SE) on the performance and carcass characteristics of broiler chicken

| Parameters | Strain | | Feed particle size | |
|---|---|---|---|---|
| | Marshal | Hubbard | 1mm | 2mm |
| **Performance Characteristics** | | | | |
| Initial weight (kg) | 0.23 ± 0.008 | 0.22 ± 0.003 | 0.22 ± 0.005 | 0.23 ± 0.006 |
| Final weight (kg) | 1.55 ± 0.03[b] | 1.69 ± 0.05[a] | 1.60 ± 0.05 | 1.64 ± 0.05 |
| Weight gain (g/b/d) | 31.47 ± 0.72[b] | 34.80 ± 1.17[a] | 32.82 ± 1.14 | 33.45 ± 1.28 |
| Feed intake (g) | 93.20 ± 1.99 | 97.69 ± 1.29 | 95.19 ± 2.09 | 95.90 ± 1.80 |
| Feed: gain | 2.97 ± 0.02 | 2.82 ± 0.07 | 2.91 ± 0.05 | 2.87 ± 0.07 |
| Protein intake (g) | 22.61 ± 0.48 | 22.34 ± 0.29 | 22.41 ± 0.33 | 22.54 ± 0.42 |
| Protein efficiency ratio | 1.39 ± 0.012[b] | 1.56 ± 0.03[a] | 1.47 ± 0.04 | 1.48 ± 0.033 |
| Mortality (%) | 3.33 ± 0.71 | 2.33 ± 0.61 | 4.00 ± 0.58[a] | 1.67 ± 0.05[b] |
| **Carcass Characteristics** | | | | |
| Live weight (kg) | 1.85 ± 0.04 | 1.86 ± 0.08 | 1.78 ± 0.05 | 1.93 ±0.06 |
| Dressed weight % | 83.35 ± 2.02 | 83.38 ± 3.05 | 81.86 ± 2.58 | 84.86 ± 2.41 |
| **Cut-up parts[1]** | | | | |
| Head | 2.42 ± 0.05[b] | 2.84 ± 0.15[a] | 2.55 ± 0.06[b] | 2.70 ± 0.19[a] |
| Neck | 4.88 ± 0.13[b] | 5.84 ± 0.10[a] | 5.41 ± 0.25 | 5.30 ± 0.23 |
| Wing | 8.39 ± 0.05[b] | 9.99 ± 0.67[a] | 9.32 ± 0.74 | 9.06 ± 0.38 |
| Thigh | 10.49 ± 0.27 | 10.54 ± 0.35 | 10.45 ± 0.29 | 10.58 ± 0.34 |
| Drumstick | 10.17 ± 0.42 | 11.01 ± 0.53 | 9.90 ± 0.19[b] | 11.29 ± 0.55[a] |
| Liver | 2.34 ± 0.15 | 2.12 ± 0.20 | 2.19 ± 0.21 | 2.27 ± 0.15 |
| Shank | 4.46 ± 0.21[b] | 5.27 ± 0.31[a] | 4.58 ± 0.13 | 5.15 ± 0.40 |
| Keel | 20.98 ± 0.88 | 18.82 ± 0.91 | 18.98 ± 1.10 | 20.81 ± 0.74 |
| Back | 14.05 ± 1.06 | 13.21 ± 0.55 | 13.23 ± 0.53 | 14.04 ± 1.07 |
| **Organs[2]** | | | | |
| Heart | 0.54 ± 0.05 | 0.48 ± 0.03 | 0.51 ± 0.05 | 0.51 ± 0.03 |
| Gizzard | 2.06 ± 0.10 | 2.27 ± 0.16 | 2.27 ± 0.17 | 2.07 ± 0.09 |
| Kidney | 0.50 ± 0.05 | 0.43 ± 0.01 | 0.50 ± 0.04 | 0.44 ± 0.04 |
| **Intestinal tract (cm)** | | | | |
| Small intestine | 127.40 ± 8.00 | 134.97 ± 8.00 | 131.53 ± 8.00 | 130.83 ± 8.00 |
| Large intestine | 14.90 ± 0.37[a] | 13.00 ± 0.37[b] | 13.43 ± 0.37[b] | 14.47 ± 0.37[a] |
| Caeca | 17.17± 0.39 | 16.17± 0.39 | 16.17± 0.39 | 17.17± 0.39 |

[a,b:] Means in the same row by factor with different superscripts differ significantly ($P<0.05$)
[1,2] Values expressed as percentages of the live weight

TABLE 3
The effects of the interaction between strain and particle size (±SE) on performance and carcass characteristics of broiler chickens

| Strain | Marshal | | Hubbard | |
|---|---|---|---|---|
| Feed particle size | 1mm | 2mm | 1mm | 2mm |
| Parameters | | | | |
| **Performance characteristics** | | | | |
| Initial weight (kg) | 0.22 ± 0.01 | 0.24 ± 0.01 | 0.23 ± 0.003 | 0.22 ± 0.005 |
| Final weight (kg) | 1.51 ± 0.04 | 1.60 ± 0.04 | 1.70 ± 0.04 | 1.67 ± 0.10 |
| Weight gain (g/b/d) | 30.64 ± 1.03 | 32.30 ± 0.89 | 35.00 ± 0.84 | 34.60 ± 2.46 |
| Feed intake (g) | 91.56 ± 2.31 | 94.84 ± 3.45 | 98.81 ± 1.87 | 96.56 ± 1.91 |
| Feed gain (g) | 3.00 ± 0.04 | 2.94 ± 0.03 | 2.82 ± 0.07 | 2.81 ± 0.14 |
| Protein intake | 22.22 ± 0.56 | 23.00 ± 0.83 | 22.60 ± 0.43 | 22.08 ± 0.44 |
| Protein efficiency ratio | 1.38 ± 0.02$^{b}$ | 1.40 ± 0.01$^{b}$ | 1.55 ± 0.04$^{ab}$ | 1.56 ± 0.08$^{a}$ |
| Mortality | 4.67 ± 0.66$^{c}$ | 2.00 ± 0.58$^{bc}$ | 3.33 ± 0.88$^{ab}$ | 1.33 ± 0.33$^{a}$ |
| **Carcass characteristics** | | | | |
| Live weight (kg) | 1.78 ± 0.06 | 1.92 ± 0.03 | 1.77 ± 0.09 | 1.95 ± 0.13 |
| Dressing percentage | 86.74 ± 2.91$^{a}$ | 79.97 ± 0.66$^{b}$ | 76.99 ± 1.07$^{b}$ | 89.77 ± 2.17$^{a}$ |
| **Cut-up part[1]** | | | | |
| Head | 2.46 ± 0.06$^{b}$ | 2.37 ± 0.06$^{b}$ | 2.64 ± 0.08$^{ab}$ | 3.03 ± 0.25$^{a}$ |
| Neck | 4.89 ± 0.17$^{b}$ | 4.86 ± 0.23$^{b}$ | 5.94 ± 0.12$^{a}$ | 5.73 ± 0.17$^{a}$ |
| Wing | 8.45 ± 0.10 | 8.33 ± 0.02 | 10.19 ± 0.42 | 9.78 ± 0.42 |
| Thigh | 11.01 ± 0.29$^{a}$ | 9.96 ± 0.11$^{b}$ | 9.89 ± 0.10$^{b}$ | 11.20 ± 0.42$^{a}$ |
| Drumstick | 9.95 ± 0.39$^{b}$ | 10.40 ± 0.83$^{b}$ | 9.85 ± 0.17$^{b}$ | 12.18 ± 0.19$^{a}$ |
| Liver | 2.53 ± 0.28 | 2.14 ± 0.02 | 1.84 ± 0.18 | 2.39 ± 0.32 |
| Shank | 4.49 ± 0.22$^{b}$ | 4.43 ± 0.41$^{b}$ | 4.67 ± 0.15$^{b}$ | 5.87 ± 0.32$^{a}$ |
| Keel | 20.93 ± 1.38$^{a}$ | 21.02 ± 1.41$^{a}$ | 17.03 ± 0.54$^{b}$ | 20.61 ± 0.82$^{ab}$ |
| Back | 14.19 ± 0.55 | 13.90 ± 2.31 | 12.26 ± 0.39 | 14.17 ± 0.65 |
| **Organs[2]** | | | | |
| Heart | 0.58 ± 0.08 | 0.50 ± 0.06 | 0.43 ± 0.05 | 0.53 ± 0.02 |
| Gizzard | 2.17 ± 0.12 | 1.95 ± 0.14 | 2.36 ± 0.34 | 2.18 ± 0.06 |
| Kidney | 0.53 ± 0.07 | 0.47 ± 0.10 | 0.04 ± 0.02 | 0.41 ± 0.00 |
| **Intestinal tract (cm)** | | | | |
| Small intestine | 140.30 ± 11.31 | 114.50 ± 11.31 | 122.77 ± 11.31 | 147.17 ± 11.31 |
| Large intestine | 14.33 ± 0.52$^{ab}$ | 15.37 ± 0.52$^{a}$ | 12.53 ± 0.52$^{c}$ | 13.47 ± 0.52$^{bc}$ |
| Caeca | 16.33 ± 0.55$^{ab}$ | 18.00 ± 0.55$^{a}$ | 16.00 ± 0.55$^{b}$ | 16.33 ± 0.55$^{ab}$ |

$^{a,b,c:}$ Means in the same row with different superscripts differ significantly ($P<0.05$)
$^{1,2}$:values expressed as percentages of live weight

TABLE 4
The main effect of strain (±SE) and feed particle size (±SE) on the morphology of ileum and jejunum in broiler chicken

| Parameters | Strain | | Feed particle size | |
|---|---|---|---|---|
| | Marshal | Hubbard | 1mm | 2mm |
| **Ileum morphology** | | | | |
| Apical width (µm) | 70.83 ± 8.44 | 60.00 ± 6.85 | 78.33 ± 7.05[a] | 52.50 ± 6.64[b] |
| Basal width (µm) | 189.58 ± 56.26 | 105.83 ± 10.25 | 107.92 ± 9.48 | 187.50 ± 56.66 |
| Villus height (µm) | 480.00 ± 74.10 | 547.50 ± 65.24 | 507.50 ± 78.60 | 520.00 ± 61.39 |
| Lamina depth (µm) | 249.17 ± 23.78[a] | 215.83 ± 20.17[b] | 241.67 ± 24.46 | 233.33 ± 20.24 |
| **Jejunum morphology** | | | | |
| Apical width (µm) | 64.17 ± 12.34 | 55.83 ± 8.21 | 71.67 ± 11.27 | 48.33 ± 8.42 |
| Basal width (µm) | 99.17 ± 11.45 | 81.67 ± 9.20 | 95.83 ± 12.22 | 85.00 ± 8.66 |
| Villus height (µm) | 504.17 ± 80.18 | 539.17 ± 68.90 | 548.33 ±82.92 | 495.00 ± 65.02 |
| Lamina propria depth (µm) | 268.33 ± 34.06 | 245.83 ± 18.73 | 258.33 ± 27.60 | 255.83 ± 27.78 |

[a,b]: Means in the same row by factor with different superscripts differ significantly (P<0.05)

apical width. Meanwhile, no significant (P>0.05) differences were recorded in all the parameters measured. Strains (Marshal MY and Hubbard) and feed particle size (1 and 2mm) gave similar result by factors.

The effects of the interaction between strain and feed particle size on the morphology of ileum and jejunum in the broiler chicken (Table 5) showed significant (P<0.05) differences in the basal width, villus height and lamina propria depth. The Marshal strain fed with 1mm feed particle size recorded the highest basal width of 272.50 µm, while the Hubbard strain fed with 2mm feed particle size had the least value of 91.66 µm. The highest value for the villus height (645.00 µm) was recorded in the Hubbard strain fed with 2mm feed particle size with the Marshal strain fed with 1mm feed particle size having the least value of 345.00µm. The Marshal strain fed with 2mm feed particle size had the highest value of 271.67µm in the lamina propria depth and the least value of 163.33 µm was recorded for the Hubbard strain fed with 1mm feed particle size. In the jejunum, no significant (P>0.05) differences detected in all the parameters measured except in the lamina propria depth with the Marshal strain fed with 2mm feed particle size recorded the highest value (350.00µm) as compared to the least value (186.67µm) recorded in the same strain fed with 1mm feed particle size.

Fig.1 shows the intestinal histomorphometry of the ileum of Marshal MY fed with feed in the particle size 1mm. It was observed that ileum is normal with good architectural display of villi. The villi were elongated proportionately and well defined. In Fig.2, the intestinal histomorphometry of the ileum of Marshal MY fed with feed in the particle size of 2mm showed abnormalities of the ileum. The villi were greatly atrophied with pieces scattered in

TABLE 5
The effect of the interaction between strain and feed particle size (±SE) on the morphology of ileum and jejunum in broiler chicken

| Strain | Marshal | | Hubbard | |
|---|---|---|---|---|
| Feed particle size | 1mm | 2mm | 1mm | 2mm |
| Parameters | | | | |
| **Ileum morphology** | | | | |
| Apical width (µm) | 70.00 ± 15.06 | 71.67 ± 9.46 | 61.67 ± 11.67 | 58.33 ± 8.33 |
| Basal width (µm) | 272.50 ± 104.03$^{a}$ | 106.67 ± 18.73$^{ab}$ | 120.00 ± 15.06$^{ab}$ | 91.66 ± 12.49$^{b}$ |
| Villus height (µm) | 345.00 ± 28.49$^{b}$ | 615.00 ± 126.72$^{ab}$ | 450.00 ± 100.83$^{ab}$ | 645.00 ± 68.98$^{a}$ |
| Lamina propria depth (µm) | 226.67 ± 31.38$^{ab}$ | 271.67 ± 36.09$^{a}$ | 163.33 ± 17.64$^{b}$ | 268.33 ± 19.40$^{a}$ |
| **Jejunum morphology** | | | | |
| Apical width (µm) | 70.00 ± 21.14 | 58.33 ± 14.47 | 45.00 ± 12.32 | 66.67 ± 9.89 |
| Basal width (µm) | 103.33 ± 17.64 | 95.00 ± 16.07 | 78.33 ± 16.00 | 85.00 ± 10.57 |
| Villus height (µm) | 391.67 ± 24.82 | 616.67 ± 150.37 | 393.33 ± 95.28 | 685.00 ± 57.49 |
| Lamina propria depth (µm) | 186.67 ± 27.78$^{b}$ | 350.00 ± 40.83$^{a}$ | 250.00 ± 27.45$^{b}$ | 241.67 ± 27.98$^{b}$ |

$^{a,b}$: Means in the same row with different superscripts differ significantly (P<0.05)

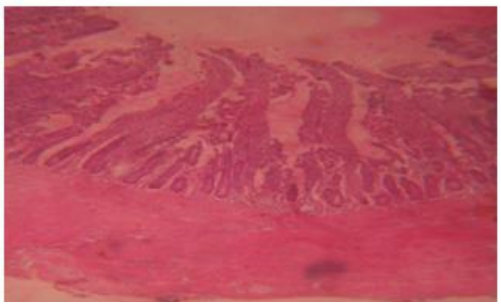

Fig.1: Intestinal Histomorphometry of the ileum of Marshal MY Broiler Chicken fed 1mm pelletized feed particle size (Magnification X10)

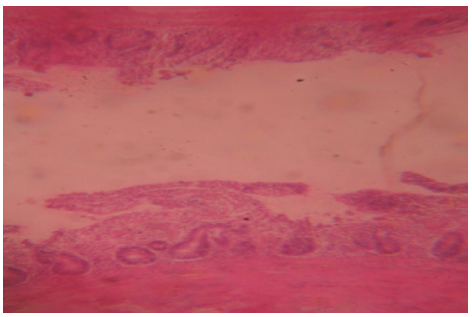

Fig.2: Intestinal Histomorphometry of the ileum of Marshal MY Broiler Chicken fed 2mm pelletized feed particle size (Magnification X10)

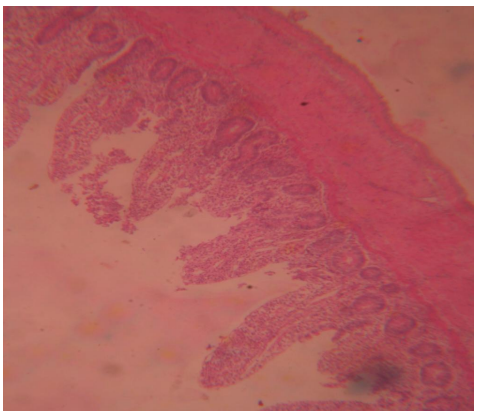

Fig.3: Intestinal Histomorphometry of Hubbard Broiler Chicken fed 1mm pelletized feed particle size (Magnification X10)

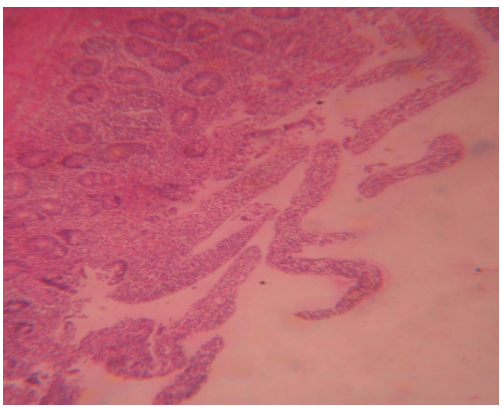

Fig.4: Intestinal Histomorphometry of Hubbard Broiler Chicken fed 2mm pelletized feed particle size (Magnification X10)

the lumen of the intestine. The intestinal histomorphometry of the ileum of Hubbard fed with feed in the particle size of 1mm shown in Fig.3 revealed that the ileum was degenerated as the villi were stumpy and not properly formed while in Fig.4, the intestinal histomorphometry of the ileum of Hubbard fed with feed om tje particle size of 2mm was normal with well-developed villi. This figure shows the best features among all the observed figures.

## DISCUSSION

The results obtained in the present study corroborated the reports of Mehaffey *et al*. (2006) who evaluated five most commercially used strains by the poultry industry in Europe and found no significant differences in the initial live body weights among broiler strains at various ages. However, there was a significant difference in the average final body weight among the strains considered, thereby corroborating the findings in the present study. The results contradicted the reports of Goliomytis *et al.* (2003) and Korver *et al.* (2004) who found that there were no significant differences between the commercial broiler strains for their final body weights at 42 days of age. The fact that the final body weight was increased in the birds fed with 2mm-sized pellets compared to 1-mm-sized pellets in the present study suggested that the 2mm pellet size is appropriate, as also reported by Cerrate *et al.* (2008).

The overall feed intakes for both the Marshal and Hubbard birds are similar. This result is also similar to those reported by Korver *et al.* (2004) whereby the overall feed intake is similar for 3 strains of broilers, but is in contrast with the findings of Abdullah *et al.* (2010) who reported that there was a significant difference in the overall feed intake between the strains, with no differences between the Hubbard and Lohman. Stickland (1995) reported that within a strain, muscle fibres increase as the average daily gain and feed conversion rate increase. It was observed that as the birds grew older and larger, they consumed more feed to meet the increasing requirement for maintenance, growth and fat deposition.

It must be recognized that not only the size of the feed particles but also the uniformity of the particles size are relevant in determining the influence of the particle size on bird's performance. Both the particle size and shape did influence the birds' performance (Axe, 1995). In addition, the birds in this study could distinguish the differences in the feed particle size by mechanoreceptors located in the beak (Gentle, 1979).

The findings of Nir *et al.* (1995) gave sustained interest in studying the effects of the particle size in the pelleted feeds on the basis that the pellets dissolve in the crop after consumption and hence, the effect of the feed particle size might be maintained even after pelleting. Reece *et al.* (1986a) found no effect on performance using maize of differing particle sizes to formulate broiler starter diets in crumble form. Similarly, Svihus *et al.* (2004a) showed no difference in any of the performance parameter when the broilers were fed pelleted feeds made

from wheat that was ground in hammer and roller mills to a range of particle sizes, and concluded that pelleting evened out the differences in particle size distribution. In addition to these findings, Cabrera (1994) found no effect of the feed particle size (1,000-400μ) on the growth performance of broiler chicks fed complex (added tallow, meat and bone meal and feather meal) diet fed in a crumb form. On the contrary, this study showed that feed form (pellets) had an impact on the performance, with pelleting improving the growth rate and particle size enhancing better protein efficiency (Engberg *et al.,* 2002; Skinner-Noble *et al.,* 2005; Greenwood *et al.,* 2005; Lemme *et al.,* 2006). Though the results of this study showed no significant effects of the feed particle sizes on the weight gain, feed intake and feed gain ratio, the results are supported by the findings of Van Biljon (2005), Galobart and Moran (2005) and Salari *et al.* (2006)) who reported that the form of diet and/or particle size had no significant effect on the weight gain, dry matter intake and feed gain. Meanwhile, Kilburn and Edwards (2001) also reported improvements in feed per gain when the diet included medium ground corn (GMD, 0.87 mm) compared to that made from very coarse corn (GMD, 2.90 mm). Reece *et al.* (1985, 1986a,b), however, found that the corn particle size (GMD, 0.68 vs. 1.29 mm) had no effect on the performance of the broilers fed crumbled or pelleted diets.

The significant difference obtained in the protein efficiency ratio with birds fed 2mm feed particle size having a higher ratio than those fed on 1 mm feed particle size could be attributable to the improvement in the feed efficiency in birds fed diets with higher particle size. Further to this, a greater proportion of coarse particulate matter resulted in a longer residence time within the gizzard, leading to enhanced digestion and thus better protein efficiency. Moreover, a greater proportion of coarse particulate matter stimulated greater gizzard activity, leading to more efficient grinding with production of greater quantities of finer particles that are more readily digested. Coarse particles may however slow the passage rate of digestion to the gizzard (Nir *et al.,* 1994a), increasing the exposure time of nutrients to digestive enzymes in the proventriculus, which in turn may improve energy utilization and nutrient digestibility (Carre, 2000). Furthermore, it has been reported that a lower pH of gizzard contents may increase pepsin activity (Gabriel *et al.,* 2003) and improve protein digestion.

Birds fed with 1mm of feed particle size recorded significantly high percentage mortality when compared to birds fed with 2mm of feed particle size. This was similar to the report by Scott (2002) who found that feed form did not affect mortality but that feeding a high-density ration resulted in a higher incidence of sudden death syndrome (SDS) compared with broilers fed a low-density ration.

Nir *et al.* (1994) suggested that the average daily feed consumption in hens is related to the particle size. In more specific, coarse particle size with denser feed bulk density promotes more feed consumption.

On the other hand, finer feed particle size decreases feed consumption due to dustiness problems (Patrick & Schaible, 1980). Hetland *et al.* (2002) reported increased feed intake when feeding diets with high inclusions of whole cereals. This is similar to the findings of this study as the birds fed with 2mm of feed particle size consumed more feed than those fed with 1mm of feed particle size.

The differences obtained in the weight gain are similar to the findings of Abdullah *et al.* (2010) who reported a significant difference in the overall average daily gain (ADG) between strains. Similarly, Korver *et al.* (2004) reported that the overall ADG (from week 1 to week 6) of 3 strains of broilers was significantly different. The result of feed gain between strains is similar to the findings of Abdullah *et al.* (2010) who reported that there was no significant difference in the overall feed conversion ratio (FCR) between the strains. In addition, Waibel *et al.* (1992) reported that if fines were fed to poultry, losses in feed conversion and rate of gain were observed. In addition, increasing the level of fines or grinding pellets has been shown to adversely affect the feed conversion (Plavnik *et al.,* 1997). However, these results contradict with the findings of Elisabeth *et al.* (1998) and Korver *et al.* (2004) who reported that the overall FCR of different strains of broiler is significantly different.

The results on the carcass evaluation contradicted the findings of Karima and Fathy, (2005) who reported that the differences in live body between breeds were found to be significant and that the proportion of meat in the valuable parts of the carcass was influenced less by diet and more by slaughter weight. Meanwhile, feed particle sizes had significant effect on the head, drumstick and large intestine length. This is similar to the findings of Ebrahimi *et al.* (2010) who reported that feed particle size had no effect on the weights of carcass, chest, femur, liver, gizzard and heart.

Past literature (see Lott *et al.,* 1992; Kilburn & Edwards, 2001) which suggested that broilers might not be able to efficiently utilize large corn particles due to underdeveloped gastrointestinal tracts contradict the findings of the present study. It, however, a showed similar finding with that of Nir *et al.* (1994a) who reported that a mash diet with large particles is better suited to the chicken's intestinal tract than a mash diet with small particles only. The authors also reported that the content weight of the gizzard was significantly less for diets containing small particles as compared with large ones, suggesting a decreased particle retention time. This contradicted the findings of this present study, as no difference was found in the gizzard weight in the two feed particle sizes.

Annison (1993) showed that the physical effect of feed such as size and fibre composition could improve the digestibility of nutrients and very fine grinded grains have had harmful effects on health and activity of broiler chicks. Feed processing, as reported in many scientific resources, could affect broiler ileum and caecum contents microflora, growth and efficiency in feed

utilization (Kenny & Kemp, 2003). These findings are corroborated by the results of the present study which has shown that the ileum morphology of the Hubbard strain on the 2 mm feed particle size had the highest villus height and a statistically similar lamina propria depth with the Marshal strain on the 2 mm feed particle size. However, the Marshal strain on 1 mm feed particle size had the highest basal height while the lowest was obtained in the Hubbard strain on 2 mm feed particle size. In addition, the jejunum morphology showed the Marshal strain on 2 mm feed particle size having the highest lamina propria depth and the lowest in the same strain on 1 mm feed particle size. This finding is in consonance with the reports of Choi *et al.* (1986) and Nir *et al.* (1994b) who revealed an increase in the broilers' digestive tract weight and in the height of jejunum and ileum through increasing the particle sizes of diet.

## RECOMMENDATION

Based on the findings of the present study, it could be recommended that:

- Pelletized feed of 2mm particle size should be adopted for broiler production from day 14.

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# The Effect of Extraction Methods on Fatty Acid and Carotenoid Compositions of Marine Microalgae *Nannochloropsis oculata* and *Chaetoceros gracilis*

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## ABSTRACT

This study was conducted to assess three extraction methods for the determination of fatty acid compositions and carotenoids (lutein, zeaxanthin, β-carotene, and α-carotene) from marine microalgae, *Nannochloropsis oculata (NO)* and *Chaetoceros gracilis* (*CG*). For this purpose, three different extraction methods for the determination of fatty acids (dichloromethane:methanol, water:propan-2-ol:hexane and direct saponification-ethanol KOH) and carotenoids (hexane:ethanol:acetone:toluene, methanol:chloroform and methanol:tetrahydrofulran) were used. Two derivatization methods using different types of catalyst (acetyl chloride and boron trifluoride) were also used for the transmethylation of the fatty acids into corresponding methyl esters. The results of the fatty acid compositions showed that *NO* had a higher amount of n-3 and n-6 polyunsaturated fatty acid (PUFA), particularly eicosapentaenoic acid (EPA) (C20:5). *CG* was predominantly high in palmitic acid (C16:0) and palmitoleic acid (C16:1). The extraction method 1 (dichloromethane:methanol) and extraction method 2 (water: propan-2-ol: hexane) with acetyl chloride-catalyzed transmethylation were found to be the best methods for the determination of fatty acid compositions in *NO* and *CG,* respectively. A significantly higher ($P<0.05$) amount of carotenoids was found in *NO* as compared to *CG* using different extraction methods. Extraction method 1 (involving saponification procedure) yielded the best result for *NO* while extraction method 3 (methanol: tetrahydrofuran with no saponification procedure) generated higher amounts of carotenoids in *CG*. Overall, this study has shown that significantly high amounts of fatty acids and carotenoids could be obtained from these microalgae using these methods.

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## INTRODUCTION

Microalgae are prokaryotic or eukaryotic photosynthetic microorganisms unified primarily by the lack of roots, leaves, and stems that characterize higher plants. They can be found almost anywhere, with water and sunlight as their fundamental requirements, including lakes, soils, rivers, hot springs, and the ocean. Microalgae contain high value compounds like fatty acids [γ- linolenic acid, arachidonic acid, eicosapentaenoic acid (EPA), docosahexaenoic acids (DHA), etc.], pigments (chlorophyll and carotenoids), vitamins (biotin, vitamins C and E, and others) (Converti *et al.,* 2009). *Chaetoceros gracillis*, a diatom in the class of Bacillariophycae and *Nannochloropsis occulata*, a unicellular green alga with spherical shape of the Eustigmatophyceae class plays an important role in the food chain system and it is also commonly used as live feed; thus, it is widely cultivated in fish hatcheries and shrimp farms (Gwo *et al.,* 2005).

DHA and EPA are constantly an area of interest in nutrition because they are essential for optimizing human health. DHA is important for the development of the brain and eyes in pre-term and young infants, as well as for supporting cardiovascular health in adults, whereas EPA is essential for the human metabolism and involved in the blood lipid equilibrium that prevents hypertriglyceridemia and anti-inflammatory activities (Kroes *et al.,* 2003; Ward & Singh, 2005; Fajardo *et al.,* 2007). Previously, fish was the principal dietary source of DHA and EPA. However, due to the serious environmental consequences and continuous exploitation, the declining sources of marine fish stocks and fish oil have prompted research into new sources of polyunsatured fatty acids (PUFAs) (Burja *et al.,* 2007). In addition, certain disadvantages of fish oil, such as the unpleasant odour, possible pollutants, and mixed fatty acid properties have also encouraged the search for alternative sources of PUFAs (Pulz & Gross, 2004).

In addition, microalgae contain a multitude of pigments, particularly chlorophyll and carotenoid. Carotenoids are essential to human health and important in commercial applications (Felti *et al.,* 2005). For example, β-carotene acts as pro-vitamin A and has been proven to prevent xeropthlamia (Puah *et al.,* 2005); astaxanthin acts as a natural colorant for muscle in marine fish and crustaceans; lutein, zeaxantin and canthaxantin for chicken skin coloration, pharmaceutical purposes, and also as a natural food additive (Del Campo *et al.,* 2000; Pulz & Gross, 2004).

Although microalgae contain important bioactive components (particularly PUFA and carotenoids), the extraction methods, especially for algae, are not well established, as there are no standard extraction methods for the determination of the fatty acid content or carotenoids in microalgae (Wiltshire *et*

*al.,* 2000). Lipids are mainly a mixture of esters, and therefore, the preparation of fatty acid methyl esters (FAME) consists essentially on the conversion of one ester to another (i.e. transesterification) by cleavage of an ester bond via an alcohol; when such an alcohol is methanol, the reaction is referred to as methanolysis or transmethylation (Liu, 1994). Transmethylation are reversible reactions which are normally accomplished in the presence of a catalyst, either an acid or a base. Reactions involving acidic catalysts require heat to accelerate the process. Commonly used acidic catalysts are (the Brønsted-Lowry acid) HCl, $H_2SO_4$, acetyl chloride and (the Lewis acid) BF3. Base-catalyzed methanolysis proceeds much more rapidly under mild temperature conditions than acid-catalyzed reactions. However, bases cannot catalyze the esterification of FFAs.

The nutritional value of microalgae is strongly dependent on its bioactive profile; therefore, methods that could get the highest value is preferred. Felti *et al.* (2005) suggested that the absence of a standard extraction method for carotenoid is actually attributed to the wide spectrum of the analyzed materials (foodstuff, plant, animal, and human samples) and the wide range of the carotenoids present. As a result, three extractions methods of fatty acid and carotenoids from marine microalgae, *Nannochloropsis oculata* and *Chaetoceros gracilis* were evaluated in the current study. The criteria used in choosing these methods were maximum extraction efficiency, ease of handling, and use of solvents of low toxicity.

## MATERIAL AND METHODS

### *Microalgae Samples*

Both the *Nannochloropsis oculata* and *Chaetoceros gracilis* samples were purchased from Reed Mariculture Inc. USA. The samples were collected using non-probability, convenient sampling method. Both the microalgae were purchased in two batches and prior to the extraction, these samples were freeze-dried, homogenized, and kept at -20$^{o}$C until further use.

### *Oil Extraction*

The first extraction method used was adopted from Cequier-Sanchez *et al.* (2008). First, 500 mg of the samples were extracted by mixing 15 ml of dichloromethane-methanol 2:1 (v/v) contained in a beaker. The mixing was performed with occasional gentle hand agitation for 2 hours. Subsequently, the samples were filtered and transferred into a new test tube to which 3.13ml of an aqueous solution of potassium chloride (0.88%, w/v) was added with strong agitation, followed by centrifugation (Universal 320/320R Benchtop Centrifuges, Hettich Instruments, Germany) at 350g at 4$^{0}$C for 5 minutes. The aqueous upper phase was discarded and the organic phase was evaporated using a rotary evaporator (Büchi Rotavapor R-200, Switzerland).

The second extraction method was adopted from Schlechtriem *et al.* (2003) with a slight modification, in which hexane was used to substitute the cyclohexane. First, 500 mg samples were weighed into the Falcon tubes and mixed with 10ml of

propan-2-ol and 12.5 ml of hexane using a vortex for 30s. The tubes were placed in an ultrasonic bath at room temperature for 15 minutes. Then, 13.75ml of water was added to obtain a mixture of water: propan-2-ol:hexane (11:8:10 v/v/v). The mixture was mixed again using the vortex for 30s. The different phases were separated by centrifugation at 1800g for 10 minutes and the organic phase was transferred into a pre-weighed Flacon tube with a dropper. The organic phase containing the lipid fraction was separated at the top of the extraction mixture (the hexane phase). Subsequently, the second extraction with 12.5ml of hexane containing 13% v/v propan-2-ol was done. The mixture was vortexed and placed into the ultrasonic bath for another 15 minutes. After centrifugation, the hexane phase was added to the first extract. The tubes were placed in a water bath ($50^0$C) for about 15 minutes and the solvent was evaporated to dryness using a rotary evaporator.

The third extraction method was adopted from Burja *et al*. (2007) with a minor modification, in which 95% ethanol was used instead of 96% ethanol. First, the 500mg samples were weighed. Then, 38ml of 3mM potassium hydroxide in ethanol (95%) was added into a 150ml beaker. The beaker was passed under the flow of nitrogen, and shaken for 1 hour in a water bath set at 60°C. Thereafter, the samples were cooled to room temperature and filtered through filter paper. The biomass was washed with 10ml of ethanol and transferred into a new beaker, to which, 10ml of water was added. Unsaponifiables were extracted by adding 20ml of hexane and gently mixing twice. After the layers were separated, the pH was adjusted to 1 (from pH 13-14) by the addition of hydrochloric acid:water (1:1, v/v). The top layer, containing the fatty acid fraction, was recovered by two rounds of the addition of 10ml of hexane and a gentle mixing. Lastly, the solvent at the top layer was evaporated to dryness using a rotary evaporator.

*Preparation of Fatty Acid Methyl Esters (FAMEs)*

This acetyl chloride-catalyzed transmethylation method was adopted from Carvalho and Malcata (2005). The lipid extracts (2mg) were subjected to acid-catalyzed transesterification by dissolving them in 2ml of a freshly prepared mixture of acetyl chloride and methanol at a ratio of 5:100 (v/v), together with 1mg of tricosanoic acid as an internal standard. The reagents were placed in Teflon-capped Pyrex tubes, and the reaction continued at 100°C for 1 hour under pure nitrogen and darkness. After cooling to 30~40°C, 1ml of the extracting solvent (isooctane containing 0.01% butylated hydroxytoluene, BHT) was added, and the FAME solvent solution was mixed using a vortex for between 5 to 30s. The purification of the solution was achieved by adding 1ml of water, causing the formation of two immiscible phases, which were then allowed to separate. Subsequently, the upper extracted solvent phase was recovered and stored in sealed glass vials at $-20^0$C until GC analysis.

The BF3-catalyzed transmethylation

method was adopted from Carvalho and Malcata (2005), which was modified by the use of 10% (v/v) of BF3 in methanol instead of 12% (v/v) of BF3 in methanol. First, all the lipid extracts (2mg) were subjected to a preliminary alkaline hydrolysis with 0.5M sodium hydroxide at 100$^0$C for 5 minutes. Subsequently, it was dissolved in 2ml of 10% (v/v) BF3 in methanol, together with 1mg of tricosanoic acid as an internal standard. The reagents were placed in the Teflon-capped Pyrex tubes, and the reaction was allowed to continue at 100°C for 30 minutes under pure nitrogen and darkness. The subsequent procedure was similar to the acetyl chloride-catalyzed transmethylation procedure, as described above.

*Gas Chromatography Analysis*

The assay of FAME was analyzed using gas chromatography (Agilent 6890, ISA Agilent Tech, USA) equipped with a split/splitless injector, and Hewlett Packard EL-980 flame ionization detector (FID). The FID system was used to separate and quantify each FAME component. FAME was separated using DB-23 column (60m x 0.25mm ID, and 0.15 µm). The chromatography data were recorded and integrated using the chemistation software (version 6). The oven temperature was programmed to hold at 50$^0$C for 1 min, before it was increased to 175$^0$C with 25$^0$C/min, held for 4 minutes, and lastly increased to 230$^0$C with 4$^0$C/min and held for 5 mins. The temperature for the injector and detector was set at 250$^0$C and 280$^0$C, respectively. One microlitre of the sample volume was injected with a split ratio of 1:50µl and a column temperature of 110$^0$C. The carrier gas was helium gas (1.0 ml/min) which was controlled at 123.4kPa/ Hz and the air used for FID was held at 275.6kPa.

*Calculation of Fatty Acid*

The identification of the fatty acid compositions for the sample was made by comparing the retention time of the sample FAMEs with those of Supelco 37 component FAMEs mixture (Sigma-aldrich, USA) for each chromatography peak. The quantification of the fatty acid was done using tricosanoic acid (C23:0) as an internal standard. The amount of the individual fatty acid was calculated using the expression: $C_i = C_p (A_i/A_p)$, where A is the chromatographic area units and C is the amount of fatty acid. Subscript p represents the internal standard and i refers to any fatty acid. The percentage of the individual fatty acid in the total amount of fats used was calculated as $C_i$/total amounts of fat x 100%.

*Carotenoids Extraction*

The first extraction method was adopted from Inbaraj *et al.* (2006). First, 400mg of freeze-dried microalgae was mixed with 12 ml hexane-ethanol-acetone-toluene (10:6:7:7 v/v/v/v) in a volumetric flask. After shaking for 1 hour, 0.8ml 40% methanolic potassium hydroxide was added and the solution was saponified at 25$^0$C in the dark for 16 hours. Then, 12ml of hexane was added to partition the carotenoids. The mixture was shaken for 1 min and 10% sodium sulfate solution was added. After

shaking for 1 minute, the upper layer was collected and the lower layer was repeatedly extracted twice with hexane. Finally, the upper extracts were pooled and evaporated to dryness using a rotary evaporator.

The second extraction method was adopted from Reboul *et al.* (2006). First, 400mg of frozen-dried microalgae was added into 8ml of methanol containing 0.57% magnesium carbonate. Subsequently, the samples were homogenised for 30s using a vortex. Then, 8ml of chloroform, containing 0.005% butylated hydroxyl toluene (BHT), was added. The samples were homogenized for 30s more in the vortex blender. After a rest of 15 minutes, 8ml of distilled water was added into the samples and centrifuged (2000g for 10 minutes). The lower phases of the samples were collected and the remaining upper phases were extracted by the addition of 6ml of tetrahydrofuran. After that, the mixture was vortexed for 30s, and 6 ml of dichloromethane was also added. It was then vortexed for another 30s, after which 4ml of distilled water was added and the mixture was further re-vortexed for 30s. After centrifugation (2000g for 10 minutes at room temperature), the lower phase was collected and pooled with the previously collected phase. Lastly, the collected lower phase solvent was evaporated to dryness using a rotary evaporator.

The final extraction method was adopted from Marinova and Ribarova (2007). First, the pigments were extracted from a 400mg sample to which 0.04 g magnesium carbonate was added, with 6ml extraction solvent methanol:tetrahydrofuran (1:1, v/v) containing 0.1% butyl hydroxytoluene (BHT). The mixture was vortexed for about 3 minutes, and then centrifuged for 3 minutes at 1400g and the supernatant was collected. The pellet was re-extracted following the same procedure until the supernatant became colourless. The combined supernatants were evaporated to dryness using a rotary evaporator.

All the extraction procedures were performed under subdued light to avoid degradation loss of the pigments. The residue was dissolved in methanol at a concentration of 100mg/ml. Prior to the HPLC analysis, the sample solution was filtered using a Whatman polytetrafluroethylene (PTFE) 0.22µm syringe filter and the filtrate was injected into a HPLC valve with a 1ml syringe.

*Analysis of Carotenoids*

The carotenoids were analyzed by using HPLC (Agilent Series 1100, Model G131 3A, Agilent Technologies, Germany) equipped with degasser, quaternary pump, auto sampler and photodiode array detector. The carotenoids were separated by HPLC using a 150 × 4.6 mm, 3 µm C30 analytical column (Waters Co., Milford, MA, USA). The mobile phase system comprised methanol–methyl tert-butyl ether (MTBE) – water (81:15:4 v/v/v) (A) and methanol/ MTBE (10:90 v/v) (B) in the following gradient conditions 100% of A and 0% B, to 50% A and 50% B in 45 minutes, followed by 100% B within 15 minutes. The column temperature was set at 25$^0$C. The volume

injected into the HPLC was set as 20µl and the flow rate during the separation was set as 1ml/min. The wavelength used for the photodiode array detector in measuring the carotenoids was 450nm. The elution time was 45 minutes for a sample and the post time was 5 minutes.

The standard for the carotenoids were prepared from a stock solution of β-carotene, α-carotene, zeaxanthin and lutein. The identification of carotenoids was made by comparing with these standards, and the spiking test was also carried out to confirm the identification of certain peaks. The carotenoids were quantified using a calibration curve that was prepared using pure standards in the range of 0.025-5µg/ml.

*Data Analysis*

The computer software Statistical Package for Social Sciences version 16 (SPSS 16) was used to analyze the data in this study. The analysis was done in triplicates and the results were expressed as mean ± standard deviation. Meanwhile, the two-way ANOVA was used to compare the differences in the mean amounts of fatty acid and carotenoid from the microalgae, *Nannochloropsis oculata* and *Chaetoceros gracilis* using various extraction methods and also two different transmethylation methods (fatty acid only). The analysis was considered at a significance value of $p<0.05$.

## RESULTS AND DISCUSSION

*Percentage of Extraction Yields (Total Oil)*

As shown in Table 1, extraction method 1 (dichloromethane:methanol) showed the highest amount of the extraction yields for both *NO* and *CG*, with the mean values of 48.61% and 36.81%, respectively. Extraction method 3 using direct saponification with ethanolic KOH gave the least amount of extraction yields for both *NO* and *CG*, with the percentage value of 10.45% and 14.67%, respectively. A two-way ANOVA was conducted to examine the sample differences and the extraction methods on the extraction yields. There was a significant interaction between the various methods used and the extraction yields, $p = .027$. However, no significant differences were seen between the two microalgae in the extraction yield. The higher extraction yields of method 1 (dichloromethane: methanol) might be attributed to the presence of methanol,

TABLE 1
Total oil yield using different extraction methods from microalgae *Nannochloropsis oculata (NO)* and *Chaetoceros gracilis (CG)*

| Extraction methods | *Nannochloropsis oculata (NO)* | *Chaetoceros gracilis (CG)* |
|---|---|---|
| Method 1(Dichloromethane:methanol) | 486 ± 236[a] | 368 ± 56[a] |
| Method 2(Water: propan-2-ol: hexane) | 366 ± 151[a] | 316 ±18[a] |
| Method 3 (Direct saponification-ethanol) | 105 ± 14[b] | 147 ± 21[a] |

Each value is the mean ± standard deviation of triplicates expressed as g $kg^{-1}$dry weight. Within a column, means followed by the same letter are not significantly different ($p>0.05$).
No significant differences were observed between *NO* and *CG*.

a primary alcohol with the most active hydroxyl group (highly polar), which could stimulate the disruption of hydrogen bonds between lipid carbonyl, hydroxyl, and the amino groups, and the compounds of the nonextractable residue (Ruiz-lopez *et al.,* 2003).

*Fatty Acid Composition of Nannochloropsis Oculata (NO) and Chaetoceros Gracilis (CG) Using Different Extraction Methods*

In terms of the extraction efficiency of the fatty acid content (weight %), extraction method 1 (dichloromethane: methanol) coupled with acetyl chloride catalyzed transmethylation appeared to be the most efficient method for *NO*, as compared to other methods (Table 2). This was because this particular method could generate higher fatty acid content than other methods using both the acetyl chloride and BF3-catalyzed transmethylation methods. Since *NO* consists of a polysaccharide cell wall, the solubility of its cell matrix towards the non-polar solvent may enable the penetration of solvents into it and subsequently allow the oil to dissolve and be extracted for transmethylation. In addition, this method is also simpler and easier in its procedure as compared to two other methods. Indeed, a previous study has shown that dichloromethane, a less hazardous solvent, was an effective extraction solvent for fatty acid research (Cequier-Sanchez, 2008).

Nevertheless, extraction method 2 (water:propan-2-ol:hexane), coupled with acetyl chloride catalyzed transmethylation, appeared to be the most suitable method for the determination of fatty acid for *CG*, particularly C16 and C18 fatty acids (Table 3). The use of additional cell disruption treatment (ultrasonic bath) in this extraction procedure was notably useful in *CG*, a genus of diatoms, as they have the unique characteristic of a silica-based rigid cell wall, which may be difficult to break (Scala *et al.,* 2002). The use of an ultrasonic bath was related to the destruction of cell walls and the enhancement of mass-transfer through the cell wall due to the collapse of the bubbles produced by cavitation (Macias-Sanchez *et al.,* 2009). In this way, its extraction efficiency could be enhanced. Moreover, the use of this particular combination of solvents was also highly recommended in terms of its safety, low toxicity, and low cost (Smedes, 1999). Although hexane was used to substitute cyclohexane in this method, their almost similar properties would not create much difference in the result.

Among the three extraction methods used, method 3, which involved a direct saponification using ethanolic potassium hydroxide, gave the least number and amount (weight %) of fatty acid compositions in both the acetyl chloride and BF3-catalyzed transmethylation methods. This result disagrees with previous study which claimed that this method was an efficient technique to increase the extraction of fatty acid from biomass (Burja *et al.,* 2007). However, a study by Wang *et al.* (2000) found a lower concentration of fatty acids on chicken egg yolk by using the direct saponification extraction method compared to other methods (direct-methylation, chloroform-

TABLE 2
Fatty acid composition of *Nannochloropsis oculata (NO)* using different extraction and derivatization transmethylation) methods

| Fatty Acid Composition | *Nannochloropsis oculata (NO)* | | | | | |
|---|---|---|---|---|---|---|
| | Acetyl chloride- catalyzed transmethylation | | | BF3-catalyzed transmethylation | | |
| | Method 1 | Method 2 | Method 3 | Method 1 | Method 2 | Method 3 |
| C8:0 | 2.78 ± 0.72[a] | ND | ND | 1.60 ± 0.06[b] | 1.78 ± 0.31[b] | ND |
| C12:0 | 2.25 ± 0.14 | ND | ND | ND | ND | ND |
| C13:0 | 9.04 ± 0.11[a] | 7.52 ± 0.03[b] | 1.80 ± 0.11[c] | 1.91 ± 0.07[d] | 1.10 ± 0.02[e] | ND |
| C14:0 | 25.62 ± 0.23[a] | 12.13 ± 0.09[b] | ND | 7.40 ± 0.04[c] | 2.98 ± 0.10[cd] | 1.43 ± 0.07[d] |
| C14:1 | ND | ND | ND | ND | ND | 3.22 ± 0.05 |
| C16:0 | 159 ± 1.56[a] | 76.12 ± 0.23[b] | 16.47 ± 0.17[c] | 60.78 ± 0.48[d] | 18.91 ± 0.1[e] | 9.62 ± 0.05[c] |
| C16:1 | 198 ± 0.61[a] | 81.32 ± 1.32[b] | 14.13 ± 0.12[c] | 71.61 ± 0.47[d] | 16.79 ± 0.2[e] | 31.36 ± 0.39[f] |
| C17:0 | 4.44 ± 1.51[a] | 4.99 ± 0.18[a] | ND | ND | ND | ND |
| C17:1 | 6.67 ± 0.26[a] | 2.50 ± 0.06[a] | ND | 2.04 ± 0.08[b] | ND | ND |
| C18:0 | 7.26 ± 0.28[a] | 8.91 ± 0.23[b] | 2.65 ± 0.31[c] | 4.24 ± 0.15[d] | 1.96 ± 0.08[e] | ND |
| C18:1n9trans | ND | ND | ND | ND | ND | ND |
| C18:1n9cis | 59.86 ± 0.83[a] | 42.71 ± 0.29[b] | 5.67 ± 0.13[c] | 19.16 ± 0.23[d] | 7.51 ± 0.40[e] | 3.27 ± 0.10[f] |
| C18:2n6cis | 43.86 ± 0.58[a] | 30.12 ± 0.12[b] | 3.79 ± 0.20[c] | 14.84 ± 0.11[d] | 6.65 ± 0.17[e] | 2.96 ± 0.10[f] |
| C18:3n6 | 6.76 ± 0.39[a] | 03.04 ± 0.03[b] | ND | ND | ND | 6.27 ± 0.18[c] |
| C18:3n3 | 2.23 ± 0.37[a] | ND | ND | ND | ND | ND |
| C20:4n6 | 34.15 ± 0.54[a] | 12.1 ± 0.40[b] | ND | 10.86 ± 0.03[c] | 1.36 ± 1.18[d] | 0.88 ± 0.76[d] |
| C20:5 | 351 ± 3.37[a] | 152 ± 0.11[b] | 19.27 ± 0.23[c] | 106 ± 0.81[d] | 34.28 ± 0.24[e] | 15.59± 0.15[e] |

Method 1: (dichloromethane: methanol), Method 2: (water:propan-2-ol:hexane), Method 3: (Direct saponification- ethanolic KOH).
Each value is the mean ± standard deviation of triplicates expressed as g $kg^{-1}$of the total oil.
Within a row, means followed by the same letter are not significantly different ($p>0.05$).
ND = not detected

TABLE 3
Fatty acid composition of *Chaetoceros gracilis (CG)* using different extraction and derivatization (transmethylation) methods

| Fatty Acid Composition | *Chaetoceros gracilis (CG)* | | | | | |
|---|---|---|---|---|---|---|
| | Acetyl chloride transmethylation | | | BF3-catalyzed transmethylation | | |
| | Method 1 | Method 2 | Method 3 | Method 1 | Method 2 | Method 3 |
| C8:0 | 3.12 ± 0.62$^{a}$ | ND | ND | 1.41 ± 0.27$^{b}$ | 0.67 ± 0.59$^{b}$ | 0.87 ± 0.76$^{b}$ |
| C12:0 | 2.03 ± 2.04 | ND | ND | ND | ND | ND |
| C13:0 | 5.83 ± 103$^{a}$ | 14.46 ± 0.07$^{b}$ | 0.93 ± 0.81$^{c}$ | 2.26 ± 0.09$^{d}$ | 1.50 ± 0.22$^{d}$ | ND |
| C14:0 | 77.03 ± 0.10$^{a}$ | 80.70 ± 0.55$^{a}$ | 11.45 ± 5.57$^{b}$ | 19.73 ± 0.10$^{c}$ | 47.34 ± 0.19$^{d}$ | 4.04 ± 0.11$^{e}$ |
| C14:1 | 2.42 ± 0.11$^{a}$ | 2.33 ± 0.02$^{a}$ | ND | 0.93 ± 0.04$^{b}$ | 1.60 ± 0.10$^{c}$ | ND |
| C16:0 | 60.60 ± 1.78$^{a}$ | 129 ± 0.47$^{b}$ | 13.52 ± 7.60$^{c}$ | 12.13 ± 0.12$^{d}$ | 70.38 ± 0.13$^{e}$ | 8.50 ± 0.10$^{c}$ |
| C16:1 | 107 ± 0.28$^{a}$ | 103 ± 0.88$^{a}$ | 19.29 ± 7.38$^{b}$ | 25.71 ± 0.55$^{c}$ | 92.70 ± 0.09$^{d}$ | 6.54 ± 0.17$^{e}$ |
| C17:0 | 20.38 ± 0.34$^{a}$ | 18.89 ± 0.29$^{ab}$ | 3.50 ± 1.59$^{c}$ | 4.80 ± 0.06$^{d}$ | 17.95 ± 0.07$^{b}$ | 1.33 ± 0.14$^{e}$ |
| C17:1 | 28.72 ± 0.22$^{a}$ | 25.55 ± 4.34$^{a}$ | 6.28 ± 2.36$^{b}$ | 6.55 ± 0.25$^{c}$ | 22 ± 0.20$^{b}$ | 2.14 ± 0.06$^{c}$ |
| C18:0 | 8.13 ± 0.04$^{a}$ | 20.95 ± 0.43$^{b}$ | ND | 1.64 ± 0.03$^{c}$ | 12.52 ± 0.12$^{d}$ | ND |
| C18:1n9trans | ND | 5.68 ± 0.28$^{a}$ | ND | ND | 6.62 ± 0.14$^{b}$ | ND |
| C18:1n9cis | 11.80 ± 0.18$^{a}$ | 173 ± 0.29$^{b}$ | 5.79 ± 0.70$^{c}$ | ND | 3.38 ± 0.19$^{d}$ | 2.42 ± 0.04$^{e}$ |
| C18:2n6cis | 7.76 ± 0.09$^{a}$ | 121 ± 0.21$^{b}$ | 3.08 ± 0.18$^{c}$ | 1.05 ± 0.02$^{d}$ | 5.13 ± 0.15$^{e}$ | 1.59 ± 0.11$^{d}$ |
| C18:3n6 | ND | ND | ND | ND | 1.22 ± 0.05$^{a}$ | ND |
| C18:3n3 | ND | 4.64 ± 0.10$^{a}$ | ND | ND | ND | ND |
| C20:4n6 | 7.83 ± 8.74$^{a}$ | ND | ND | ND | 2.44 ± 0.11$^{b}$ | ND |
| C20:5 | 18.05 ± 0.35$^{a}$ | 14.93 ± 0.28$^{ab}$ | 3.82 ± 3.32$^{c}$ | 3.52 ± 0.06$^{d}$ | 17.45 ± 0.12$^{b}$ | ND |

Method 1: (dichloromethane: methanol), Method 2: (water:propan-2-ol:hexane), Method 3: (Direct saponification- ethanolic KOH).
Each value is the mean ± standard deviation of triplicate expressed as g $kg^{-1}$of total oil.
Within a row, means followed by the same letter are not significantly different ($p$>0.05).
ND = not detected.

methanol extraction, and postextraction saponification), although the reason was unknown.

Acetyl chloride-catalyzed transmethylation generated a higher amount (weight %) of fatty acid compared to BF3-catalyzed transmethylation in both the microalgae. This might be due to the highly basic condition of acetyl chloride, which could cause severe disruption to cell integrity, making *in situ* methyl ester derivation efficient (Tran *et al.,* 2009). Furthermore, the use of acetyl chloride-catalyzed transmethylation procedure has several advantages as compared to the most commonly performed methanolic BF3 method, such as longer shelf-life (without the need for refrigeration), lower cost, and smaller amount of catalyst required (5% acetyl chloride versus 10% BF3) (Carvalho & Malcata, 2005).

### *Percentage of Extraction Yields (Carotenoids)*

Table 4 shows that extraction method 1 (he xane:ethanol:acetone:toluene) generated the highest extraction yields for both *NO* (74.54 ± 4.75%) and *CG* (69.28 ± 14.71%). This was probably due to the longer period of contact time (1 hour) between the cellular component to be extracted and the solvent mixtures in extraction method 1 as compared to the other two methods (Henriques *et al.*, 2007). The two-way ANOVA showed significant differences between the samples ($p = 0.022$) on the extraction yields. Overall, it could be seen that all the extraction methods used generated higher extraction yields in *NO* than in *CG*. However, the difference in the extraction yields was small among these microalgae, particularly between extraction methods 2 and 3.

### *Carotenoids Concentration of the Different Extracts of Nannochloropsis Oculata (NO) and Chaetoceros Gracilis (CG)*

As shown in Table 5, β-carotene was found to be the highest, followed by zeaxanthin, α-carotene, and lutein in the *NO* using different extraction methods. However, extraction methods 1 and 2 were the only methods that could detect lutein

TABLE 4
Extraction yield of carotenoids using different extraction methods from microalgae *Nannochloropsis oculata (NO)* and *Chaetoceros gracilis(CG)*

| Extraction methods | *Nannochloropsis oculata (NO)* | *Chaetoceros gracilis (CG)* |
|---|---|---|
| Method 1 (Saponification) (hexane:ethanol:acetone:toluene) | 745 ± 48[a] | 693 ± 147[a] |
| Method 2 (No saponification) (methanol:chloroform) | 682 ± 25[a] | 515 ±12[b] |
| Method 3 (No saponification) (methanol:tetrahydrofuran) | 636 ± 39[a] | 502 ± 25[a] |

Each value is the mean ± standard deviation of triplicates expressed as g $kg^{-1}$dry weight.
Within a row, means followed by the same letter are not significantly different ($p>0.05$).
No significant differences were observed between the 3 extraction methods.

and α–carotene in *NO*, respectively, while zeaxanthin was not detected in extraction method 2. For zeaxanthin and β-carotene contents in *NO*, extraction method 1, which involved the saponification step, generated the highest concentration (µg/100g dry weight) as compared to the other methods. The functions of saponification include hydrolyzing the carotenoid esters and removing the chlorophyll and unwanted lipids on microalgae, which may interfere with chromatographic separation (Howe *et al.*, 2006). Since microalgae were high in their lipid content, saponification was necessary to achieve better results (better identification and higher concentration) as compared to the other two methods, which do not employ saponification.

Just like *NO*, *CG* was found to be the highest in the amount (g/kg dry weight) of β-carotene, followed by lutein and zeaxanthin using different extraction methods. However, α-carotene was not detected in *CG* with either of these extraction methods. This does not indicate the absence of α-carotene in *CG* because the failure to detect it might be due to other possible reasons such as the presence of light and oxygen while handling the samples or storing that would have contributed to its degradation. As shown in Table 6, extraction method 2 was the only method that could not

TABLE 5
Carotenoid concentrations (g $kg^{-1}$ dry weight) of *Nannochloropsis oculata (NO)* using different extraction methods

| Carotenoids | Method 1 ( Saponification) (hexane:ethanol:acetone: toluene) | Method 2 (No saponification) (methanol : chloroform) | Method 3 (No saponification) (methanol: tetrahydrofuran) |
|---|---|---|---|
| Lutein | 1.55 ±0.01 | ND | ND |
| Zeaxanthin | $3.74 \pm 0.03^{a}$ | ND | $2.67 \pm 0.08^{b}$ |
| β-carotene | $9.58 \pm 0.002^{a}$ | $9.46 \pm 0.05^{b}$ | $8.69 \pm 0.04^{c}$ |
| α-carotene | ND | 2.16 ± 0.004 | ND |

Each value is the mean ± standard deviation of triplicates expressed as g $kg^{-1}$ dry weight.
Within a row, means followed by the same letter are not significantly different ($p>0.05$).
ND = not detected.

TABLE 6: Carotenoid concentrations (g $kg^{-1}$dry weight) of *Chaetoceros gracilis (CG)* using different extraction methods

| Carotenoids | Method 1 ( Saponification) (hexane:ethanol:acetone: toluene) | Method 2 (No saponification) (methanol:chloroform) | Method 3 (No saponification) (methanol: tetrahydrofuran) |
|---|---|---|---|
| Lutein | $1.33 \pm 0.003^{a}$ | ND | $1.57 \pm 0.02^{b}$ |
| Zeaxanthin | $0.58 \pm 0.01^{a}$ | $0.75 \pm 0.003^{b}$ | $8.68 \pm 0.02^{c}$ |
| β-carotene | $7.945 \pm 0.002^{a}$ | ND | $8.08 \pm 0.01^{b}$ |
| α-carotene | ND | ND | ND |

Each value is the mean ± standard deviation of triplicates expressed as g $kg^{-1}$dry weight.
Within a row, means followed by the same letter are not significantly different ($p>0.05$).
ND = not detected.

detect the presence of lutein and β-carotene in *CG*. Hence, extraction method 2 was less suitable for the determination of carotenoid for *CG* as compared to extraction methods 1 and 3. However, extraction method 3 could generate a higher concentration of carotenoids (g/kg dry weight) compared to extraction method 1. In the present study, the saponification step in extraction method 1 might not have much impact on carotenoids determination of *CG* since the absence of the saponification step in extraction method 3 yielded a better result for carotenoids.

Moreover, the different cell matrix of these microalgae might have contributed to the difference in the concentrations of carotenoid in them. As described earlier in the determination of the fatty acid composition, the cellular structure of *NO* was distinctly different to *CG*. Hence, *CG* with its unique characteristic of a silica-based rigid cell wall might cause incomplete extraction of the biochemical compounds by the solvents alone, without any additional treatment (e.g. ultrasound bath, enzymes, microwave-assisted, etc.). This is in accordance with a published study, whereby an efficient disruption treatment of the membrane was required in order to achieve the efficient extraction of carotenoids as there was no standard technique can guarantee a maximization of the extraction yield (Valduga *et al.*, 2009). Unlike *CG*, the polysaccharide cell wall of *NO* might also be easier to penetrate using compatible solvents, and subsequently allow the extraction of the desired biochemical compounds.

## CONCLUSION

The comparison of various extraction methods on both fatty acids and carotenoids revealed that they produced extracts with different characteristics as well as quantitative differences. For fatty acid determination, the utilization of method 1 (dichloromethane:methanol) appeared to be the most efficient method for *NO*. Nevertheless, extraction method 2 (water:propan-2-ol:hexane), which involved additional treatment (ultrasonic bath), appeared to be a more suitable method for fatty acid determination in *CG*. As for carotenoids, extraction method 1, which uses the saponification step to remove chlorophyll, unwanted lipids and the involvement of more solvent mixtures (2 polar and 2 non-polar solvents), generated the highest concentration (µg/100g dry weight) in *NO*. However, extraction method 3 generated the highest concentrations in *CG*. Overall, this study has shown that using the right extraction method, high amounts of fatty acids and carotenoids could be obtained from the microalgae.

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# Soil Factors Influencing Heavy Metal Concentrations in Medicinal Plants

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## ABSTRACT

This study was conducted with the aim of finding soil factors which influence heavy metals uptake by medicinal plants. The heavy metal concentrations in medicinal plants at 3 different sites (different soil types) and the soils on which the plants grow were analysed. From the correlation analysis, soil properties affect all of the heavy metal concentrations in soils, meanwhile, only Cu and Se concentrations in soils affect their uptake by plants. However, this depends on plant parts (root and foliar), and the soil types. Principal component analysis (PCA) was also conducted to ascertain any patterns in the soil samples in relation to soil chemical characteristics and reinforce the findings from the correlation analysis. From the principal component analysis in this study, total Pb and As concentrations in medicinal plants were correlated with their concentrations in soils; however, they vary according to the soil types.

*Keywords:* Heavy metals, medicinal plants, soil properties, agriculture input, correlation analysis, principal component analysis

## INTRODUCTION

Medicinal plants play an important and vital role in traditional medicines and are widely consumed as home remedies (Ajasa *et al*., 2004). A survey carried out by World Health Organization (WHO) indicated that about 70-80% of the world population rely on non-conventional medicines, mainly of herbal sources in their primary healthcare (WHO, 2002). The Secretariats of the Convention on Biological Diversity (CBD) also reported US$60 billion sales in the global herbal medicine markets in 2000 (Ang & Lee, 2006). Meanwhile, in Malaysia, the latest

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report shows that the total sales for local herbal products reached approximately RM100 million for the year 2000. In 2005, these sales increased to RM500 million and RM2.5 billion during 2010 (Hassan, 2008). In recent decades, the use of phytopharmaceuticals and herbal medicines has increased worldwide due to several reasons, among them are the side-effects which are often lower than those presented when synthetic drugs are employed, as well as due to the higher costs of many conventional pharmaceutical formulations (Mamani *et al*., 2005). However, the vast majorities of the medicinal herbal products are unlicensed and are not required to demonstrate efficiency, safety or quality. Unknown effects of some of medicinal herbs have been observed. Several examples are allergic reactions, toxic reactions, mutagenic effects, drug interaction, drug contamination, and mistaken plant identities (Basgel & Erdemoglu, 2006).

One obvious safety issue related to the medicinal plants is the possibility that some herbal medicines contain heavy metals (Ernst, 2002). Although the phase three registration of traditional medicines was implemented on 1st January, 1992, there are still many unregistered traditional medicines rampantly available in the Malaysian market with contaminations of heavy metals above the permissible limits. All products registered with the Drug Control Authority (DCA) Malaysia will have to carry a unique product registration number. The unregistered traditional medicines or counterfeits are a serious threat to public health since these drugs are manufactured illegally and contain levels of chemical compounds which, once consumed, could result in serious unknown side effects or even death (Ang, 2008).

Metals are probably the oldest known toxins to man (Mamani *et al*., 2005). The eight most common heavy metal pollutants listed by the Environment Protection Agency (EPA) are arsenic, cadmium, chromium, copper, mercury, nickel, lead and zinc (Athar & Vohora, 1995). Poisonings associated with the presence of toxic metals in medicinal plants have been reported in Asia, Europe and the United States (Olujohungbe *et al*., 1994; Dunbabin *et al*., 1992; Kakosy *et al*., 1996; Markowitz *et al*., 1994). Their basic source, for man, is the food chain (Lozak *et al*., 2002). Ingestion of heavy metals through medicines and food can cause accumulation in organisms, producing serious health hazards such as injury to the kidneys, symptoms of chronic toxicity, renal failure and liver damage (Abou-Arab *et al*., 1999). Individuals generally use herbal medicines for prolonged period to achieve a desirable effect. Prolonged consumption of such herbal medicines may induce chronic or subtle health hazards (Shailendra & Sahadeb, 2002).

Heavy metals in soils originate either from weathering of parent materials and/or from numerous external contaminating sources (Fergusson & Kim, 1991). Plants are an important link for transferring trace elements from soils to man. The level of essential elements in plants varies, in which the content is being affected by the

geochemical characteristics of a soil and the ability of plants to selectively accumulate some of these elements. Bioavailability of the elements depends on the nature of their association with the constituents of a soil (Lozak *et al*., 2002). Their availability for plant absorption can be affected by factors including pH, Eh, CEC and organic matter content of soils and concentrations of the competing trace elements (Weiping *et al*., 2008). Plants readily assimilate through the roots such elements which dissolve in water and occur in ionic forms (Lozak *et al*., 2002). High levels of toxic metals can also occur during medicinal preparations or processing when they are used as active ingredients, as in the case of Pb and Hg in some Chinese, Mexican and Indian medicines (Levitt, 1984; Chan *et al*., 1993) or when the plants are grown in polluted areas, such as near roadways or metal mining and smelting operations (Pip, 1991). In addition, high levels can be found when agricultural expedients are used, including cadmium containing fertilizers, organic mercury or lead based pesticides, and contaminated irrigation water (Abou-Arab *et al*., 1999).

In this study, the heavy metals and their concentrations at 3 different sites (different soil types) and the soils on which the plants grow were analysed. The aim of this study was to identify the soil factors affecting heavy metals uptake by correlating the soil factors with the plant data.

## MATERIALS AND METHODS

### *Study Area*

The soil and plant samples in this study were randomly collected from the FRIM External Research Station, Setiu, Terengganu (17 samples), Felda Agricultural Services Sdn. Bhd, Jengka 25 Bandar Jerantut, Pahang (16 samples) and MARDI Jalan Kebun, Klang, Selangor (24 samples), with each site having its own management practice. The samples were collected using paired sampling with the soil samples taken adjacent to the medicinal plants which were sampled. At each point, three soil auger borings were composited into one sample. It is noted that each location has a different soil type. For the location at Setiu, Terengganu, the soil type is of the Beach Ridges Interspersed with Swales (BRIS) soil from the Jambu series (Spodic Quartzipsamment), whereas for Jengka 25, Pahang, the soil type is of the Durian series (Plinthaquic Paleudult). As for the location at Jalan Kebun, Klang, the soil is of peaty type.

The history of fertilization for each site also differs. The *Tongkat Ali* collected from Setiu, Terengganu was fertilized with the NPK fertilizer. However, there were also medicinal plants that were unfertilized or considered as growing naturally or wild. The medicinal plants collected from Jengka 25, Pahang were all fertilized with ammonium sulphate (AS), Christmas Island Phosphate Rock (CIRP), muriate of potash (MOP), kieserite and compost. Meanwhile, the medicinal plants from Jalan Kebun, Klang, were fertilized with chicken dung only. The medicinal plants collected were

grouped based on the plant parts utilized for medicinal purposes, which are the foliar and roots. This was done in this study because the distribution of heavy metals is different for different plant parts.

*Soil and Plant Samples*

Aqua-regia digestion (Black *et al*., 1965) was used to extract the total heavy metals in the soil samples, and these elements were determined using the PE 5100 atomic absorption spectrophotometer (AAS). Meanwhile, to determine the total heavy metals in the plant tissue, the dry ashing method (Leo & James, 1973) was used and determined by the Zeeman 4100ZL graphite furnace atomic absorption spectrophotometer. Soil properties data such as pH, EC, CEC, organic carbon (determined by the Walkley and Black method) and clay content (determined by the pipette method) were also required for the correlation analysis, and all the methods were referred to Black *et al*. (1965).

## RESULTS AND DISCUSSION

Chemical properties and heavy metal concentrations of the soils under study are given in Table 1 and Table 2, respectively. All of the soils in these 3 locations differed in their total heavy metal concentration.

The soil pH values were below 7.0 (4.52-6.71), indicating the acidic nature of the soils for all the 3 locations. As mentioned by Bang (2002), acidity is the main factor that controls metal mobility in soils. Generally, soils of Malaysia are acidic because they are highly weathered. The total heavy metal concentrations in soils sampled from Jalan Kebun, Klang, which used only the chicken dung fertilizer, were highest for Cu (49.29 mg $kg^{-1}$), Zn (84.45 mg $kg^{-1}$), Ni (13.88 mg $kg^{-1}$), As (12.56 mg $kg^{-1}$) and Cd (0.72 mg $kg^{-1}$). Meanwhile, for Jengka 25, Pahang, which used a compound fertilizer for fertilization, had the highest Pb (56.77 mg $kg^{-1}$) and Se (7.98 mg $kg^{-1}$) total concentrations. The soil samples collected from Jalan Kebun, Klang, also showed that their chemical properties such as pH, EC, CEC and organic carbon were the highest among all the locations. The sandy BRIS soil sampled from Setiu, Terengganu, exhibited lower concentrations of heavy metals as compared to the other 2 locations.

Tables 3 (a) – (d) show the heavy metal concentrations (mg $kg^{-1}$ dry weight basis) in the medicinal plants sampled from Setiu, Terengganu, Jengka 25, Pahang and Jalan Kebun, Klang. The concentrations obtained were compared to the maximum permissible concentrations as stated in the Malaysian Herbal Monograph (2009), which are 10, 5.0 and 0.3 mg $kg^{-1}$ for Pb, As and Cd, respectively. On the other hand, for Cu and Zn, the concentration values were compared to the maximum permissible concentrations as stated in the Malaysian Food Act (1983) and Malaysian Food Regulation (1985).

Based on the data as shown in Table 3(a), in comparing between the *Tongkat Ali* from Setiu, Terengganu and Jengka 25, Pahang, the *Tongkat Ali* from the latter site tended to have higher heavy metal concentrations except for Ni and Se. However, in terms of maximum permissible concentrations,

TABLE 1
Chemical properties of the soils at different locations.

| Locations | Soil properties | | | | |
|---|---|---|---|---|---|
| | pH | EC ($dScm^{-1}$) | CEC ($cmolkg^{-1}$) | Organic Carbon(%) | Clay Content (%) |
| Setiu, Terengganu | 3.49 – 5.22 | 10.4 – 88.8 | 1.26 – 13.0 | 0.46 – 6.70 | 0 – 1.64 |
| Mean ± SE, n=17 | 4.37 ± 0.13 | 30.87 ± 5.92 | 5.39 ± 0.92 | 3.37± 0.58 | 0.27 ± 0.12 |
| Jengka 25, Pahang | 4.22 – 6.43 | 23.4 – 217.3 | 2.45 – 15.06 | 0.05 – 2.09 | 21.04 – 57.01 |
| Mean ± SE, n=16 | 5.42 ± 0.19 | 74.42 ± 13.05 | 7.71 ± 0.98 | 0.73 ± 0.11 | 33.30 ± 3.13 |
| Jalan Kebun, Klang, Selangor | 5.98 – 7.30 | 235 - 2780 | 21.40 – 59.10 | 4.74- 6.38 | 0 |
| Mean ± SE, n=24 | 6.71 ± 0.13 | 654.97 ± 135.28 | 42.31 ± 2.04 | 5.91 ± 0.06 | 0 |

SE: Standard Error

TABLE 2
Total heavy metals concentration ($mgkg^{-1}$) in the soil samples at different locations.

| Locations | Heavy metals concentration in soil ($mgkg^{-1}$)* | | | | | | |
|---|---|---|---|---|---|---|---|
| | Cu | Zn | Ni | Pb | As | Cd | Se |
| Setiu, Terengganu | 1.87 – 3.73 | 5.73 – 11.47 | 0.28 – 4.33 | 0.70 – 4.35 | 0.16 – 2.86 | 0.01 – 0.42 | 0.06 – 0.78 |
| Mean ± SE, n=17 | 2.60 ± 0.13 | 7.80 ± 0.44 | 1.63 ± 0.32 | 2.88 ± 0.42 | 1.09 ± 0.22 | 0.09 ± 0.02 | 0.28 ± 0.05 |
| Jengka 25, Pahang | 4.53 – 21.20 | 12.67 – 59.07 | 3.65 – 9.26 | 29.01 – 118.52 | 0 – 26.27 | 0.11 – 0.85 | 0.39 – 51.83 |
| Mean ± SE, n=16 | 12.07 ± 1.23 | 28.19 ± 3.70 | 6.37 ± 0.36 | 56.77 ± 6.91 | 9.72 ± 2.95 | 0.46 ± 0.07 | 7.98 ± 4.18 |
| Jalan Kebun, Klang, Selangor | 33.04 – 69.72 | 50.76 – 141.12 | 7.32 – 17.87 | 1.50 – 25.36 | 4.37 – 36.65 | 0.36 – 0.93 | 0.18 – 2.29 |
| Mean ± SE, n=24 | 49.29 ± 2.06 | 84.45 ± 4.67 | 13.88 ± 0.52 | 7.15 ± 1.20 | 12.56 ± 1.56 | 0.72 ± 0.03 | 1.07 ± 0.11 |

SE: Standard Error
*$95^{th}$ percentile concentration levels for:
Cu: 50 $mgkg^{-1}$; Zn: 95 $mgkg^{-1}$; Ni: 45 $mgkg^{-1}$; Pb: 65 $mgkg^{-1}$; As: 60 $mgkg^{-1}$; and Cd: 0.30 $mgkg^{-1}$

Cd concentration in a few of the *Tongkat Ali* samples from Setiu, Terengganu and all of the *Tongkat Ali* samples from Jengka 25, Pahang exceeded the reference limit. Other than that, Pb concentration in most of the *Tongkat Ali* sampled from Jengka 25, Pahang, was also found to exceed the reference limit.

Table 3(b) shows the heavy metal concentrations in the leafy medicinal plants sampled from Setiu, Terengganu. As observed, Cu, Zn, Pb and Cd concentrations in certain medicinal plants already exceeded the maximum permissible concentrations. For the heavy metal concentrations in the leafy medicinal plants sampled from Jengka

TABLE 3(a)
Heavy metal concentrations (mgkg$^{-1}$, dry weight basis) in Tongkat Ali (*Eurycoma longifolia*) samples at different locations.

| | Sample | Elements* | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Cu | Zn | Ni | Pb | As | Cd | Se |
| Setiu, Terengganu | Tongkat Ali Control R2 | 9.33 | 22.33 | 0.00 | 2.93 | 0.05 | 0.00 | 2.29 |
| | Tongkat Ali Control R3 | 10.33 | 13.00 | 1.92 | 2.08 | 0.10 | 0.00 | 2.68 |
| | Tongkat Ali Control R4 | 10.67 | 22.33 | 0.98 | 1.20 | 0.10 | 0.00 | 2.39 |
| | Tongkat Ali NPK R1 | 9.33 | 24.33 | 21.85 | 1.27 | 0.01 | 0.00 | 1.75 |
| | Tongkat Ali NPK R2 | 8.00 | 22.00 | 10.91 | 1.68 | 0.23 | 0.52 | 0.22 |
| | Tongkat Ali NPK R3 | 8.33 | 26.33 | 10.41 | 1.97 | 0.22 | 2.06 | 0.42 |
| | Tongkat Ali NPK R4 | 9.67 | 25.67 | 0.00 | 1.29 | 0.10 | 2.88 | 0.06 |
| | Tongkat Ali (W) | 9.67 | 15.00 | 0.00 | 3.97 | 0.05 | 0.00 | 0.48 |
| | Mean ± SE | 9.42 ± 0.32 | 21.37 ± 1.72 | 5.76 ± 2.82 | 2.06 ± 0.34 | 0.11 ± 0.03 | 0.68 ± 0.40 | 1.2 ± 0.39 |
| Jengka 25, Pahang | Tongkat Ali R1 | 16.00 | 23.50 | 3.07 | 22.07 | 0.70 | 1.11 | 0.02 |
| | Tongkat Ali R2 | 15.00 | 34.00 | 2.83 | 4.73 | 0.32 | 0.77 | 0.18 |
| | Tongkat Ali R3 | 17.00 | 35.00 | 2.07 | 50.15 | 0.34 | 1.38 | 0.16 |
| | Tongkat Ali R4 | 13.00 | 26.50 | 3.54 | 11.07 | 0.33 | 0.68 | 0.16 |
| | Mean ± SE | 15.25 ± 0.85 | 29.75 ± 2.82 | 2.88 ± 0.31 | 22.0 ± 0.04 | 0.42 ± 0.09 | 0.99 ± 0.16 | 0.13 ± 0.04 |

SE: Standard Error
*Maximum permitted concentration for:
Cu: 30 mgkg$^{-1}$; Zn: 40 mgkg$^{-1}$; Pb: 10 mgkg$^{-1}$; As: 5.0mgkg$^{-1}$ and Cd: 0.3 mgkg$^{-1}$.

TABLE 3(b)
Heavy metal concentrations (mgkg$^{-1}$, dry weight basis) in the leafy medicinal plants sampled from Setiu, Terengganu

| Sample | Elements | | | | | | |
|---|---|---|---|---|---|---|---|
| | Cu | Zn | Ni | Pb | As | Cd | Se |
| Cucur Atap (*Baeckea frutescens*) (W) | 25.53 | 33.00 | 6.91 | 12.43 | 0.28 | 0.48 | 2.78 |
| Kerbau Amok (*Schefflera ridleyi*) (W) | 27.63 | 30.67 | 3.22 | 5.89 | 0.43 | 1.03 | 3.28 |
| Mata Ayam (*Ardisia crenata*) (W) | 25.93 | 44.00 | 2.99 | 11.62 | 1.51 | 0.22 | 1.35 |
| Mas Cotek (*Ficus deltoidea*) (W) | 21.53 | 174.7 | 4.05 | 15.70 | 0.45 | 0.20 | 0.60 |
| Senduduk (*Melastoma malabathricum*) 1 (W) | 41.23 | 46.33 | 3.23 | 35.09 | 1.70 | 0.43 | 0.65 |
| Senduduk (*Melastoma malabathricum*) 2 (W) | 39.17 | 102.7 | 2.47 | 7.88 | 1.14 | 0.34 | 0.87 |
| Gelam (*Melaleuca cajputi*) (W) | 21.37 | 35.00 | 3.15 | 8.89 | 0.87 | 0.32 | 1.92 |

TABLE 3(b) (continue)

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Kapal terbang (*Chromolaena odorata*) (W) | 75.90 | 60.00 | 5.45 | 13.41 | 1.66 | 2.18 | 0.88 |
| Kemunting (*Catharanthus roseus*) (W) | 21.40 | 63.33 | 2.67 | 8.38 | 0.29 | 0.21 | 5.20 |
| Mean ± SE | 33.30 ± 5.87 | 65.52 ± 15.53 | 3.79 ± 0.49 | 13.25 ± 2.92 | 0.93 ± 0.20 | 0.60 ± 0.21 | 1.95 ± 0.52 |

SE: Standard Error (W): Wild plant
*Maximum permitted concentration for:
Cu: 30 $mgkg^{-1}$; Zn: 40 $mgkg^{-1}$; Pb: 10 $mgkg^{-1}$ ; As: 5.0 $mgkg^{-1}$; and Cd: 0.3 $mgkg^{-1}$

TABLE 3(c)
Heavy metal concentrations ($mgkg^{-1}$, dry weight basis) in the leafy medicinal plants from Jengka 25, Pahang.

| Sample | Elements | | | | | | |
|---|---|---|---|---|---|---|---|
| | Cu | Zn | Ni | Pb | As | Cd | Se |
| Kacip Fatimah (*Labisia Pumila*) R1 | 18.00 | 38.50 | 1.43 | 6.58 | 0.59 | 1.47 | 0.44 |
| Kacip Fatimah R2 | 14.50 | 27.00 | 2.64 | 2.01 | 1.33 | 1.80 | 0.60 |
| Kacip Fatimah R3 | 15.00 | 26.00 | 2.24 | 5.82 | 0.30 | 1.94 | 0.20 |
| Kacip Fatimah R4 | 16.00 | 24.50 | 2.76 | 2.58 | 0.18 | 0.19 | 0.25 |
| Mas Cotek (*Ficus deltoidea*) R1 | 16.00 | 16.50 | 0.96 | 1.46 | 0.33 | 1.37 | 0.15 |
| Mas Cotek R2 | 12.50 | 14.50 | 1.01 | 2.39 | 0.25 | 0.12 | 0.23 |
| Mas Cotek R3 | 9.50 | 12.00 | 0.66 | 2.08 | 0.28 | 0.08 | 0.03 |
| Mas Cotek R4 | 9.50 | 15.00 | 0.45 | 2.48 | 0.45 | 0.09 | 0.19 |
| Misai Kucing (*Orthasiphon stamineus*) R1 | 17.50 | 35.00 | 0.68 | 2.79 | 0.00 | 0.09 | 0.01 |
| Misai Kucing R2 | 18.00 | 38.50 | 1.67 | 0.55 | 0.00 | 0.09 | 0.11 |
| Misai Kucing R3 | 24.00 | 42.50 | 1.96 | 3.02 | 0.00 | 0.10 | 0.13 |
| Misai Kucing R4 | 19.00 | 32.00 | 5.71 | 4.79 | 0.00 | 0.14 | 0.37 |
| Mean ± SE | 15.79 ± 1.18 | 26.83 ± 3.06 | 1.85 ± 0.42 | 3.05 ± 0.52 | 0.31 ± 0.11 | 0.62 ± 0.22 | 0.23 ± 0.05 |

SE: Standard Error
*Maximum permitted concentration for:
Cu: 30 $mgkg^{-1}$; Zn: 40 $mgkg^{-1}$; Pb: 10 $mgkg^{-1}$; As: 5.0 $mgkg^{-1}$; and Cd: 0.3 $mgkg^{-1}$.

TABLE 3(d)
Heavy metal concentrations ($mgkg^{-1}$, dry weight basis) in the medicinal plants from Jalan Kebun, Klang, Selangor.

| Sample | Elements | | | | | | |
|---|---|---|---|---|---|---|---|
| | Cu | Zn | Ni | Pb | As | Cd | Se |
| Ginseng Jepun (*Panax japonica*) | 8.35 | 56.70 | 0.10 | 0.74 | 0.96 | 0.14 | 0.79 |
| Tetulang @ hempedu ular | 7.95 | 35.80 | 0.00 | 0.61 | 0.88 | 0.06 | 0.46 |
| Ketumbar jawa (*Eryngium foetidum L.*) | 10.35 | 40.15 | 1.65 | 0.82 | 0.58 | 0.08 | 0.04 |

TABLE 3(d) (continue)

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Naga buana (*Phyllanthus pulcher*) | 11.10 | 48.30 | 4.24 | 1.29 | 0.37 | 0.08 | 0.31 |
| Tebu badak | 8.60 | 29.20 | 2.88 | 1.71 | 0.36 | 0.13 | 0.05 |
| Kunyit hantu (*Curcuma aeruginosa*) | 5.45 | 17.70 | 3.08 | 1.27 | 0.86 | 0.09 | 0.11 |
| Asparagus (*Asparagus officinalis*) | 6.80 | 37.85 | 2.80 | 0.14 | 0.49 | 0.01 | 0.00 |
| Lemayung hijau (*Zingiber zerumbet*) | 6.10 | 40.85 | 2.29 | 0.28 | 0.22 | 0.18 | 0.44 |
| Pegaga brunei (*Centella asiatica*) | 5.25 | 34.90 | 2.50 | 0.68 | 0.53 | 0.08 | 0.15 |
| Daun Mengkudu (*Morinda citrifolia*) | 6.35 | 33.90 | 2.58 | 0.93 | 0.20 | 0.10 | 0.19 |
| Kaduk (*Piper sarmentosum roxb*) | 10.15 | 42.10 | 2.73 | 0.82 | 0.21 | 0.09 | 0.87 |
| Bangun-bangun (*Solenostemon amboinicus*) | 8.10 | 56.70 | 2.31 | 1.28 | 0.38 | 0.07 | 0.45 |
| Jintan hitam (*Nigella Sativa*) | 2.70 | 27.20 | 2.51 | 0.55 | 0.64 | 0.36 | 0.23 |
| Sabung nyawa (*Gynura procumbens*) | 7.15 | 31.55 | 2.83 | 0.26 | 0.51 | 0.09 | 0.11 |
| Tembaga suasa (*Hanguana malayana (Jack) Merr.*) | 5.70 | 49.50 | 0.81 | 0.59 | 0.33 | 0.12 | 0.00 |
| Cekur jantan (*Kaempferia galangal*) | 3.15 | 27.95 | 0.71 | 0.77 | 1.27 | 0.12 | 0.00 |
| Selasih serai (*Ocimum Sanctum*) | 4.05 | 87.55 | 0.89 | 1.03 | 0.70 | 0.25 | 0.00 |
| Pegaga melayu (*Centella asiatica*) | 4.10 | 79.10 | 0.73 | 0.59 | 0.41 | 0.70 | 0.00 |
| Pegaga nyonya (*Centella asiatica*) | 3.75 | 29.55 | 0.92 | 5.52 | 0.70 | 0.54 | 0.00 |
| Cekur mas (*Kaempferia galangal*) | 8.80 | 25.85 | 0.83 | 5.18 | 0.42 | 0.26 | 0.00 |
| Mata pelanduk (*Ardisia crenata*) | 6.65 | 31.80 | 0.88 | 0.30 | 1.06 | 0.08 | 0.00 |
| Pudina (*Mentha arvensis*) | 14.10 | 34.90 | 0.91 | 0.54 | 0.86 | 0.14 | 0.00 |
| Beremi (*Limnophila aromatic*) | 9.00 | 37.95 | 0.97 | 0.34 | 0.49 | 0.08 | 0.27 |
| Kesum (*Polygonum minus huds*) | 6.90 | 22.85 | 4.18 | 4.05 | 1.27 | 0.13 | 0.00 |
| Mean ± SE | 7.11 ± 0.56 | 40 ± 3.38 | 1.85 ± 0.25 | 1.26 ± 0.30 | 0.61 ± 0.06 | 0.17 ± 0.03 | 0.19± 0.05 |

SE: Standard Error

*Maximum permitted concentration for:

Cu: 30 $mgkg^{-1}$; Zn: 40 $mgkg^{-1}$; Pb: 10 $mgkg^{-1}$; As: 5.0 $mgkg^{-1}$; and Cd: 0.3 $mgkg^{-1}$.

25, Pahang, Table 3(c) shows that only a few medicinal plants exceeded the limit for Cd concentration. Conversely for the other samples, their heavy metal concentrations were still under the permissible levels. Table 3(d) shows the heavy metal concentrations in the leafy medicinal plants from Jalan Kebun, Klang. From the table, it shows that some of the medicinal plants exceeded the maximum permissible concentrations, which were Zn and Cd.

Correlation analysis was carried out in order to see the relationship between heavy metal concentrations in the soil samples with the soil properties, or the soil factors that influence or control heavy metal concentrations in soils. The correlation analysis between the soil properties (i.e.

pH, EC, CEC, organic carbon, and clay content) with heavy metal concentrations in soils grown with the *Tongkat Ali* are shown in Table 4 (a). As observed in this table, Cu and Zn concentrations in the soils sampled from Jengka 25, Pahang were negatively correlated with CEC and organic carbon of the soils, respectively. Notably, arsenic concentration exhibited a positive correlation with organic carbon of the soils, while the other elements were not significantly correlated with any soil properties of the soils from Jengka 25, Pahang. Table 4 (b) shows the correlation coefficients between soil properties with heavy metal concentrations in soils grown with leafy medicinal plants. From this table, the pH values of soils from Setiu, Terengganu were found to affect most of heavy metal concentrations (Cu, Ni, Pb, As and Se) in the soil samples from there. Furthermore, organic carbon was also found to affect Pb concentration inversely. For the soil samples from Jengka 25, Pahang, all the soil properties were found to affect heavy metal concentrations in the soil samples. As observed, the pH of the soils affected Cu, Zn, Pb and Cd concentrations positively. Moreover, electrical conductivity also affected Zn, Ni, Pb and Cd concentrations of the soils from Jengka 25. Besides, clay content was also positively correlated with Cu, Zn, Ni, Pb and Cd concentrations in the soils from Jengka 25, Pahang, whereas organic carbon showed a positive correlation with Se concentration in that soils. For the site at Jalan Kebun, Klang, the properties of the soils did not show any significant correlation with heavy metal concentrations in the soils, except for EC, which was positively correlated with As concentration in the peaty type soil.

TABLE 4(a)
Correlation coefficients (r) between soil properties with heavy metals concentration in soil samples grown with Tongkat Ali (*Eurycoma longifolia*) at different locations.

| Location | Soil properties | Correlation coefficients, r | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Cu | Zn | Ni | Pb | As | Cd | Se |
| Setiu, Terengganu (n=8) | pH | 0.01 | -0.54 | 0.15 | 0.07 | 0.44 | -0.16 | 0.41 |
| | EC | 0.20 | 0.26 | -0.09 | -0.12 | -0.34 | 0.17 | -0.03 |
| | CEC | 0.30 | 0.41 | -0.14 | -0.17 | -0.42 | 0.11 | -0.01 |
| | Organic carbon | 0.30 | 0.43 | -0.24 | -0.25 | -0.52 | 0.08 | -0.02 |
| Jengka 25, Pahang (n=4) | pH | 0.21 | 0.42 | 0.44 | 0.96 | -0.37 | 0.13 | -0.75 |
| | EC | -0.74 | -0.88 | -0.13 | -0.59 | 0.80 | -0.32 | 0.65 |
| | CEC | -0.99* | -0.93 | 0.10 | 0.16 | 0.77 | -0.59 | 0.01 |
| | Organic carbon | -0.92 | -0.98* | 0.28 | -0.18 | 0.97* | -0.22 | 0.51 |
| | Clay content | 0.23 | 0.11 | -0.91 | -0.78 | -0.32 | -0.38 | 0.17 |

* Significantly correlated ($p<0.05$)

TABLE 4(b)
Correlation coefficients (r) between soil properties with heavy metal concentrations in the soil samples grown with the leafy medicinal plants at different locations.

| Location | Soil properties | Correlation coefficients, r | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Cu | Zn | Ni | Pb | As | Cd | Se |
| Setiu, Terengganu (n=9) | pH | 0.34 | 0.67* | 0.75* | 0.85* | 0.86* | 0.64 | 0.81* |
| | EC | -0.33 | -0.30 | -0.31 | -0.51 | -0.33 | -0.38 | -0.39 |
| | CEC | -0.37 | -0.50 | -0.39 | -0.58 | -0.54 | -0.45 | -0.43 |
| | Organic carbon | -0.55 | -0.54 | -0.19 | -0.69* | -0.51 | -0.61 | -0.59 |
| | Clay | -0.09 | 0.09 | 0.01 | 0.06 | 0.13 | 0.06 | -0.22 |
| Jengka 25, Pahang (n=12) | pH | 0.71* | 0.66* | 0.53 | 0.75* | 0.38 | 0.79* | -0.04 |
| | EC | 0.50 | 0.84* | 0.72* | 0.73* | 0.46 | 0.73* | -0.19 |
| | CEC | 0.08 | -0.26 | -0.55 | -0.19 | -0.37 | 0.05 | -0.03 |
| | Organic carbon | -0.12 | 0.03 | 0.08 | 0.26 | -0.11 | 0.24 | 0.84* |
| | Clay content | 0.63* | 0.85* | 0.73* | 0.88* | 0.28 | 0.73* | -0.16 |
| Jalan Kebun, Klang (n=24) | pH | 0.01 | 0.03 | -0.09 | -0.01 | -0.14 | 0.26 | -0.25 |
| | EC | 0.14 | 0.23 | -0.02 | 0.20 | 0.63* | 0.13 | 0.004 |
| | CEC | 0.34 | 0.13 | -0.40 | 0.08 | -0.15 | -0.16 | -0.37 |
| | Organic Carbon | 0.12 | 0.17 | -0.09 | -0.09 | 0.12 | 0.09 | 0.05 |

* Significantly correlated ($p<0.05$)

The correlation analyses between the heavy metal concentrations in the *Tongkat Ali* and leafy medicinal plants with soil properties (pH, EC, CEC, organic carbon, clay content) are shown in Tables 5(a) and(b), respectively. From Table 5(a), the pH value of the soils from Setiu, Terengganu was positively correlated with Zn concentration in the *Tongkat Ali*, which means Zn concentration in the plants would increase with the increase in the soil pH. Meanwhile, Pb concentration in the plants increased with the increase in EC and CEC of the BRIS soil. For the *Tongkat Ali* sampled from Jengka 25, Pahang, the EC of the soils were found to have a positive correlation with As and a negative correlation with Se concentration in the *Tongkat Ali*. The correlation analysis between the soil properties with heavy metal concentrations in the leafy medicinal plants is shown in Table 5(b). From the table, there was no correlation observed between the soil properties with heavy metal concentrations in the leafy medicinal plants collected from Setiu, Terengganu. Meanwhile, for the medicinal plants collected from Jengka 25, Pahang, Pb concentration showed a positive correlation with pH and EC of the soils. Other than EC and pH of the soils, clay content in the soils of Jengka 25, Pahang, also affected the Cd concentration in the

TABLE 5(a)
Correlation coefficients (r) between soil properties with heavy metal concentrations in Tongkat Ali (*Eurycoma longifolia*) at different locations.

| Location | Soil properties | Correlation coefficients, r | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Cu | Zn | Ni | Pb | As | Cd | Se |
| Setiu, Terengganu (n=8) | pH | -0.49 | 0.72* | 0.32 | -0.43 | 0.36 | 0.30 | 0.06 |
| | EC | 0.19 | -0.57 | -0.31 | 0.76* | -0.29 | -0.24 | -0.25 |
| | CEC | 0.24 | -0.66 | -0.32 | 0.72* | -0.23 | -0.20 | -0.21 |
| | Organic carbon | 0.26 | -0.59 | -0.26 | 0.63 | -0.22 | -0.16 | -0.23 |
| Jengka 25, Pahang (n=4) | pH | 0.40 | 0.86 | -0.79 | 0.65 | -0.70 | 0.47 | 0.69 |
| | EC | 0.15 | -0.83 | 0.34 | -0.08 | 0.98* | 0.14 | -0.98* |
| | CEC | 0.82 | -0.22 | -0.45 | 0.56 | 0.79 | 0.79 | -0.78 |
| | Organic carbon | 0.45 | -0.65 | -0.01 | 0.39 | 0.92 | 0.55 | -0.94 |
| | Clay content | -0.43 | -0.30 | -0.59 | -0.91 | 0.21 | -0.71 | -0.15 |

* Significantly correlated ($p<0.05$)

TABLE 5(b)
Correlation coefficients (r) between soil properties with heavy metal concentrations in the leafy medicinal plants at different locations.

| Location | Soil properties | Correlation coefficients, r | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Cu | Zn | Ni | Pb | As | Cd | Se |
| Setiu, Terengganu (n=9) | pH | 0.53 | -0.30 | 0.27 | 0.14 | 0.41 | 0.47 | -0.33 |
| | EC | -0.23 | -0.05 | -0.51 | -0.04 | 0.21 | -0.23 | -0.02 |
| | CEC | -0.34 | 0.46 | -0.40 | -0.17 | 0.01 | -0.47 | -0.19 |
| | Organic carbon | -0.15 | -0.01 | 0.37 | 0.46 | -0.10 | -0.05 | -0.04 |
| | Clay | 0.07 | 0.14 | -0.49 | 0.09 | 0.19 | -0.11 | -0.33 |
| Jengka 25, Pahang (n=12) | pH | 0.15 | 0.47 | 0.30 | 0.63* | 0.32 | 0.40 | 0.48 |
| | EC | 0.09 | 0.22 | 0.20 | 0.58* | 0.18 | 0.55 | 0.35 |
| | CEC | 0.53 | 0.39 | 0.45 | -0.45 | -0.44 | -0.43 | -0.22 |
| | Organic carbon | -0.14 | 0.12 | -0.44 | -0.26 | -0.01 | -0.07 | -0.29 |
| | Clay content | 0.09 | 0.21 | 0.36 | 0.44 | 0.49 | 0.64* | 0.58 |
| Jalan Kebun, Klang (n=24) | pH | 0.12 | -0.35 | 0.12 | 0.13 | 0.01 | -0.33 | 0.34 |
| | EC | 0.016 | -0.06 | 0.04 | 0.14 | -0.17 | 0.22 | -0.26 |
| | CEC | -0.35 | -0.33 | 0.41* | 0.38 | -0.14 | 0.05 | -0.16 |
| | Organic Carbon | -0.51* | 0.09 | 0.17 | 0.07 | -0.37 | 0.05 | 0.06 |

* Significantly correlated ($p<0.05$)

medicinal plants. The cation exchange capacity of the soils from Jalan Kebun, Klang, also revealed a positive correlation with Ni concentration in the leafy medicinal plants collected from there, while Cu concentration was negatively correlated with organic carbon of the soils.

The correlation analyses between the total heavy metal concentrations in the soils with the heavy metal concentrations in the *Tongkat Ali* and leafy medicinal plants are shown in Tables 6(a) and (b), respectively. In the *Tongkat Ali* samples, as shown in Table 6(a), there was no correlation between the heavy metal concentrations in the soils and in the *Tongkat Ali* being observed. Meanwhile, as revealed in Table 6(b), the data shows that only Cu and Se concentrations in the soils collected from Jalan Kebun, Klang, had a correlation with their concentration in the medicinal plants.

The principal component analysis (PCA) of the soil samples (Fig.1) shows that Cu concentration in the soils was correlated

TABLE 6(a)
Correlation coefficients (r) between heavy metal concentrations in soil with heavy metals concentrations in Tongkat Ali (*Eurycoma longifolia*) at different locations.

| Correlation coefficients, r | Location | |
|---|---|---|
| | Setiu, Terengganu | Jengka 25, Pahang |
| Cu in soil with Cu in plant | -0.26 $^{ns}$ | -0.75 $^{ns}$ |
| Zn in soil with Zn in plant | -0.59 $^{ns}$ | 0.57 $^{ns}$ |
| Ni in soil with Ni in plant | 0.08 $^{ns}$ | -0.21 $^{ns}$ |
| Pb in soil with Pb in plant | 0.03 $^{ns}$ | 0.82 $^{ns}$ |
| As in soil with As in plant | 0.29 $^{ns}$ | 0.83 $^{ns}$ |
| Cd in soil with Cd in plant | 0.59 $^{ns}$ | -0.33 $^{ns}$ |
| Se in soil with Se in plant | -0.46 $^{ns}$ | -0.59 $^{ns}$ |

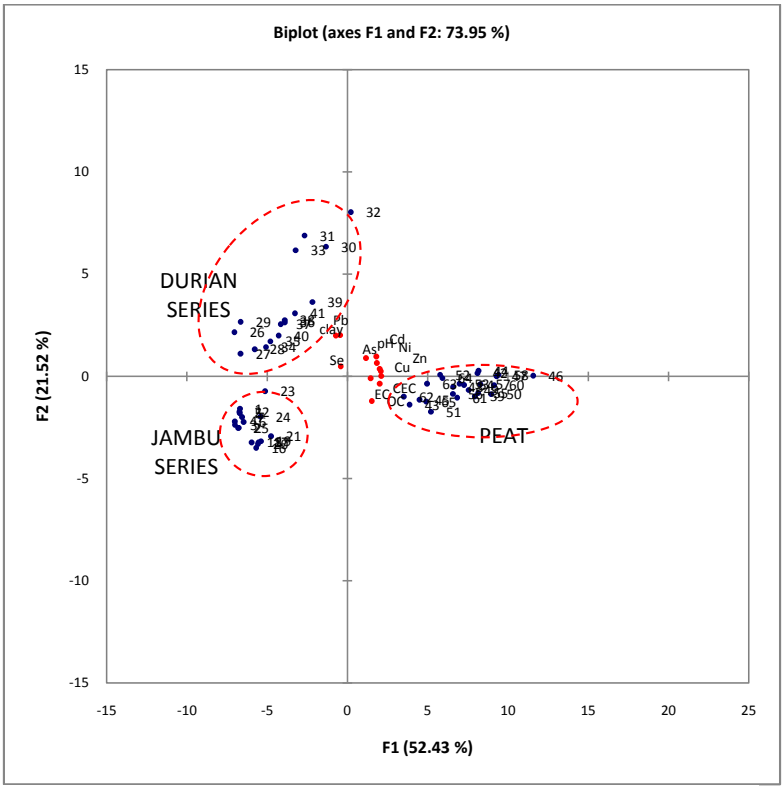
$^{ns}$ Not significant ($p>0.05$)

TABLE 6(b)
Correlation coefficients (r) between heavy metal concentrations in the soil with heavy metal concentrations in the leafy medicinal plants at different locations.

| Correlation coefficients, r | Locations | | |
|---|---|---|---|
| | Setiu, Terengganu | Jengka 25, Pahang | Jalan Kebun, Klang, Selangor |
| Cu in soil with Cu in plant | 0.60 | 0.52 | -0.45* |
| Zn in soil with Zn in plant | -0.23 | 0.13 | 0.40 |
| Ni in soil with Ni in plant | 0.043 | 0.18 | -0.25 |
| Pb in soil with Pb in plant | 0.02 | 0.25 | -0.01 |
| As in soil with As in plant | 0.66 | -0.01 | -0.33 |
| Cd in soil with Cd in plant | -0.18 | 0.29 | 0.36 |
| Se in soil with Se in plant | -0.31 | -0.34 | -0.48* |

* Significantly correlated ($p<0.05$)

with EC, CEC and organic carbon of the soils, according to the quartile of which it fell into. Whereas, most heavy metals like Zn, Ni, Cd and As were correlated with the pH of the soils. Selenium and Pb in the soils were shown to be correlated with each other, and its concentration was affected by the clay content of the soils. Soil types also had an influence on the heavy metal concentrations in the soil samples. As observed in Fig.1, the soil samples from Jalan Kebun, Klang tended to have high levels of Cu, Zn, Ni, As, and Cd. Besides, the peaty type soils from Jalan Kebun, Klang, also recorded the highest levels of pH, EC, CEC and organic carbon. Meanwhile, the Durian series soil samples from Jengka 25, Pahang, showed the highest levels of clay, Pb and Se, whereas the BRIS soil from Setiu, Terengganu, was characterized to have relatively lower values of heavy metal concentrations and soil properties.




**Legend:**

| | | | |
|---|---|---|---|
| pH: Soil pH | EC: Soil EC | CEC: Soil CEC | OC: Organic Carbon |
| Clay: Clay content | Cu: Cu conc. in soil | Zn: Zn conc. in soil | |
| Ni: Ni conc. in soil | Pb: Pb conc. in soil | As: As conc. in soil | |
| Cd: Cd conc. in soil | Se: Se conc. in soil | | |

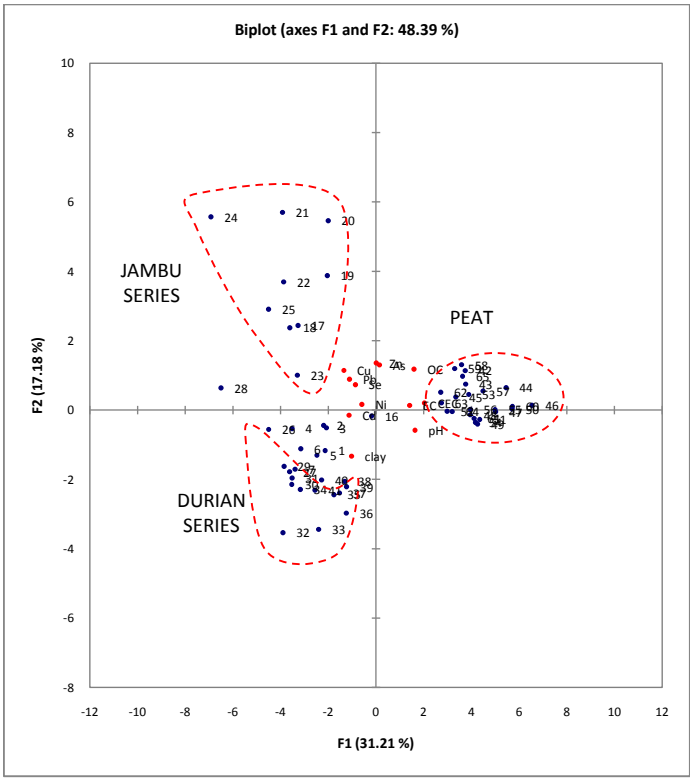
**SAMPLES FROM:**

| | | |
|---|---|---|
| 1-25: Jambu series soil | 26-41: Durian series soil | 42-65: Peat |

Fig.1: The principal component plot of the heavy metal concentrations in the soil and chemical properties of the soils sampled in this study

The principal component analysis (PCA) between the soil properties with the heavy metal concentrations in the medicinal plants was also conducted (Fig.2). From this analysis, Zn and As concentrations in the medicinal plants were influenced by pH, EC, CEC and OC of the soils, and this can be clearly observed in the samples from Jalan Kebun, Klang. In addition, cadmium concentration in the medicinal plants sampled from Jengka 25, Pahang grown in the Durian series soil was also found to correlate with the clay content of the soils.

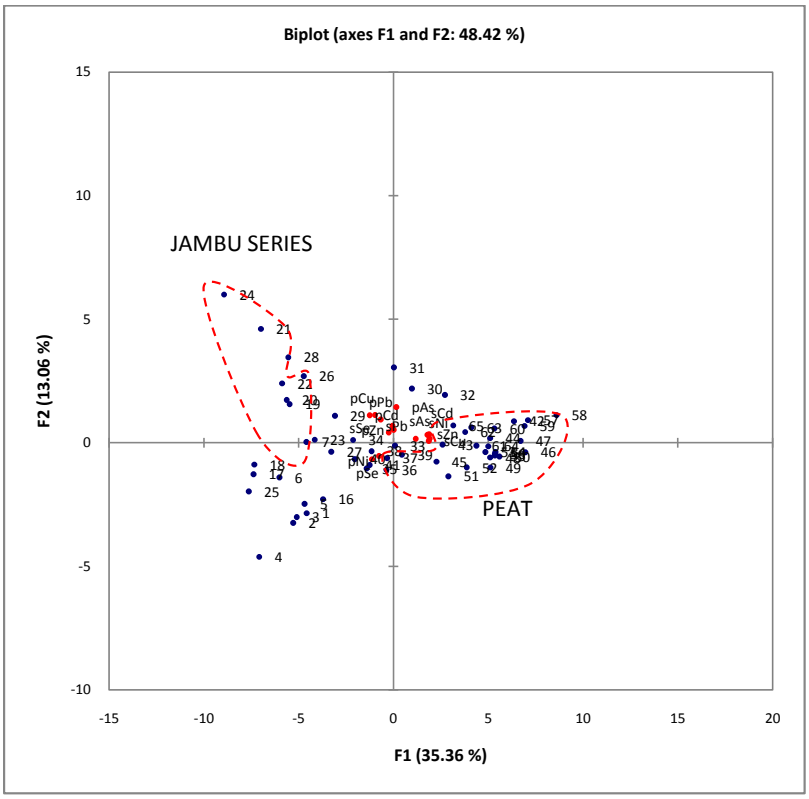
The principal component analysis (PCA) of the heavy metal concentrations in the medicinal plants and soils (Fig.3) showed that only As concentration in the soils was correlated with its concentration in the medicinal plants, and this applies to the medicinal plants grown in the peat soil from Jalan Kebun, Klang. Furthermore, from this analysis, Pb concentration in the




**Legend:**
pH: Soil pH EC: Soil EC CEC: Soil CEC OC: Organic Carbon
Clay: Clay content Cu: Cu conc. in plants Zn: Zn conc. in plants
Ni: Ni conc. in plants Pb: Pb conc. in plants As: As conc. in plants
Cd: Cd conc. in plants Se: Se conc. in plants

**SAMPLES FROM:**
1-25: Jambu series soil 26-41: Durian series soil 42-65: Peat

Fig.2: The Principal component plot of the heavy metal concentrations in the medicinal plant and chemical properties of the soils sampled in this study




**Legend:**

| | | |
|---|---|---|
| pCu: Cu conc. in plant | pZn: Zn conc. in plant | pNi: Ni conc. in plant |
| pPb: Pb conc. in plant | pAs: As conc. in plant | pCd: Cd conc. in plant |
| pSe: Se conc. in plant | sCu: Cu conc. in soil | sZn: Zn conc. in soil |
| sNi: Ni conc. in soil | sPb: Pb conc. in soil | sAs: As conc. in soil |
| sCd: Cd conc. in soil | sSe: Se conc. in soil | |

**SAMPLES FROM:**

1-25: Jambu series soil 26-41: Durian series soil 42-65: Peat

Fig.3: The principal component score plot of the heavy metal concentrations in the medicinal plants and heavy metal concentrations in the soils sampled in this study

soils was also found to correlate with its concentration in the medicinal plants, and this applies to the samples collected from Setiu, Terengganu, and a few plant samples from Jengka 25, Pahang, grown on the Durian series soil.

## CONCLUSION

In this study, the random sampling of the medicinal plants was necessary in order to investigate the sources of heavy metals. The sources of medicinal plants nowadays are no more found from natural resources or those that grow in the wild since they

have been domesticated. Thus, apart from the soils where the medicinal plants are planted, the sources of heavy metals can also come from fertilizers, pesticides or other sources. In this research, from all the 3 study areas where the medicinal plants were collected, each area had different levels of heavy metal concentrations. All the locations was found to have different soil types, therefore, they varied in soil properties. The principal component analysis between the soil properties with the heavy metal concentrations in the medicinal plants showed that all the soil properties determined affected Zn, As and Cd concentrations in the medicinal plants, which depended on the soil types where the plants were grown. From the principal component analysis in this study, the concentrations of Pb and As in the plants were also correlated with the heavy metals in soils; however, there was a variation according to soil types. Notably, various plants have their own ability to take up heavy metals from soils. Hence, this ability may have contributed to the elevated level of heavy metal concentrations in medicinal plants, other than their concentration in soils.

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# Ribosomal DNA Analysis of Marine Microbes Associated with Toxin-producing *Pyrodinium bahamense* var. *compressum* (Böhm), a Harmful Algal Bloom Species

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## ABSTRACT

Blooms of the toxic alga, *Pyrodinium bahamense* var. *compressum* (Böhm), have become a problem in Malaysia over the past three decades. The alga is a causative agent of paralytic shellfish poisoning, a potentially fatal neurological disorder. Past research suggest that bacteria-algae association may play a direct or indirect role in toxin production. As such, ribosomal DNA-based restriction enzyme analysis for the identification of bacteria associated with *Pyrodinium* sps. was undertaken. A total of 16 bacterial isolates were successfully obtained from the clonal cultures of *Pyrodinium* sps. The diversity of the extracellular bacteria associated with *Pyrodinium bahamense* var. *compressum* was limited to the Phyla Proteobacteria and Actinobacteria. The major bacterial species identified included *Alcanivorax* spp. and *Hyphomonas* spp., whereas *Kocuria* spp., *Nesterenkonia* spp., *Alteromonas* spp., *Roseobacter* spp., *Xanthomonas* spp., and *Acinetobacter* spp. were identified as minor isolates. The identified bacterium *Hyphomonas* spp. exhibited high sequence identity with an unknown bacterium strain, SCRIPPS_739, in the GenBank database that is known to be associated with toxic and non-toxic dinoflagellates, *Alexandrium* spp. and *Scrippsiella trochoidea*, respectively.

*Keywords:* Bacteria, harmful algal bloom, paralytic shellfish poisoning, *Pyrodinium bahamense* var. *compressum*, ribosomal DNA

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## INTRODUCTION

In Malaysia, toxic harmful algal bloom (HAB) only occurs in the coastal waters of

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west Sabah, where the causative organism is the dinoflagellate, *Pyrodinium bahamense* var. *compressum* (*Pyrodinium)*. *Pyrodinium* blooms are very common in the Southeast Asian region, and where the blooms have often been reported in the waters of Malaysia (Anton *et al*., 2008), Brunei (Seliger, 1989), Indonesia (Wiadnyana, 1996) and the Philippines (Azanza-Corrales *&* Hall, 1993). The photosynthetic alga is one of the causative agents of paralytic shellfish poisoning (PSP), a potentially fatal neurological disorder.

Paralytic shellfish toxins consist of saxitoxin and at least 20 other chemically related derivatives, which block sodium channels in mammalian nerve cells, and thus prevent the flow of signals along the neuron (Gallacher *et al*., 1997; Plumley *et al*., 1999). Initially, marine dinoflagellates were considered to be the sole contributor for toxin production during a toxic algal bloom; however, according to Plumley *et al*. (1999), it is postulated that certain marine bacteria attached to or associated with algae may able to synthesize toxins and/or influence the toxicity of the algae.

Bacteria-algae interactions play an important role in HAB dynamics, where it has been postulated that they are regulators in the processes of algal bloom initiation, maintenance and decline (Ferrier *et al*., 2002). The diversity of bacteria associated with microalgae belongs to two bacterial phyla, namely, the Proteobacteria (α-Proteobacteria and γ-Proteobacteria) and the Cytophaga-Flavobacter-Bacteroides (Alverca *et al*., 2002).

In addition, it has been suggested that bacteria-algae association may play a direct or indirect role in toxin production. These toxin-producing bacteria can autonomously produce toxin and have the ability to metabolize the toxins, converting them from one derivative to another (Córdova *et al*., 2003). Thus, bacteria may be involved in both the production and modification of these toxins. Bacteria that are involved in PSP toxin production have been identified in dinoflagellate *Alexandrium lusitanicum* (Plumley *et al*., 1999) and *Alexandrium* sp. (Gallecher *et al*., 1997).

Due to the close relationships between algae and bacteria, it is necessary to identify the genetic diversity of the bacteria to better understand the occurrence of toxic blooms and to assess the bacteria-algae association in the marine ecosystems. In this study, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of 16S ribosomal DNA (rDNA) gene was used to investigate the bacterial population associated with the toxic dinoflagellate, *Pyrodinium bahamense* var. *compressum*.

## MATERIALS AND METHOD

The culture and isolation of *Pyrodinium bahamense* var. *compressum* were carried out based on the methods described by Guillard (1975) and Guillard and Morton (2004). All the cultures and isolation procedures were carried out under aseptic condition to prevent contamination. The isolated *Pyrodinium* cells were transferred through ten drops of sterile f/2 media (≈

20 µL) for serial washing, and this was done to do away with those marine bacteria that were not associated with *Pyrodinium bahamense* var. *compressum*.

Then, the cultures were checked for bacterial growth after two weeks from the initial isolation of *Pyrodinium* cells. Approximately 100 µl of *Pyrodinium* cultures (≈ 200 cells/ml) were spread on the surface of marine agar media (Difco, USA) and kept at 37°C for overnight incubation. All bacterial isolation and culture procedures were performed in a laminar flow cabinet under sterile conditions.

Preparation of pure bacteria culture was done based on the streak plate method (Beveridge & Daview, 1983), where the isolated bacteria were then used for *gram-staining* and 16S rDNA PCR amplification. The *gram-staining* method was used to differentiate bacteria into two major groups, namely, *gram-positive* and *gram-negative* bacteria, based on the method described by Beveridge and Daview (1983).

The bacterial genomic DNA was obtained by lysing the bacterial cells at 100°C. PCR was then carried out in a 20 µl reaction mixture containing 1 U Taq DNA polymerase (Promega, USA), 1 x PCR buffer (Promega, USA), 3.75 mM $MgCl_2$ (Promega, USA), 500 µM dNTPs (Promega, USA), 1.25 µM each primer and 1 µl of the supernatant of the lysed bacterial cells (≈ 50 ng genomic DNA). Universal PCR primers, 27F (5'-AGAGTTTGATCMTGGCTCAG -3') and 1492R (5'- TACGYTACCTTGTTACGACT-3') were used for the 16S rDNA amplification of the marine bacteria. The PCR amplification was performed as follows: 2 min of initial denaturation step at 94°C, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 1 min. A final extension at 72°C for 2 min was also included. The PCR was carried out on a PTC-200 thermal cycler (Bio-Rad, USA). After gel electrophoresis, the resulting PCR bands within the expected size of 1,500 bp were excised and purified by using the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's protocol.

The purified PCR products were digested with six restriction enzymes (RE), *Hae*III, *Dpn*II, *Alu*I, *Rsa*I, *Bam*HI and *Xho*I (New England Biolabs, USA). The RE analysis was carried out in a 10 µl reaction mixture containing 1 x restriction enzyme buffer, 10µg/µl BSA, 5U of RE and 5µl of DNA template. A total of 20 µl of mineral oil was added to prevent evaporation of the sample. The RE digestion was carried out in a PTC-200 thermal cycler (Bio-Rad, USA). The reaction conditions of each RE were according to the manufacturer's protocol.

The purified PCR products (≈ 20 ng/µl) were then sequenced using the BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems, USA). Sequencing was performed using the ABI 3130 Genetic Analyzer (Applied Biosystems, USA).

Meanwhile, the analysis of the 16S rDNA sequences of the bacteria isolates was conducted using the software Lasergene 6.1 (DNASTAR, Inc., USA). The rDNA sequences of the bacteria isolates were

then aligned with DNA sequences of the marine bacteria obtained from GenBank (www.ncbi.nih.gov). The construction of phylogenetic tree (neighbour-joining) for the assembled sequences was performed using MEGA 4.0 software (Tamura *et al*., 2007), whereby the sequences of the other bacteria were also retrieved and incorporated: *Alteromonas* sp., DQ412075; *Hyphomonas* sp., AY258084; *Alcanivorax venustensis*, DQ768632; *Roseobacter* sp., EF512125; *Acinetobacter* sp., DQ366086; *Xanthomonas* sp., DQ213024; *Luteimonas aestuarii*, EF660758; *Lysobacter gummosus*, AB161361; *Stenotrophomonas* sp., AM400231; *Kocuria rhizophila*, AY030315; *Nesterenkonia* sp., AY914062.

## RESULTS AND DISCUSSION

A total of 16 individual bacterial colonies were successfully isolated from the *Pyrodinium* cultures. The isolated bacterial colonies (Pyro-Bac) were labelled as 19A, 22A, 25A, 27A, 27B, 27C, 27D, 27E, 27F, 27G, 28A, 29A, 30B, B5, B9 and B18. The majority of the Pyro-Bac samples were *gram negative*, except for two Pyro-Bac, 27A and B5, which were stained in purple colour (*gram positive*). In addition, the majority of the Pyro-Bac samples were rod-like or bacillus shape, except for three Pyro-Bac (27A, 27E and B5), whereby their morphology was cocoid or spherical shape and sometimes short rod-like shape could also be seen.

The PCR amplification was conducted using the universal ribosomal DNA (rDNA) primers 27F and 1492R, which yielded positive results of a single band at the expected size of approximately 1.5 kb. It is an alternative way for identifying bacteria, which does not require prior knowledge in the bacterial characteristics. Compared to the conventional morphological and metabolic identification techniques, 16S rDNA sequence-based bacterial identification is simpler and accurate because it is based on highly conserved stretches of DNA sequences. Meanwhile, the 16S rDNA gene has always been the choice for bacterial identification by bacteriologists because it has a large and authentic sequence database, where comparisons of the sequences around the world could be done and the BLAST search programme could also be used even by users with limited expertise in the field of bacterial systematic (Mehnaz *et al*., 2006).

Restriction enzymes *Hae*III, *Dpn*II, *Alu*I and *Rsa*I yielded positive results, whereas for RE *Bam*HI and *Xho*I, no clear restriction patterns were observed. The four restriction enzymes had successfully produced eight riboprint (riboprint A – H) according to their restriction patterns (Fig. 1). The major population of bacteria associated with *Pyrodinium bahamense* var. *compressum* belonged to riboprint C, representing seven Pyro-Bac samples. This was followed by riboprints D with three Pyro-Bac samples. The remaining 6 riboprint had one Pyro-Bac sample each.

According to Clark (1997), twelve enzymes with four-based recognition sequence could yield up to 15 % of the interested gene sequence without necessary cloning or DNA sequencing. This is because

rDNA fingerprinting is a cost-effective method meant to evaluate the DNA sequence variation without DNA sequencing (Clark, 1997; Weising *et al*., 2005). The main advantages of rDNA fingerprinting are cost-effectiveness and high reproducibility. However, there are also some drawbacks, such as tedious experimental procedures and the requirement of microgram amounts of relatively pure and intact DNA (Weising *et al*., 2005).

The 16 Pyro-Bac samples were then subjected to direct sequencing to confirm their identity. The restriction enzyme analysis data should be combined with the sequencing data to produce a better data comparison and interpretation (Dowling *et al*., 1996). The sequences of the Pyro-Bac samples were obtained (GenBank accession nos: EF688604 to EF688619) after the PCR amplification using the universal rDNA primers, 27F and 1492R.

The sequencing results showed that the diversity of the extracellular bacteria associated with *Pyrodinium* was limited to the Phyla Proteobacteria (α- proteobacteria and γ-proteobacteria) and Actinobacteria, which are similar to the findings by Alverca *et al*. (2002) and Azanza *et al*. (2006). Alverca and colleagues reported that γ-proteobacteria was found outside the dinoflagellate, *Gyrodinium instriatum*, whereas β-proteobacteria and *Cytophaga-Flavobacterium-Bacteroides* were observed in the cytoplasm and nuclear; however, no α-proteobacteria was detected either free-living or intracellular (Alverca *et al*., 2002).

An investigation of the bacteria associated with *Pyrodinium bahamense* var. *compressum* was also conducted by Azanza and co-workers (2006). They investigated bacterial endosymbionts of *Pyrodinium bahamense* var. *compressum,* where bacteria from the phyla Proteobacteria, Actinobacteria and Firmicutes were identified. The identified microorganisms included *Moraxella* spp., *Bacillus* spp., *Erythrobacter* spp., *Micrococcus* spp., *Pseudomonas putida* and *Dietzia maris*. Meanwhile, a comparison with the findings

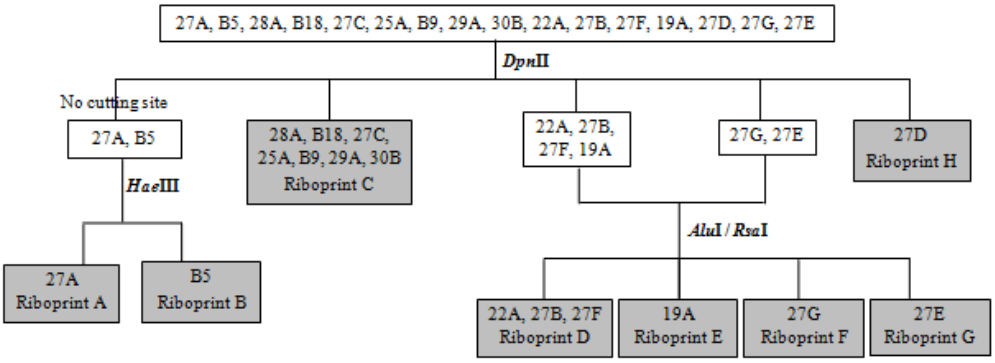


Fig.1: Division of the 16 Pyro-Bac samples based on the restriction patterns produced by the restriction enzymes *Hae*III, *Dpn*II, *Rsa*I and *Alu*I

of Azanza *et al*. (2006) showed that both proteobacteria and actinobacteria were also identified in this study; however, the identity of the microorganisms was different. This can be attributed to the presence of two different groups of microorganisms predominating intracellularlly and extracellularly, as mentioned in the findings of Alverca *et al*. (2002).

The major bacteria associated with *Pyrodinium* was from the genus *Alcanivorax* (97 to 100% identity), which belongs to the γ-proteobacteria class. This common marine bacterium has been previously described by Fernandez-Martinez *et al*. (2003) and Liu and Shao (2005), where γ-proteobacteria was isolated from the Mediterranean Sea and Bohai Sea, respectively. Another major bacterium associated with *Pyrodinium* was from the genus *Hyphomonas* (98 to 99% identity), belonging to α-proteobacteria class. In fact, the three isolates (22A, 27B, 27F) exhibited high sequence identity with an unknown bacterium strain, SCRIPPS_739, that is associated with toxic and non-toxic dinoflagellates, *Alexandrium spp*. and *Scrippsiella trochoidea* (GenBank accession no.: AF359546), as reported by Hold *et al.* (2001).

The minor bacteria isolates identified in this study were *Alteromonas* spp., *Xanthomonas* spp., *Acinetobacter* spp., *Roseobacter* spp., *Kocuria* spp., and *Nesterenkonia* spp. (one isolate each). *Alteromonas* spp., *Xanthomonas* spp., *Acinetobacter* spp., and *Roseobacter* spp. belonging to the Proteobacteria class, whereas *Kocuria* spp. and *Nesterenkonia* spp. belonging to the Actinobacteria class. Bacteria from the genus *Alteromonas* (Pyro-Bac 19A) and *Roseobacter* (Pyro-Bac 27D) have been reported to be associated with the harmful algal bloom species of the genera *Alexandrium*. Jasti *et al*. (2005) investigated that the genera *Roseobacter* showed a higher degree of association with the PSP toxin-producing dinoflagellate, *Alexandrium* spp. than with other bacterial groups, whereby *Alteromonas* spp. was one of the identified bacteria associated with *Alexandrium*. However, no toxicity test was carried out by in their studies (Jasti *et al*., 2005).

The same findings were also observed by Wichels *et al*. (2004), whereby both *Roseobacter* and *Alteromonas* bacteria were isolated from the toxic *Alexandrium tamarense* blooms off the Orkney Isles and the First of Forth of Scotland. Gallacher *et al*. (1997) provided strong evidence that a range of bacterial species isolated from the *Alexandrium* spp. cultures were capable of autonomous production of paralytic shellfish toxin. However, the identity of each bacteria species remains unknown.

The *Roseobacter* clade of marine bacteria was also found to be associated with the harmful alga *Pfiesteria*, one of the major producers of dimethylsulfoniopropionate (DMSP). It has been suggested that *Roseobacter* bacteria benefit from the association with DMSP-producing dinoflagellates because of the high metabolic rate at which *Roseobacter* can degrade them (Miller & Belas 2004).

The *gram-negative* bacteria strain, Pyro-Bac 27G, isolated in the study

exhibited a high identity (>95%) with a few different bacteria such as *Xanthomonas*, *Luteimonas*, *Lysobacter*, *Stenotrophomonas*, and several unknown gammaproteobacteria. Therefore, the identity of the Pyro-Bac 27G isolate remains uncertain. Pyro-Bac 27E (γ-proteobacteria) and 27A (Actinobacteria) were considered as contaminated, whereby the species were distributed in widespread, diverse habitats (Bull *et al*., 2005). The remaining bacteria isolate, Pyro-Bac B5, was identified as *Nesterenkonia* (95 to 98% identity), whereby the bacterium is a common marine actinobacterium and has previously been isolated from Lake Abjata in Ethiopia (Delgado *et al*. 2006).

According to Fox *et al*. (1992), 16S rDNA sequence identity may not necessarily be sufficient to guarantee species identity. Fox and colleagues had compared the sequences of three different psychrophilic *Bacillus* strains. The strains exhibited more than 99.5% sequence identity and the results could be regarded as identical. In contrast, previously published DNA-DNA hybridization results have convincingly established that the three strains did not belong to the same species. These results emphasize that the identity of the 16S rDNA sequence is not a good criterion to guarantee species identity. Although 16S rDNA sequences could be routinely used to distinguish and establish relationships between the genera and well-resolved species, very diverged species might not be recognizable (Fox *et al*., 1992). For this reason, all the sequences of the sixteen Pyro-Bac samples were identified only to its genus level.

The molecular phylogenetic tree of the partial 16S rDNA of bacteria associated with *Pyrodinium bahamense* var. *compressum*, together with sequences of other marine microbes obtained from the GenBank, was constructed and is shown in Fig.2. The tree was constructed using the Neighbour-Joining (NJ) algorithm.

Eight clusters of the bacterial groups (clusters A to H) were identified according to the phylogenetic NJ tree. The phlylogenetic results were corroborated by the ribotyping restriction fragment patterns analysis data as well as the sequencing data. The phylogenetic tree also distinctively separated the three bacterial phyla, the Actinobacteria, the α-Proteobacteria and the γ-Proteobacteria. Similar results were also reported by Wichels *et al*. (2004), who investigated the bacterial diversity in toxic *Alexamdrium tamarense* blooms in the Scotland waters and the phylogenetic tree they constructed also separated the tree into two phyla Proteobacteria (α and γ subdivisions) and Bacteroids.

According to Erko Stackebrandt (2002), the available phylogenetic branching pattern reflects the actual situation in nature quite incompletely. Phylogenetic reconstructions are based on the similarities from only a few nucleotides and thus can be considered as an approximation. The gradually emerging 16S rRNA tree is probably best considered as presenting a hypothesis about the relationships which should be tested on the basis of supporting data. The phylogenetic branching pattern serves as an aid to recognize the clusters of phylogenetically related strains but the delineation of

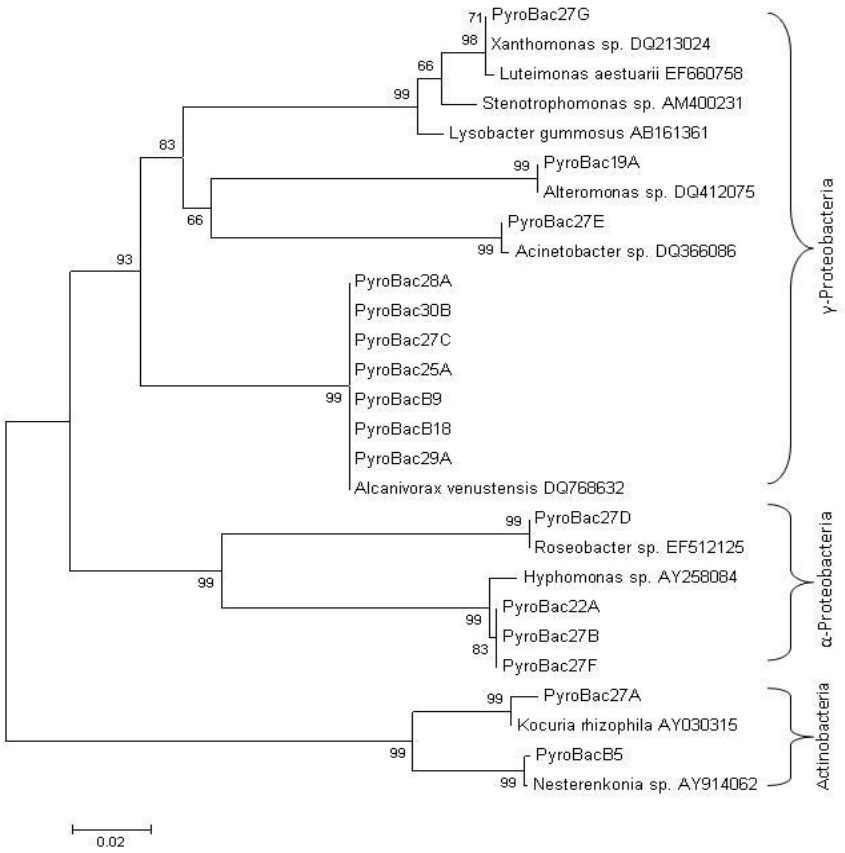


Fig.2: Molecular phylogenetic tree (neighbour-joining) of the partial 16S rDNA for bacteria associated with *Pyrodinium bahamense* var. *compressum*

phylogenetically neighbouring clusters is predominantly made on the basis of morphology, biochemical properties and episematic molecules (Stackebrandt, 2002).

## CONCLUSION

The diversity of cultivable extracellular marine microbes associated with Malaysian *Pyrodinium bahamense* var. *compressum* strain has been shown to be limited to the Phyla Proteobacteria (α-proteobacteria and γ-proteobacteria) and Actinobacteria. Majority of the bacterial isolates are *gram-negative* rods which are common in the marine environment. Some of the isolates (*Hyphomonas* spp., *Roseobacter* spp. and *Alteromonas* spp.) were shown to be associated with other toxin-producing HAB species, such as *Alexandrium spp*. and *Scrippsiella trochoidea*. Although toxicity assessments were not part of this study, the species have previously been described as producing toxin. Therefore, an approach combining the information in this study with toxin detection methods could add much pertinent information regarding the participation of the bacteria in the event of harmful algal bloom. Further studies using

metagenomic techniques can be used in the future to characterize a complete diversity of the marine microbes that are associated with *Pyrodinium bahamense* var. *compressum* as to represent both culturable and non-culturable bacteria groups, and thereby enlarging the limited information available about the natural bacterial environment associated with dinoflagellates. In addition, studies on the interactions between algae and bacteria are beneficial to better understand the way both organisms interact during algal bloom initiation, maintenance and decline.

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# Desorption Isotherm Model for a Malaysian Rough Rice Variety (MR219)

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## ABSTRACT

Moisture desorption model of long grain Malaysian rough rice variety (MR219) was experimentally obtained using the static gravimetric method for different combinations of temperature (40, 45, 50, 55°C) and water activities (0.0507 to 0.9331). Five most commonly used models with three parameters namely modified Chung–Pfost, modified GAB, modified Halsey, modified Henderson and modified Oswin and a four parameters model (Zuritz *et al*., 1978) were determined for their ability to fit the experimental data using non linear regression techniques. Comparisons between all models were made on the basis of standard error of estimate (SEE), residual sum squares (RSS) and residual plots. Based on the results of this study, the Zuritz *et al*., modified Chung–Pfost and modified GAB models could be useful to predict the desorption EMC of MR219. The modified Halsey, modified Henderson and modified Oswin presented a poor fitting to the experimental data. In addition, the model by Zuritz *et al*. was found to be the most appropriate equation for representing the desorption isotherm model for MR219 at the range of temperatures from 40°C to 60°C.

*Keywords:* Equilibrium moisture content, desorption, static gravimetric, fit ability, rough rice

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## INTRODUCTION

Rough rice is known as a hygroscopic material that can gain or lose moisture even though its surrounding air conditions

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undergo changes. A product can gain or lose its moisture when it is subjected to a continual supply of air at constant temperature, humidity and vapour pressure (p), until the vapour pressure of moisture in the product becomes equal to p. In this state, moisture content (MC) of a product is known as equilibrium moisture content (EMC) (Champagne, 2004). There is a relationship between the MC of a product and its equilibrium relative humidity (ERH) at a constant temperature which can be shown by an isotherm curve. It is necessary to have information on the adsorption or desorption isotherms of a particular food. These isotherms can be applied in food processing and engineering to improve product quality (Delgado & Sun, 2002b). The EMC affects the rate of moisture transfer from kernels to the surrounding air which can affect rice milling quality (Fan *et al*., 2000., Kunze, 1979). Chen (1997) and Bonazzi, *et al*. (1997) claimed that variety, temperature and relative humidity were the main factors that lead head rice yield (HRY) to decrease. Adsorption and desorption are important factors that can affect fissure formation in the rice kernel, and the subsequent HRY during drying and storage. The EMC is developed as a result of an interaction between the material and the environmental condition (Molna´r, 2007):

$$\overline{X}^{*} = \overline{X}^{*}(p_v, \mathrm{T}) \qquad [1]$$

MC of products can be changed because of the condition ($p_v$, T) dominating on the surface of the products. After an adequately long time with stable limit conditions an internal moisture diffusion balance occurred until the EMC is reached. The sorption equilibrium is a condition where the moisture adsorption and desorption does not occur in a product. The equilibrium vapour pressure (EVP) is the vapour pressure at which the sorption equilibrium takes place at a certain level of product MC ($\overline{X}$) at a certain temperature (T). EVP can be expressed as follows:

$$p_v^{*} = p_v^{*}(\mathrm{T})_{\overline{X}} \qquad [2]$$

In drying research, it is necessary to have enough information on EVP at a constant temperature. Therefore, when T is constant $p_v^{*}$ is constant, the equilibrium relative vapour content is applied in drying process as a characteristic of the vapour pressure (Molna´r, 2007):

$$\psi = \frac{p_v}{p_{ov}^{*}} \qquad [3]$$

Generally, the sorption isotherm ($\overline{X}^{*} = \overline{X}^{*}(p_v)_T$), sorption isobars ($\overline{X}^{*} = \overline{X}^{*}(\mathrm{T})_{p_v}$), and sorption isosteres ($p_v = p_v(\mathrm{T})_{x^*}$) are applied in drying process. They are derived from the sorption equilibrium function ($\overline{X}^{*} = \overline{X}^{*}(p_v, \mathrm{T})$). The sorption isotherms are most frequently applied to describe the sorption behaviour of a material. Sorption isotherms are determined from point to point (Molna´r, 2007):

$$\overline{X}_1^{*}(\psi_1)_T, \overline{X}_2^{*}(\psi_2)_T \cdots \overline{X}_n^{*}(\psi_n)_T, \qquad [4]$$

Each pair of values for determining a point is in general the result of a measurement. The elements of this measurement are as follows:

1. Presentation of the pair of values to be measured on the condition that T is constant.
2. Measurement of the value of $p_v$, and $\psi$ should be done during or at the end of the sorption measurement.
3. The EMC ($\overline{X}^*$) of sample should be determined at the end of the sorption measurement.

Gal (1981) reviewed different methods and finally classified them into three basic techniques, namely, manometric, gravimetric and other special methods. Meanwhile, Molna´r (2007) stated that the gravimetric technique is the common method used for EMC determination. In this technique, saturated salt solutions or sulfuric acid dilutions at different concentrations are used to maintain constant relative humidity in closed still moist air at certain temperature (Gal, 1981) and a thermostat is used to control the air temperature (Molna´r, 2007). Champagne (2004) stated that different rice varieties equilibrate to slightly different moisture contents in a given environment. The same rice variety also equilibrates to slightly different MC depending on whether the grain is adsorbing or desorbing moisture while approaching the equilibrium state. Chio *et al*. (2010) reviewed different rice hygroscopic equilibrium studies and finally concluded that rice type and variety affect EMC/ERH relationship significantly. The researchers added that since new varieties are introduced by rice industry, it is necessary to obtain suitable EMC/ERH equation for each to improve rice processing and storage. Since the EMC model is an important parameter in computer drying simulation, selecting a proper model can help to achieve good results. It must be noted that all EMC/ERH models applied in this study have been recommended by previous researchers for a limited range of temperature (10°C - 40°C). In addition, Zuritz *et al*. (1978) conducted an equilibrium isotherm for a medium grain. Therefore, the main goal of this study was to obtain a desorption equilibrium isotherm in the higher range of temperature (40°C – 60°C) for a selected Malaysian long rice variety (MR 219) using the existing models.

## MATERIALS AND METHOD

Long grain rough rice, MR219 with approximately 18% moisture content (d.b.) was used in this experimental study. The gravimetric method was chosen to find suitable EMC equation. Sulphuric acid solutions were used to provide constant relative humidity. The following equation was applied to estimate ERH or water activity (Molna´r, 2007):

$$\log a_w = (a_1 - \frac{a_2}{T}) + \log(\frac{133.3224}{P_0}) \quad [5]$$

Where:

T= absolute temperature (K)

$P_0$ = vapor pressure of water (Pa)

The empirical constant values ($a_1$ and $a_2$) which depend on the sulfuric acid weight percentage are presented by Molna´r (2007). Water activity ranges from 0.9331 to 0.0507 was achieved by different concentrations of sulphuric acid from 10% to 70% (w/w). About 20 g of rough rice samples were put inside a cloth net and suspended from jars covers above 125 ml sulfuric acid solution. The jars with rough rice samples were put in temperature controlled incubator set at 40, 45, 50, 55, 60 ºC. The measurement of samples masses commenced after 2 weeks. Sample masses were checked again every 72 h. Depending on the relative humidity and temperature, different times were required for samples to reach an equilibrium condition. The maximum time for the samples to reach equilibrium was 28 days. Moisture content of each sample was measured in duplicates by drying 13-15 g samples for 24 h in an oven set at 130°C (Jindal & Siebenmorgen, 1987). The following equation was used to calculate the EMC of the samples (ASAE, 2007):

$$MC(d.b.) = \frac{W_e - W_d}{W_d} \quad [6]$$

Where:

$W_e$ = Sample weight at equilibrium before oven drying

$W_d$ = Sample weight at equilibrium after oven drying

The experiments for each temperature and water activity were carried out in triplicate. Five three-parameter equations that had been recommended by the ASAE standard D245.5 (ASAE, 2007) were used to determine their fit ability in this study. They can be expressed as follows:

Modified Chung–Pfost equation (Pfost *et al*., 1976; ASAE, 2007; Basunia & Abe, 2001; Choi *et al*., 2010):

$$M_e = \frac{\ln A}{B} - \frac{1}{B}.\ln[-(T + C).\ln RH] \quad [7]$$

Modified Halsey equation (Iglesias & Chirife, 1976; ASAE, 2007; Basunia & Abe, 2001; Choi *et al*., 2010):

$$M_e = [-\frac{\exp(A + B.T)}{\ln RH}]^{1/C} \quad [8]$$

Modified Henderson equation (Thompson *et al*., 1968, ASAE, 2007; Basunia & Abe, 2001; Choi *et al*., 2010):

$$M_e = [-\frac{\ln(1 - RH)}{A.(T + C)}]^{1/B} \quad [9]$$

and the modified Oswin's equation (Oswin, 1946; ASAE, 2007; Basunia & Abe, 2001; Choi *et al*., 2010):

$$M_e = (A + B.T).\left[\frac{1 - RH}{RH}\right]^{-1/C} \quad [10]$$

The developed and modified GAB equation is as follows (Jayas & Mazza, 1993):

$$M_e = \frac{A.B.(\frac{C}{T}).RH}{(1 - B.RH)[1 - B.RH + (\frac{C}{T}).B.RH]} \quad [11]$$

Where,

$M_e$ = Equilibrium moisture content (EMC, d.b., %)

RH = Equilibrium relative humidity (ERH, decimal)

T = temperature (°C)

A, B & C = parameters

A four-parameter equation by Zuritz *et al.* (1978) was also used for fitting the data. This particular equation was developed by Zuritz *et al.* (1978) for medium grain variety (CSM 5) at drying air temperature range of 10°C - 40ºC and Rh of 11.2% - 87.9%. It can be expressed as follows:

$$M_e = \exp\left[\frac{\log\left[-\frac{(\log(1-RH).T)}{(1-\frac{T}{T_c})^A.B}\right]}{C.T^D}\right] \quad [12]$$

Where,

$M_e$ = EMC (% d.b.)

RH = ERH (decimal)

T = Temperature (ºK)

$T_c$ = Critical temperature of water=647.1 (ºK)

A, B, C & D = Equation parameters

The parameters in the sorption equations were estimated using non-linear regression analysis by applying the Lab Fit V7.2.44 software. Meanwhile, the sum of square error (SEE) was used to measure the accuracy of the model and the coefficient of determination ($R^2$) to show the variability between the predicted and measured data. In a good mathematical model, the coefficient of determination should be close to 1 (one) and sum of square errors should be close to 0 (zero). The suitability of the equations was evaluated and compared using residual sum of square (RSS), standard error of estimate (SEE) and coefficient of determination ($R^2$):

$$R^2 = \frac{S_t - SEE}{S_t} \quad [13]$$

Where,

$$S_t = \sqrt{\frac{\sum_{j=1}^{N}(\bar{y} - y_i)^2}{N-1}} \qquad \bar{y} = \frac{\sum_{j=1}^{N} y_i}{N}$$

$$SEE = \sqrt{\frac{\sum_{j=1}^{N}(y_{jcal} - y_{j\exp})^2}{df}}$$

The residual sum square (RSS) is defined as:

$$RSS = \sum_{j=1}^{N}(y_{jcal} - y_{j\exp})^2 \quad [14]$$

df and N are the degree of freedom and the number of data points, respectively, and $y_{j\exp}$ and $y_{jcal}$ are experimental and calculated values of y, respectively. The equation giving the smallest RSS and SEE and the highest $R^2$ value are considered to be the best fitted equation.

## RESULTS AND DISCUSSION

Table 1 shows the desorption isotherm values for MR 219 at ERH of 93.31% to 5.07% and temperature of 40ºC – 60ºC with equilibrium moisture content ($M_e$) in decimal and dry basis.

The experimental data were used to estimate parameters of isotherms equations of Zuritz, modified Chung-Pfost, modified GAB, modified Oswin, modified Halsey and modified Henderson. Table 2 shows the estimated parameters of these models in which the EMC was taken as the dependent variable. Meanwhile, the corresponding correlation coefficient between the experimental and predicted data ($R^2$), the standard error of estimate (SEE) and residual sum of square (RSS) which indicate the fitting ability of each equation are shown in Table 2. As illustrated, the correlation coefficients are very high in most cases ($R^2 > 0.97$).

The Zuritz model obtained the highest $R^2$, the least SEE and the least RSS followed by modified Chung- Pfost, modified Gab and modified Oswin at selected temperature range (40°C – 60°C) and ERH (93.31% to 5.07%). According to Chen and Morey (1989) and Aviara *et al*. (2004), statistical parameters like $R^2$ or SEE may not be

TABLE 1
Desorption isotherm values for MR219 at different ERH (%) and temperatures.

| T= 40 º C | | T= 45 º C | | T= 50 º C | | T= 55 º C | | T= 60 º C | |
|---|---|---|---|---|---|---|---|---|---|
| ERH | $M_e$ | ERH | $M_e$ | ERH | $M_e$ | ERH | $M_e$ | ERH | $M_e$ |
| 92.97 | 0.2094 | 92.90 | 0.176 | 92.94 | 0.168 | 93.08 | 0.1587 | 93.31 | 0.1436 |
| 86.42 | 0.1755 | 86.44 | 0.1631 | 86.56 | 0.1483 | 86.78 | 0.1375 | 87.08 | 0.1193 |
| 73.96 | 0.1441 | 74.01 | 0.1413 | 74.14 | 0.1234 | 74.35 | 0.1089 | 74.63 | 0.0954 |
| 56.96 | 0.1167 | 57.18 | 0.108 | 57.46 | 0.1027 | 57.80 | 0.0862 | 58.19 | 0.0754 |
| 36.51 | 0.0852 | 36.89 | 0.0767 | 37.32 | 0.0688 | 37.77 | 0.0595 | 38.26 | 0.0520 |
| 17.86 | 0.0600 | 18.26 | 0.055 | 18.68 | 0.0414 | 19.11 | 0.0382 | 19.57 | 0.0321 |
| 5.07 | 0.0284 | 5.32 | 0.0235 | 5.59 | 0.0209 | 5.86 | 0.0191 | 6.15 | 0.0165 |

TABLE 2
The estimated coefficients and the statistical parameters for selected mathematical sorption equations

| Equations | parameters | | | | $R^2$ | SEE | RSS |
|---|---|---|---|---|---|---|---|
| | A | B | C | D | | | |
| Zuritz | $-.25239\times10^{2}$ | $9.480\times10^{-8}$ | $1.1801\times10^{6}$ | -2.307 | 0.9956 | 0.0037 | 0.1875 |
| Modified Chung-Pfost | 107.048 | 24.277 | -28.320 | - | 0.9899 | 0.0078 | 0.2013 |
| Modified GAB | 463.148 | 1.068 | $1.701\times10^{-2}$ | - | 0.9871 | 0.0091 | 0.2392 |
| Modified Oswin | 0.1654 | $-1.673\times10^{-3}$ | 3.282 | - | 0.9726 | 0.0094 | 0.2650 |
| Modified Halsey | -4.417 | $-5.396\times10^{-2}$ | 2.630 | - | 0.9321 | 0.0147 | 0.4229 |
| Modified Henderson | $1.797\times10^{12}$ | 1.932 | $2.247\times10^{10}$ | - | 0.9063 | 0.0169 | 0.9519 |

sufficient evidence for the goodness of fit of a moisture sorption isotherm model based on experimental data. Therefore, the nature of the residual plots should be considered. The residual plots of the Zuritz *et al*. modified Chung-Pfost and modified Gab models were random distributions over the range of temperature and EMC tested in this study. As shown in Fig.1 to Fig.3, the modified Oswin, modified Halsey and modified Henderson models presented a patterned distribution for MR219 under the tested conditions in this study.

Based on the results shown in Table 2 and the residual plots, the Zuritz *et al*. modified Chung- Pfost, and the modified Gab models could be useful for predicting the EMC and its temperature dependence. From the three models, the Zuritz *et al*.'s model was the most appropriate model to predict moisture desorption isotherms of MR219 at the temperature ranges from 40°C to 60°C. The desorption isotherms obtained in this study have a sigmoid shape (Fig.4). Under fixed relative humidity condition with increasing air temperature, the EMC decreases. In addition, at fixed air temperature, EMC decreases with decreasing relative humidity.

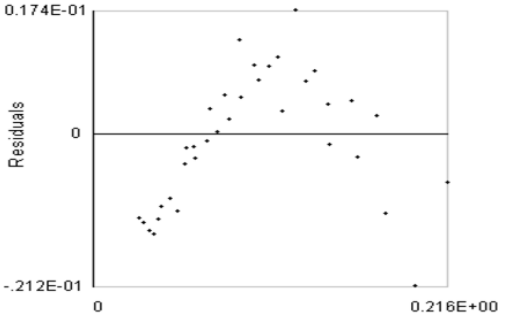


Fig.1: Residuals versus predicted EMC for the modified Oswin's equation

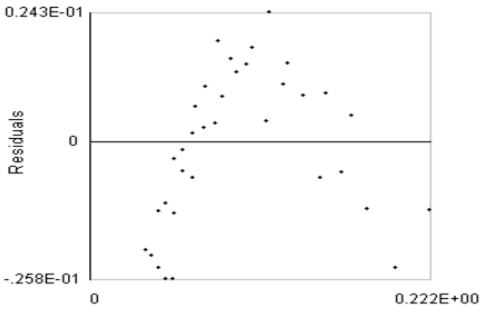


Fig.2: Residuals versus predicted EMC for modified Halsey equation

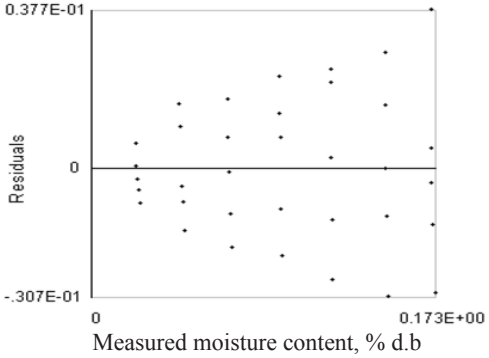


Fig.3: Residuals versus predicted EMC for modified Henderson equation

## CONCLUSION

The desorption isotherm obtained in this study presented a sigmoid shape. The results illustrate that the EMC of rough rice decreases with an increase in temperature at constant ERH. The correlation coefficient ($R^2$) obtained for all the models was in the range of 0.9956 – 0.903. Among the moisture isotherm equations tested in this study, the four-parameter moisture isotherm equation of Zuritz *et al*. was identified as the most appropriate equation to represent EMC of the Malaysian rice variety (MR219) at the temperature ranges from 40°C to 60°C. Three parameter equations of the modified Chung-Pfost and the modified Gab were also found acceptable. However, the modified Oswin, modified Halsey and modified Henderson presented a poor fitting ability to the experimental data.

## NOTATIONS

A, B, C, D = Coefficients
EMC = Equilibrium moisture content
ERH = Equilibrium relative humidity
EVP = Equilibrium vapor pressure
$M_e$ = Equilibrium moisture content
P = Partial pressure
$R^2$ = Correlation coefficient
RSS = Residual sum square
SEE = Standard error of estimate
T = Temperature
X = Moisture content of material
$\psi$ = Relative equilibrium vapor pressure

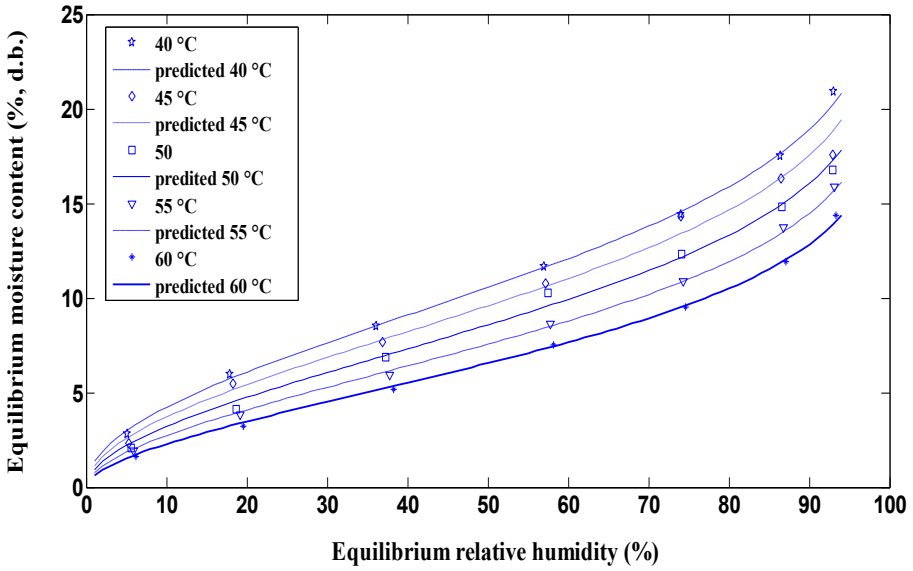


Fig.4: The experimental and Zuritz model predicted the desorption isotherms for MR219 at 40, 45, 50, 55, and 60°C

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# Evaluation on the Properties of Mentarang (*Pholas orientalis*) Protein Hydrolysate

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## ABSTRACT

Mentarang (*Pholas orientalis*) protein hydrolysate was produced by hydrolysis with Alcalase 2.4L using pH-stat method. The muscle was initially hydrolysed for 2 hours at 3% enzyme-substrate ratio, $60^0$C and pH 8.5, centrifuged and freeze dried. The yield, composition and functional properties of the resulting hydrolysate were determined. A reasonably high yield was achieved which is 11.03%. The hydrolysate was characterized by high protein content (43.0%) and yellowish in colour (L* = 72.98, a* = 0.42, b* = 15.15). It contains high amount of essential amino acids (45.62%) with alanine and serine as the dominant amino acids. The protein hydrolysate had an excellent solubility (92.32%) and an emulsifying stability index of 36.13 min. Foaming properties decrease significantly ($p<0.05$) with increasing time of foam. In view of these beneficial properties, mentarang (*Pholas orientalis*) hydrolysate has the potential for application as a natural additive in food.

*Keywords:* Mentarang (*Pholas orientalis*), hydrolysate, alcalase, solubility, colour, hydrolysis

## INTRODUCTION

Mentarang (*Pholas orientalis*) is a bivalve species enclosed by two thin elongated shells. In western countries it is also known as 'anglewing' clam (Jesse *et al*., 2006). It can be found in muddy shore and live in low temperature water for example in estuary. In Malaysia, this species can be found easily in Sabak Bernam, Selangor. *Pholas orientalis* is highly favoured because of its excellent flavour and attractive milky white shell.

Protein hydrolysate refers to compound produced by the hydrolysis of high protein food such as milk, egg, fish and meat with acid, alkali or enzyme. In general, protein

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hydrolysate is obtained by mixing the protein raw material with water and incubated at specific time and temperature before the enzyme, acid or alkaline is introduced. Hydrolysate contains a mixture of amino acids and peptides. Some studies have shown that hydrolysate can contribute to water holding, texture, gelling and whipping properties when added to food (Kristinsson, 2007; Wasswa *et al*., 2007b; Wasswa *et al.,* 2008). Addition of hydrolysate from salmon reduced water loss after freezing (Kristinsson, 2000a). Hydrolysates have also been proven to have good foaming and emulsifying properties. Therefore, it may be used as emulsifying and emulsion stabilizing ingredients in a variety of products as well as aids in the formation and stabilisation of foam-based products (Kristinsson, 2007).

Most hydrolysate was produced using sea species because sea species are easy to find, breed in thousands, and almost all sea species are edible and 'halal'. Many bivalve species including mentarang has limited uses and only introduced as seafood meal. In Malaysia, they are only sold and consumed in some areas and not very popular among many people, even though this seafood species contains a very rich source of protein. Thus, hydrolysate was prepared in this study to expand the utilization of bivalves. In addition, the determination of compositional and functional properties of hydrolysate derived from this bivalve species may lead to the discovery of new ingredient to food or pharmaceutical product. The objective of this study is to determine the compositions and the functional properties of mentarang (*Pholas orientalis*) hydrolysate produced by hydrolysis using alcalase.

## MATERIALS AND METHOD

### *Materials*

Mentarang was bought from Pantai Remis, Selangor, Malaysia and immediately placed in ice and transported to the laboratory. Upon arrival, the flesh was removed manually from the shell, washed and then minced using a blender.

Alcalase (with a declared activity of 2.4 AU/g and a density of 1.18 g/ml) is a bacterial endoproteinase from a strain of *Bacillus Licheniformis* was purchased from Science Technic Sdn. Bhd. Malaysia.

### *Preparation of Mentarang (Pholas orientalis) Protein Hydrolysate*

Mentarang hydrolysate was prepared according to the method by Adler-Nissen (1986), with a slight of modification. Thirty grams of minced mentarang meat was suspended in 120 ml of distilled water. The mixture was incubated in a circulated water bath at $60^0$C. The pH of the mixture was adjusted to pH 8.5 and constantly maintained during hydrolysis using 1.0 N NaOH. Once the pH and temperature have stabilized, alcalase at enzyme-substrate ratio of 3% was added and the reactions continued for 2 hours. The enzymatic reaction was terminated by placing the samples in a water bath at $90^0$C for 15 min with occasional agitation. This was followed by centrifugation at 14000g for 10 min. Supernatants obtained were freeze-dried using the SANYO-Biomedical freeze dryer.

*Determination of the Percent Degree of Hydrolysis (DH %)*

Degree of hydrolysis (DH) is defined as the percentage ratio between the number of peptide bonds cleaved (h) and the total number of peptide bonds in the substrate studied ($h_{tot}$) (Adler-Nissen, 1986). The degree of hydrolysis was determined based on the consumption of base necessary for controlling the mixture's pH during the batch assay as in the equation below:

$$DH\% = \frac{\beta \text{ x } N_{\beta}}{\alpha \text{ x } M_P \text{x } h_{tot}} X100$$

Where:

$\beta$ = Volume of 1.0N NaOH

$N_{\beta}$ = Normality of NaOH

$\alpha$ = Average degree of dissociation of the $NH_3$ groups

$M_P$ = Mass of protein in g

$h_{tot}$ = Total number of peptide bonds in the protein substrate (mmol/g protein)

*Yield*

The yield was determined by the ratio of the mass of hydrolysate and the total weight of the fresh mentarang muscle. The yield obtained was calculated as follows:

$$\text{Yield } (\%): \frac{\text{Weight of powdered hydrolysate x 100}}{\text{Wet weight of fresh mentarang muscle}}$$

*Determination of Mentarang (Pholas orientalis) Protein Hydrolysate Compositions*

**Moisture Content**

Moisture content was determined according to the (AOAC, 2005) by placing approximately 2 g of minced mentarang or hydrolysate sample into a pre-weighed aluminium dish. The sample was dried in a forced-air convection oven at $105^0$C until a constant weight was achieved. Moisture content was calculated as follows:

$$\% \text{ Moisture } (wt/wt) = \frac{\text{Weight of wet sample } - \text{ Weight of dry sample}}{\text{Weight of wet sample}} X100$$

**Determination of Fat Content**

The extraction and determination of the fat content from the minced muscle or the hydrolysate sample were performed using the Soxhlet extraction method (AOAC, 2005).

**Protein Concentration**

Protein concentration was determined using the Lowry method based on the modified procedure of Hartree (1972).

**Colour Measurement**

The colour of powdered hydrolysate was measured by chromameter CR400 (Konica Minalto). L*, a*, and b* parameters indicate brightness, redness and yellowness, respectively. The measurement was performed in triplicate.

**Amino Acid Analysis**

Sample preparation was conducted by hydrolysis with 6 M HCl at $110^0$C for 24 hours and derivatisation using phenyl isothiocyanate prior to AccQ Tag HPLC analysis. The total amino acid was analysed by the AccQ Tag method using an AccQ Tag column (3.9 x 150mm) at a flow rate of 1 ml/min with UV detection. The mobile phase used is AccQ Taq Eluent A that consists of 100ml Eluent A and 1000ml deionized water, while AccQ Tag Eluent B consists of 60% acetonitrile and 40% deionized water or 60% acetonitrile. The total running time per injection was 50 minutes.

*Determination of Functional Properties*

**Solubility**

Solubility was determined following the procedure of Morr (1985) with slight modification. Protein hydrolysate (0.5 g) was dispersed in 50 ml of 0.1 M NaCl. The mixture was stirred for 1 hour at room temperature and then centrifuged using a Kubota 5420 centrifuge at 2600g for 30 min. The supernatant was filtered through Whatman filter paper No.1 and the nitrogen content in the total fraction and in the soluble fraction was analysed by Lowry method. Solubility was calculated as follows:

$$\text{Solubility } (\%) = \frac{\text{Protein content in supernatant}}{\text{Total protein content in the sample}} \text{ x } 100$$

**Emulsifying Stability**

The emulsion stability index (ESI) was determined according to the method of Pearce and Kinsella (1978) with a slight modification. Soybean oil (10 ml) and 30 ml of protein hydrolysate solution (3g in 100ml) was mixed and homogenised using homogenizer at a speed of 20,000 rpm for 1 min using IKA T25 digital ULTRA-TURRAX homogenizer. Aliquot of the emulsion (50 µl) was pipetted from the bottom of the container at 0 and 10 min after homogenisation and diluted 100-fold using 0.1% SDS solution. The absorbance of the diluted solution was measured at 500 nm. The absorbance, measured immediately ($A_0$) and 10 min ($A_{10}$) after emulsion formation was used to calculate the emulsion stability index (ESI), as follows:

$$\text{ESI (min)} = A_0 \text{ x } \Delta t/\Delta A$$

Where $\Delta A = A_0 - A_{10}$ and $\Delta t = 10$ min.

**Foaming Stability**

Foaming stability of protein hydrolysate was analysed according to the method of Shahidi *et al*. (1995). Twenty millilitres of protein hydrolysate solution was homogenized in a 50 ml cylinder at a speed of 16,000 rpm to incorporate the air for 1 min. The total volume was measured at 0, 0.5, 5, 10, 40, and 60 min after whipping. Foaming stability was expressed as foam expansion at 60 min. Foam stability was calculated according to the following equation (Sathe & Salunkhe, 1981):

$$\text{Foam stability (\%)} = [(A - B)/B] \text{ x } 100$$

Where;

A = volume after whipping (ml) at different time (0, 0.5, 5, 10, 40, 60)

B = volume before whipping (ml)

**Water Holding Capacity**

Water-holding capacity (WHC) was determined using the centrifugation method according to Diniz and Martin (1997). The samples (0.5 g) of hydrolysate was dissolved with 20 ml water in centrifuge tubes and dispersed with a vortex mixer for 30s. The dispersion was allowed to stand at room temperature for 6 hours, and it was subsequently centrifuged at 2800g for 30 min. The supernatant was filtered through Whatman filter paper No. 1 and the volume recovered was accurately measured. The difference between the initial volume of distilled water added to the protein sample and the volume of the supernatant were determined. The results were reported as ml of water absorbed per gram of protein sample.

$$\text{WHC } (\text{ml/g}) = \frac{\text{Initial volume of distilled water } - \text{ Volume of supernatant } (\text{ml})}{\text{Weight of hydrolysate } (\text{g})}$$

## RESULTS AND DISCUSSION

*Percent Degree of Hydrolysis (%DH)*

During hydrolysis, mentarang muscle mixture changed into brownish red. After centrifugation, the supernatant was clear, reddish in colour and had a sticky characteristic. After freeze drying the hydrolysate had a light yellowish and strong fishy odour. The freeze-dried hydrolysate turned into dark yellowish powdered when stored for long period at room temperature for more than a month. The degree of hydrolysis using alcalase at $60^0$C was 9.44%. Shahidi *et al*. (1995) reported a higher degree of hydrolysis (22%) during the hydrolysis of capelin protein at $65^0$C using alcalase. According to Guerard *et al*. (2002) a reduction in the reaction rate may be due to the limitation of the enzyme activity by formation of reaction products at high degrees of hydrolysis. However, the decrease in hydrolysis rate may also be due to a decrease in the concentration of peptide bonds available for hydrolysis, enzyme inhibition and enzyme deactivation. The research by Mahmodreza *et al*. (2009) revealed that the degree of hydrolysis increase at increasing hydrolysis time and temperature. Bhaskar *et al.* (2008) also observed higher degree of hydrolysis at elevated temperatures. In addition, alkaline proteases like alcalase have been reported to exhibit higher activities than neutral or acid proteases, such as papain or pepsin (Rebeca *et al*., 1991; Sugiyama *et al*., 1991).

*Compositions, Yield and Colour*

The compositions of mentarang (*Pholas orientalis*) and its hydrolysate are given in Table 1. The yield obtained was reasonably high (11.03%). The yield can be improved by properly monitoring the hydrolysis conditions. The low moisture content of the hydrolysate enhanced the stability of the hydrolysate to be stored for a prolonged period of time. Hydrolysis of mentarang muscle in this study increased the protein concentration in hydrolysate. The increasing protein content was a result of the solubilisation of protein during hydrolysis and the removal of insoluble undigested non-protein substance (Benjakul & Morrissey, 1997). A previous report has

shown that the sample containing high amount of lipids contained low percentages of solubilised protein (Slizyte *et al*., 2005). Mentarang muscle contains only 2.82% fat content, thus the protein content obtained in the hydrolysate was considerably high.

Hydrolysate contains lower fat content than the mentarang muscle. The low fat content could be due to centrifugation, where some of the fats are separated while others may have entrapped in the pellet after the centrifugation. Decreasing lipid content in the protein hydrolysates might significantly increase stability of the materials towards lipid oxidation, which may also enhance the product stability (Kristinsson & Rasco, 2000b).

Colour influences the overall acceptability of food products. Hydrolysis of mentarang (*Pholas orientalis*) produced hydrolysate that is light yellow in colour. Wasswa *et al*., (2007a) stated that increased hydrolysis time resulted in increased enzymatic browning reaction. Enzymatic reactions are assumed to have contributed to the reduction in the luminosity, giving a darker appearance at high degree of hydrolysis (Wasswa *et al*., 2007b).

*Amino Acid Composition*

The amino acids composition of the freeze-dried mentarang hydrolysate is presented in Table 2. The hydrolysate contains high amount of alanine followed by serine, histidine, threonine and glutamine. Mentarang hydrolysate may probably exhibit some antioxidant activity. This was due to the high content of histidine and alanine, the amino acids known to contribute to antioxidant activity (Mendis *et al*., 2005). High antioxidant activity of histidine-containing peptide was attributable to the imidazole ring of histidine that enables histidine to chelate metal ions and trap lipid radicals (Uchida & Kawakishi, 1992). The higher content of total hydrophilic amino acids (aspartic acid, glutamic acid, glycine, alanine, threonine and serine) as compared to total hydrophobic amino acids is consistent with the high solubility characteristic of the hydrolysate. The ratio of essential amino acid to non-essential amino acid is 0.75. Thiansilakul *et al*. (2007) obtained the ratio of 0.92. Fish and shellfish have been reported to contain high essential amino acid to non-essential amino acid ratio

TABLE 1
Yield, compositions and colour of mentarang (*Pholas orientalis*) hydrolysate

| Composition | mentarang (*Pholas orientalis*) | hydrolysate |
|---|---|---|
| Yield (%) | | 11.03 ± 1.38 |
| Moisture content (%) | 79.60 ± 4.37 | 9.51 ± 1.51 |
| Fat content (%) | 2.82 ± 1.84 | 0.81 ± 0.56 |
| Protein (%) | 13.72 ± 0.56 | 43.0 ± 0.04 |
| Colour | | |
| L* | | 72.98 ± 0.06 |
| a* | | 0.42 ± 0.01 |
| b* | | 15.15 ± 0.01 |

(Iwasaki & Harada, 1985). Based on the amino acids profile, mentarang hydrolysate is high in nutritional value.

*Functional Properties*

**Solubility**

Solubility is one of the most important physicochemical and functional properties of protein hydrolysates (Kristinsson & Rasco, 2000a). In general, the degradation of protein into smaller peptides leads to more soluble products (Gbogouri *et al*., 2004). The smaller peptides are expected to have more polar residues, increasing hydrophilicity through an increased ability to form hydrogen bonds with water (Sathivel *et al*., 2005). The balance of hydrophilic and hydrophobic forces of peptides is another crucial influence on solubility increments (Gbogouri *et al*., 2004). Protein hydrolysate from *Pholas orientalis* shows high solubility at pH 8.5 (Table 3). Salmon head hydrolysate produced using Alcalase 2.4L was reported to show more than 75% solubility at 11.5% to 17.3% DH (Gbogouri *et al*., 2004). Due to its high solubility, *Pholas orientalis* hydrolysate was presumed to have a low molecular weight and was hydrophilic in nature and with 92% solubility this suggested that the hydrolysate is very soluble in aqueous system. This shows that protein hydrolysate derived from *Pholas orientalis* can be a good food additive because it is easy to dissolve. The high nitrogen solubility of protein hydrolysate indicates potential applications in formulated food systems by providing attractive appearance and smooth mouth feel to the product (Peterson, 1981).

**Emulsifying Properties**

Emulsifying stability index of protein hydrolysate ten minutes after homogenization was 39.18 min (Table 3). This value shows that protein hydrolysate produced from *Pholas orientalis* at 9.44% degree of hydrolysis can slightly emulsify with oily food. Emulsifying stability of hydrolysates decreased with the increase in hydrolysis due to the presence of small peptides,

TABLE 2
Amino acids composition of mentarang (*Pholas orientalis)* hydrolysate

| Essential amino acid | Content (%) | Non-essential amino acids | Content (%) |
|---|---|---|---|
| Val | 3.39 ± 1.23 | Asp | 4.22 ± 2.08 |
| His | 14.10 ± 0.71 | Ser | 15.71 ± 0.42 |
| Met | 3.29 ± 2.15 | Glu | 9.24 ± 1.32 |
| Thr | 13.02 ± 0.87 | Gly | 8.57 ± 3.98 |
| Lys | 1.58 ± 0.33 | Ala | 17.97 ± 1.01 |
| Ile | 5.75 ± 4.00 | Pro | 2.19 ± 1.40 |
| Leu | 4.49 ± 2.32 | Cys | 0.97 ± 1.10 |
| | | Tyr | 2.30 ± 0.64 |
| Total | 45.62 | Total | 61.17 |

which are less effective in stabilizing emulsions (Wasswa *et al*., 2007a; Wasswa *et al*., 2007b). In addition, an increase in hydrolysis also reduced the hydrolysate emulsifying capacity and stability due to lesser surface hydrophobicity (Kristinsson & Rasco, 2000b). A peptide should have a minimum length of more than 20 amino acid residues in order to possess good emulsifying and interfacial properties and it has been shown that large peptides of about 20,000 Da contributed to the high emulsifying capacity of hydrolysate (Lee *et al*., 1987; Kristinsson & Rasco, 2000b). The pH of protein solutions during emulsification affects their emulsifying properties via charge effects (Nielsen, 2001). Meanwhile, an addition of salt improves the emulsion properties of peptide fractions (Turgeon *et al*., 1992). Several previous studies have reported that excessive hydrolysis brings about the loss of emulsifying properties (Gbogouri *et al*., 2004; Kristinsson & Rasco, 2000b; Klompong *et al*., 2007), while at limited degree of hydrolysis, the hydrolysates have an exceptional emulsifying stability (Kristinsson & Rasco, 2000b). Several factors such as blending speed, protein source, temperature, pH, type of oil added and water content influence emulsion capacity. In this study the degree of hydrolysis obtained was reasonably low (9.44%), and thus, good emulsifying properties was achieved. Nalinanon *et al*. (2011) obtained emulsifying stability index of 14.1 min at 10% DH using pepsin. This shows that the specificity of enzyme may also influence emulsifying properties.

TABLE 3
Functional properties of mentarang (*Pholas orientalis*) hydrolysate

| Functional properties | |
|---|---|
| Solubility (%) | 92.32 ± 2.10 |
| Emulsifying stability (min) | 39.13 ± 0.72 |
| Foaming properties (%) | |
| 0 min | $78.00^{a}$ ± 1.15 |
| 0.5 min | $76.00^{a}$ ± 7.21 |
| 5 min | $56.67^{b}$ ± 1.15 |
| 10 min | $26.67^{c}$ ± 1.15 |
| 40 min | $22.00^{c}$ ± 2.00 |
| 60 min | $6.67^{d}$ ± 3.06 |
| Water holding capacity (ml/g) | 3.92 ml/g ± 0.98 |

## Foaming Properties

Foaming properties are physicochemical characteristics of proteins to form and stabilise foams (Thiansilakul *et al.,* 2007). Enzymatic hydrolysis of protein can improve foaming characteristics (Adler-Nissen, 1986). However, this depends on the degree of hydrolysis achieved which is influenced by several factors including the type of enzyme, pH, duration of hydrolysis and temperature. Foam stability for powdered mentarang (*Pholas Orientalis*) hydrolysate at different times after homogenization is shown in Table 3. In general, there was a significant decrease ($p < 0.05$) in foaming properties with time. Thiansilakul *et al*. (2007) who studied on the effect of different protein concentration observed that foam stability increases with the increasing in protein concentration. High degree of hydrolysis has been shown to reduce foaming properties of hydrolysate. Klompong *et al*. (2007), who studied on the effect of 5% to 25% degree of hydrolysis (DH) during the production of yellow-

striped trevally hydrolysate, found that the highest foaming capacity was at 5% DH, while the lowest at 25% DH. Shahidi *et al*. (1995) reported that a good foaming for capelin protein hydrolysate was produced at 12% DH. This finding suggested that a prolonged hydrolysis could reduce foaming stability since microscopic peptides do not have the strength needed to maintain stable foam. In order for a hydrolysate to present good foamability, it must have among other factors, a good balance of surface, a molecular hydrophibicity, as well as net charge and charge distribution (Damodaran, 1997; Pacheco-Aguilar *et al*., 2008). Molecular weight and charge of peptides may be different for hydrolysate produced at different conditions which will affect the foaming properties (Van Der van *et al*., 2002; Klompong *et al*., 2007).

### Water Holding Capacity

Water holding capacity (WHC) for powdered mentarang hydrolysate is 3.92mg/ml (Table 3). In general, water holding capacity of partly unfolded and hydrolysed proteins is greater than that of the native proteins due to an increase in surface area to mass ratio with an exposure of some previously buried hydrophobic groups (Damodaran, 1997). Several studies have shown that hydrolysates have excellent water holding capacity and can increase the cooking yield when added to minced meat (Kristinsson & Rasco, 2000b; Shahidi *et al*., 1995). The presence of polar groups such as COOH and $NH_2$ that increased during enzymatic hydrolysis had a substantial effect on the amount of adsorbed water (Kristinsson & Rasco, 2000b). Wasswa *et al*. (2007) showed a similar result of water holding capacity at 10% degree of hydrolysis with 3.8 ml/g. WHC increased with an increased in degree of hydrolysis (Wasswa *et al*., 2007). This shows that protein hydrolysate derived from *Pholas Orientalis* has the ability to hold water molecules well.

## CONCLUSION

The protein hydrolysate derived from mentarang (*Pholas orientalis*) muscle serve as a good source of desirable amino acids. This protein hydrolysate could be used as an emulsifier, foaming agent as well as water holding agent. Mentarang hydrolysate has the potential to be used as natural additive, possessing functionality properties in food systems. Based on the findings of this study, it can be stated that the functionalities and compositions of protein hydrolysate derived from *Pholas Orientalis* using alcalase are reasonably acceptable.

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## REFEREES FOR THE PERTANIKA JOURNAL OF TROPICAL AGRICULTURAL SCIENCE

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*Our goal is to bring high quality research to the widest possible audience*

**Journal of Tropical Agricultural Science**

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Revised: February 2013

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*Pertanika* began publication in 1978 as the Journal of Tropical Agricultural Science (JTAS).

In 1992, a decision was made to streamline *Pertanika* into **3 journals**. i.e.,

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2. Journal of Science and Technology (JST)
3. Journal of Social Sciences and Humanities (JSSH)

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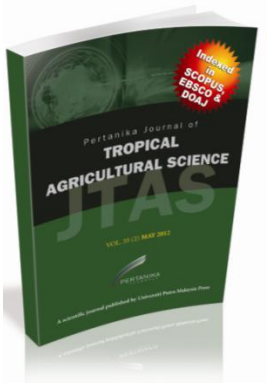


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