Renewable Bioresources
ISSN 2052-6237 | Volume 3 | Article 2

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Research Open Access

Optimisation of phenolic extraction and quantification of phenolics in palm kernel cake

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Abstract

This study was designed to determine the types of phenolics in palm kernel cake (PKC) and optimise the extraction of phenolics. The phenolics of PKC were extracted in free methanol-soluble and cell wall-bound forms. Optimisation of phenolic extraction from PKC was carried out by investigating the effects of pretreatment, different solvents (water, methanol, ethanol and acetone), extraction temperatures (25℃, 60℃ and 80℃) and extraction durations (15, 30, 60 and 120 minutes). The results indicated that 70.44% (500.9 µg/g of dry weight) of the phenolics in PKC were in free methanol-soluble form whereas 29.56% (210.2 µg/g of dry weight) was cell wall-bound phenolics. The optimum phenolics extraction conditions for PKC suggested in this study are by using water with 1:10 material to solvent ratio, stirring at 250 rpm, 25°C for 30 minutes. The ultra-performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry (UPLC-ESI-MS/MS) analysis showed that p-hydroxybenzoic and protocetechuic acids were the predominant phenolics in methanol extract. Meanwhile, p-hydroxybenzoic, protocatechuic as well as shikimic acids appeared to be the predominant phenolics in water extract.

Keywords: Palm kernel cake (PKC), phenolic extraction, free soluble phenolics, bound phenolics

Introduction

Phenolics are known as secondary metabolites and are found in plants. Some of these compounds have been found to exhibit a wide range of biological and physiological properties such as anti-oxidant, anti-artherogenic, anti-inflammatory and anti-thrombotic activities. It has been reported that phenolics extracted from fruits were used in treatment or prophylaxis of cardiovascular diseases, colon cancer and digestive health [1]. It has also been observed that tea polyphenols show numerous anti-oxidant, anti-bacterial, anti-cancerous activities as well as reducing blood cholesterol [2-6].

Furthermore, agro-industrial by-products including rapeseed, rice hulls, apple peels and grape seeds were reported as rich sources of phenolics [7-10]. By-product from the olive oil industry is another potential source for phenolics. It has been disclosed in US20080014322 that olive polyphenol concentrates were derived from the by-product of olive oil extraction [11].

Similar to olive fruits, the oil palm fruit is a rich source of phenolics. Ofori-Boateng and Lee reported that 225 kg palm phytochemicals was generated from 21.63 tonnes of by-products per hectare of oil palm cultivation every year which bringing in revenue over USD420000 to the industry [12]. Oil palm phenolics extracted from palm oil mill effluent (POME) was reported to contain large amount of phenolics including caffeoylshikimic acid (10800±2400 mg/kg), p-hydroxybenzoic acid (7000±1000 mg/kg) and protocatechuic acid (6000±100 mg/kg) [13]. Furthermore, the process for extraction of phenolics from POME has been patented [14]. The palm phenolics have been used in treatment of cancer [15], prevention of neurodegenerative ailments [16], food preservatives [17] and cosmeceutical applications [18]. There were a few studies carried out on extraction, analysis and quantification of phenolics from oil palm fruits as well as its anti-oxidant properties [19-21].

Palm kernel cake (PKC) is the by-product derived from the kernel of the oil palm fruit after extraction of kernel oil. It has been used as animal feed which is a relatively low value product. There was a research by Mohammad Zarei et al., worked on PKC protein hydrolysate which was found to possess antioxidant activity [22]. In agreement with this finding, Oskoueian et al., demonstrated PKC extract contains 18.2% phenolics (12.3% hydroxybenzoic acid and 5.9% 4H-pyran-4-one) which is most likely contributing to its hepatoprotective activity in heat-induced chicken hepacytes [23]. A few of studies have been done on PKC

doi: 10.7243/2052-6237-3-2

extract as well as hydrolysate. Most of these reported studies assessed its antioxidant and antibacterial activities [22-24]. To date, there has been no analysis on phenolic content of PKC reported. Hence, this study was carried out to identify the major free and bound phenolics in PKC and optimise the extraction of phenolics.

Materials and methods

Plant material

Palm kernel cake (PKC) was obtained from the palm kernel crushing plant in Carey Island, Malaysia. The PKC used in this study was derived from a conventional mechanical screw press oil extraction method.

Extraction of free methanol soluble-phenolics from PKC

Extraction of free methanol-soluble phenolics was adapted from Alu'datt et al., [25] and carried out in triplicate. One gram of PKC was extracted with 25mL methanol for 1 hour in a water bath (25°C) and centrifuged at 10,000×g for 10 minutes. The solid residue was re-extracted with 25mL methanol for 1 hour at 60°C and centrifuged at 10,000×g for 10 minutes. The supernatants were filtered through 0.2µm GH Polypro (GHP, hydrophilic polypropylene) membrane (Pall Corporation, NY, US) and kept in -20°C freezer for further analysis.

Extraction of cell wall bound phenolics from PKC

The extraction of cell wall bound phenolics was adapted from Alu'datt et al., [25] and carried out in triplicate. The residue remaining after methanol extraction at 60°C (Section 2.2) was hydrolysed using 25mL of 0.1M sodium hydroxide at 25°C for 12 hours and then followed by acid hydrolysis using 25mL of 0.1M hydrochloric acid at 25°C for 12 hours. The extract was centrifuged at 10,000×g for 10 minutes and then filtered through 0.45µm GHP membrane before undergoing freeze-drying. The dried supernatant was re-suspended with 25mL methanol at 25°C for 30 minutes and then centrifuged at 10,000×g for 10 minutes. The methanol extracted- supernatant was then filtered through 0.2µm GHP membrane and stored in a -20°C freezer for further analysis.

Optimisation of phenolics extraction from PKC

Single factor experiments were used to investigate the parameters for phenolic compounds from PKC. Four parameters were studied, namely, with or without pretreatments, different solvents system, extraction temperature and extraction duration. Firstly, the effect of pretreatments (sonication and hexane wash) was investigated. For hexane treatment, 10g of PKC was washed with 30mL hexane and followed by centrifugation (10,000xg, 10 minutes). The hexane treated PKC was air-dried and then extracted with methanol at 25°C for 30 minutes. For sonication treatment, 10g of PKC was added with 100mL methanol and then sonicated at 25°C for 15 minutes. The subsequent parameter studies were carried out without pretreatment. Mono-solvent system (water, methanol, ethanol and acetone) and binary solvent system (80% aqueous methanol, 80% aqueous ethanol and 80% aqueous acetone) were investigated. At this step, the extraction time and extraction temperature were kept constant at 25°C for 30 minutes. Following this was the study of the effect of extraction duration by varying the extraction duration from 15 to 120 minutes using 80% aqueous methanol which was chosen in the initial step and kept the extraction temperature constant at 25°C. Lastly, the effect of extraction temperature was investigated using 80% aqueous methanol and extraction time determined in the earlier part of this study (15 minutes) with extraction temperature ranged from 25 to 80°C. In these parameter studies, single extraction process was used. The phenolic extraction of PKC was carried out at a PKC to solvent ratio of 1:10 and stirring at 250rpm. The solid-solvent mixture was centrifuged at 10,000×g for 10 minutes to remove solid residue. The resulting extracts were filtered through 0.2µm GHP membrane and stored in -20°C freezer for further analysis. All experiments were carried out in triplicate.

Determination of total phenolic content

The total phenolic content was determined using Folin-Ciocalteu assay [26]. The extracts (50µL; Section 2.2-2.4) were added with 450µL of Folin-Ciocalteu reagent (0.2N). The mixture was homogenised for 3 min and then 500µL of aqueous sodium carbonate (7.5%, w/v) was added. The absorbance was measured at 725 nm after 1 h of incubation at 25°C in a waterbath. Gallic acid was used as a reference standard to construct a standard curve ranging from 250 to 1000 mg/L, whereas methanol was used as a blank. The phenolic content of each extract was expressed as milligrams of gallic acid equivalents per gram of plant sample (mg of GAE/g).

Ultra-performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry (UPLC-ESI-MS/MS) analysis of phenolics

An Acquity UPLC system coupled to Xevo triple quadrupole mass spectrometer with Zspray electrospray ionisation (ESI) was used for analysis of phenolics in the resulting extracts. An Acquicty UPLC BEH C18 (100mm x i.d. 2.1mm, 1.7µm) column was used in liquid chromatography (LC) part of the UPLC-ESI-MS/MS analysis. The mass spectrometry analysis was performed in both negative and positive modes using the multiple reactions monitoring (MRM). The conditions of mass spectrometry were as following: capillary voltage, 3kV; desolvation gas temperature, 350°C; desolvation gas flow, 800 L/h; nebuliser flow, 7 bar. The nebulising and collision gas used in mass spectrometry analysis was nitrogen. The corresponding cone voltage and collision energy of 20 phenolic standards were optimised. Data acquisition and processing were performed using MassLynx V4.1 and TargetLynx respectively. The mobile phase was a binary solvent system consisting of solvent A (water with 0.1% formic acid) and solvent B (methanol with 0.1% formic acid). The UPLC gradient was:

 doi: 10.7243/2052-6237-3-2

0-4 minutes, 11.5-50% B; 4-12 minutes, 50-11.5% B; 12-15 min, 11.5% B for final washing and equilibration of the column for the next run. The flow rate was 0.3 mL/min and the injection volume was 3µL.

Results and discussion

Extraction of free soluble and cell wall bound phenolics of PKC

In this analysis, methanol was used to extract free methanol soluble phenolics. The first step was extraction of the PKC using methanol at 25°C. This was followed by methanol extraction at 60°C to increase the solubility of other remaining free soluble phenolics into the solvent. The solid residue after extraction was then subjected to basic hydrolysis as well as acidic hydrolysis to break down intermolecular bonds in the PKC and release the cell wall bound phenolics. Based on the total phenolics extracted from PKC as depicted in **Table 1**, the amount of free methanol-soluble phenolics was 70.44% (500.9 µg/g of dry weight), of which 21.24% was extracted at 25°C and 49.2% at 60°C. Subsequently, 29.56% (210.2 µg/g of dry weight) of phenolics were extracted from the remaining solid residue after hydrolysis. Among the detected phenolics, the most abundant phenolics found were p-hydroxybenzoic acid (527.21 µg/g of dry weight) followed by protocatechuic acid (80.1 µg/g of dry weight). A small amount of d-glucuronic and quinic acid were only detected in methanol extract of PKC whereas salicylic and sinapic acid were only detected in the extract of PKC cell wall. Recently, a considerable amount of literature has been published on phenolics from oil palm fruit and vegetation liquor. Most of these studies identified phenolics and assessed its antioxidant activity [**8**,**11**-**21**]. Our finding was in agreement with some other previous studies where p-hydroxybenzoic acid was the most dominant compound in mesocarp of oil palm fruit and *Cocos nucifera* [**21**,**27**,**28**].

Effect of pretreatment on phenolic extraction

After crushing palm kernel, the resulting PKC contains approximately 10% (w/w) oil. Hexane, a non-polar solvent was used to remove oil from PKC for the purpose of increasing phenolic extraction yield. There was no improvement of extraction yield using hexane pretreatment (data not shown). This might be due to oil content was too small to have any significant effect on the extraction process. On the other hand, sonication was attempted to determine if breaking intermolecular bonds would release the phenolics from PKC. In this study, PKC was sonicated for 15 minutes at 48 kHz using NEY ultrasonik 208H. Total phenolic content slightly decreased from 5.24±0.07 to 5.13±0.08 mg GAE/g of dry weight after sonication (data not shown). This study has been unable to demonstrate that sonication increases efficiency of phenolic extraction as reported in a few of previous studies [**29**-**31**]. This rather contradictory result may indicate that sonication

Table 1. Free methanol soluble and cell wall bound phenolics of PKC.
The free soluble phenolics was extracted using methanol at a PKC to solvent ratio of 1:10 and stirring at 250rpm. The PKC was extracted at 25°C for 1 hour and then re-extracted at 60°C for 1 hour. To extract cell wall bound phenolics, the residue was subjected to basic and acidic hydrolysis followed by methanol extraction as detailed in section 2.3. The phenolics in extracts were determined using UPLC-ESI-MS/MS. Values are presented as means of three replicates±standard deviation. The total phenolic content was the sum of individual phenolics identified in the extracts.

	Content (µg/g of dry weight)			
Phenolic	**Free methanol soluble phenolics**		**Cell wall bound phenolics**	**Total**
	25°C	**60°C**		
Caffeic acid	0.60±0.03	1.02±0.05	0.99±0.06	2.61
d-Glucuronic acid	1.01±0.05	Not detected	Not detected	1.01
Ferulic acid	0.63±0.04	0.94±0.02	10.53±0.46	12.10
Glutaric acid	1.45±0.11	2.44±0.14	3.67±0.27	7.56
Protocetechuic acid	25.67±1.69	43.75±3.80	10.68±0.64	80.10
P-Coumaric acid	0.89±0.02	1.65±0.11	7.88±0.53	10.42
P-Hydroxybenzoic acid	99.83±0.69	268.71±2.35	158.67±6.72	527.21
Quinic acid	4.70±0.45	4.99±0.38	Not detected	9.69
Salicylic acid	Not detected	Not detected	0.16±0.04	0.16
Shikimic acid	6.95±0.32	8.93±0.72	1.51±0.38	17.39
Sinapic acid	Not detected	Not detected	1.51±0.06	1.51
Syringic acid	1.15±0.02	1.91±0.07	1.83±0.07	4.89
Vanillic acid	8.20±0.10	15.58±0.25	12.77±0.37	36.55
Total phenolics	151.08	349.92	210.20	711.20
Total phenolics (%)	21.24	49.20	29.56	100

doi: 10.7243/2052-6237-3-2

duration and intensity appear to be important in this study. Fifteen minutes of sonication might not be sufficient to give any effect on phenolic extraction. However, too long sonication at overly strong intensities might cause degradation to phenolics.

Effect of solvent type on phenolic extraction

Solvents with different polarities can have large effects on phenolic extraction efficiency. The hydroxyl group, length of hydrocarbon and molecular size of a phenolic compound determine its solubility in solvent [32]. In this study, we have investigated the effects of a mono-solvent system and a binary solvent system on phenolic extraction of PKC. In the absence of water, higher phenolics were extracted using methanol compared to ethanol and acetone (**Figure 1a**).

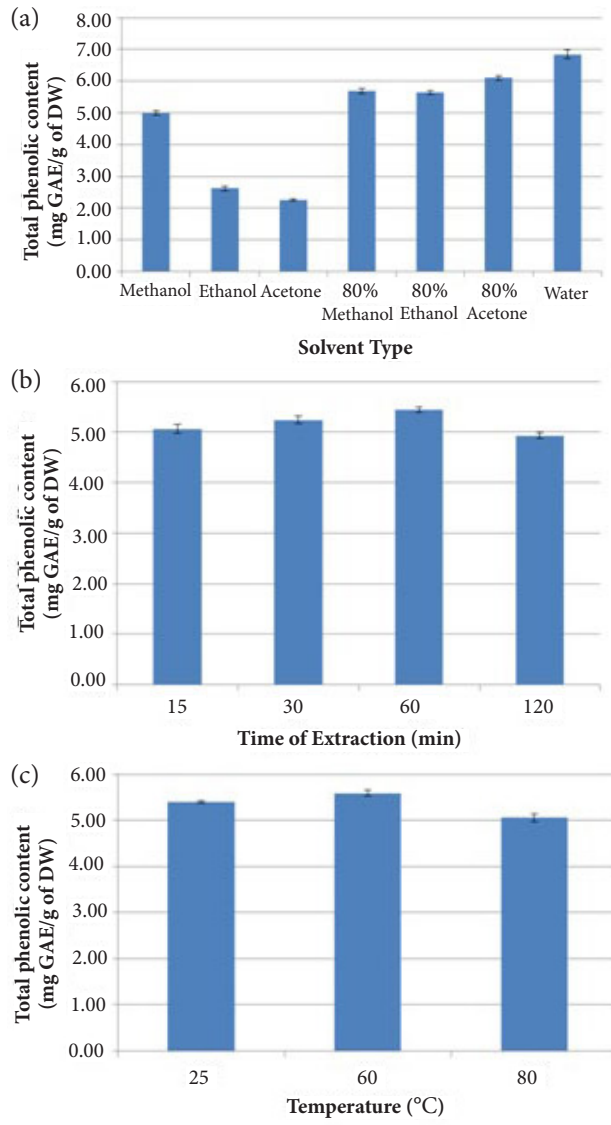

Figure 1. (**a**) Effect of solvent type, (**b**) Time of extraction and (**c**) Temperature on total phenolic content of palm kernel cake extracts.

Relative polarity of water, methanol, ethanol and acetone is 1, 0.762, 0.654 and 0.355 respectively [33]. This showed that increasing polarity of solvents increases phenolics yield. Furthermore, addition of water, which is a strong polar solvent, to methanol, ethanol and acetone increases polarity of the solvent system. Therefore, binary solvent systems (80% aqueous methanol, 80% aqueous ethanol and 80% acetone) extracted considerably higher phenolics than mono-solvent systems (methanol, ethanol and acetone). The highest phenolics yield was obtained using 80% aqueous acetone (6.10±0.08 mg GAE/g of dry weight) whereas acetone extract contained the lowest phenolics (2.25±0.04 mg GAE/g of dry weight). These results are consistent with those of other studies [34-36] and suggest a higher yield of phenolics using binary solvent system as compared to mono-solvent system.

Furthermore, water was also evaluated for its efficacy in extracting phenolics as compared to organic solvent in this study. Contrary to earlier findings [34-36], however, water alone extracted more phenolics from PKC (6.84±0.14 mg GAE/g of dry weight) compared to other solvents or mixtures. In accordance with the present results, previous studies have demonstrated that water could be an adequately good solvent for extracting phenolics from apple peel [37] and edible wild type mushroom [38]. Spigno et al., reported that higher water content in the extraction system might improve efficiency of phenolic extraction but it also increased concomitant extraction of other compounds and impurities [39]. A recent finding from Mohammedi and Atik not only supports Spigno's idea but they also found that water is only capable of extracting water-soluble compounds [40]. In this study, water was found to be the best solvent to extract phenolics from PKC. However, it might be cost-prohibitive from the industrialization point of view due to its difficulty to isolate phenolics from water and high cost of evaporation for water removal. Therefore, we should consider the purpose of extraction, polarity of solvent, interested and undesirable extractives, cost, safety as well as environmental concern when selecting an extraction solvent system. In this case, 80% aqueous ethanol could be used because its ability to extract considerable amount of phenolics from PKC. Besides, ethanol is considered as a green solvent which has low toxicity and being accepted for human consumption.

Effect of extraction duration on phenolic extraction

Extraction duration is another crucial parameter influencing phenolic extraction. There are a large number of published studies describing the effect of extraction duration on phenolic extraction from plant materials [35,36,38,39,41]. These studies suggested optimum duration of extracts could range from a few minutes to a few hours. Almost every study has related solubility to Fick's second law of diffusion which states that a final equilibrium between the solute concentrations in the solid matrix (plant matrix) and in the bulk solution (solvent) might be reached after a certain time.

doi: 10.7243/2052-6237-3-2

In our current study, a small increment (7.5%) was observed when increasing the extraction duration from 15 minutes to 60 minutes, whereas phenolic yield was slightly decreased when the extraction was prolonged to 120 minutes (**Figure 1b**). A possible explanation for the observed small increment might be well explained by Fick's second law of diffusion that phenolics started to be in equilibrium with solvent and this point, increasing extraction time was unable to extract more phenolics. Moreover, longer extraction durations increase the chances of oxidation of the phenolics due to light and oxygen exposure which explained the reason of decreased phenolic yield when prolonging extraction duration to 120 minutes. Therefore, phenolic extraction duration from plant materials should be carefully controlled in order to prevent oxidation of the phenolics.

Effect of extraction temperature on phenolic extraction

The effects of temperature on phenolic extraction from PKC are depicted in **Figure 1c**. The phenolic yield was slightly increased from 5.40±0.03 to 5.59±0.07 mg GAE/g of dry weight when extraction temperature increased from 25°C to 60°C. This result may be explained by the fact that higher temperatures promote diffusivity of solvent into plant matrix as well as higher solubility of phenolics into the solvent. However, some phenolics are more easily oxidised and hydrolysed at higher temperatures. Our study showed that 9.5% of decrease on phenolic yield when elevating temperature to 80°C. These results agree with the findings of other studies in which phenolic yield was increased proportionally with increasing extraction temperature while further increasing the temperature beyond certain values might denature phenolics [**35**,**36**,**38**,**39**,**41**,**42**].

Effect of extraction solvent on phenolic composition

Water, ethanol or water/ethanol mixture are the most widely used solvents because of their low toxicity and high extraction yield. In addition, the solvent mixture polarity can be modulated by using different ratios of water to ethanol. Therefore, water, ethanol and 80% aqueous ethanol were chosen for further analysis on the composition of phenolics using UPLC-ESI-MS/MS. The results are depicted in **Figure 2**. The phenolic content was the sum of individual phenolics identified in the extracts and was categorised into four groups which included p-hydroxybenzoic, protocetechuic, shikimic acids and others (caffeic, d-glucuronic, ferulic, glutaric, p-coumaric, syringic and vanillic acids). In accordance with our previous result using Folin-Ciocalteu assay (**Figure 1b**), this result demonstrated the extraction of phenolics increased with an increase of water content in the extraction solvent. As shown in **Figure 2**, by increasing water content in ethanol to 20%, the amount of phenolics was increased from 8.33 to 329.58 µg/g of dry weight. Further increase in the amount of phenolics to 579.23 µg/g of dry weight was observed when water was used as extraction solvent. Results also showed that p-hydroxybenzoic and shikimic acids were the two major compounds that contributed to this significant increment on phenolic extraction due to their higher solubility in water. In this analysis, our finding corroborates the ideas of Dent et al., who suggested that the amount of water in water/organic solvent mixtures has higher impact on the extraction of polyphenols than the solvent itself [**36**].

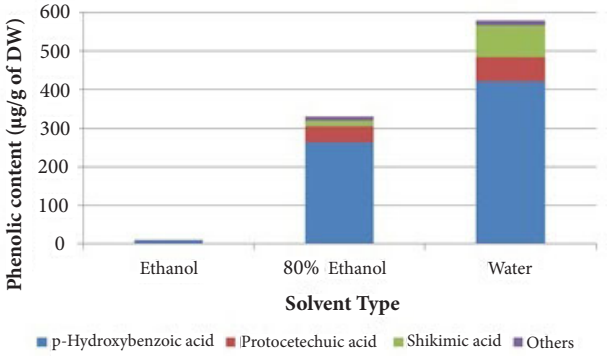

Figure 2. Effect of solvent type on phenolic composition. The phenolic content was the sum of individual phenolics identified in the extracts.

Conclusion

There was 2,516,664 tonnes of PKC generated from Malaysia oil palm industry in year 2013 and this amount is increasing annually [**44**]. Thereby, PKC could be a new potential source of phenolics and it represents an opportunity to transform an agro-industrial waste into a range of phenolic-containing products. This is the first study reporting phenolic content in PKC and suggested that 70.44% of the phenolics in PKC were in free methanol-soluble form whereas 29.56% was cell wall-bound phenolics. This study also demonstrated that pretreatment is not necessary for phenolic extraction from PKC. Water was shown to be a good solvent to extract the considerable amount of phenolics present in PKC. Longer extraction and higher temperature do not improve the extraction process; rather they appear to decrease the yield of phenolics. In summary, this study revealed an optimum phenolics extraction conditions for PKC: water, 25°C and 30 minutes at 1:10 material to water and stirring at 250rpm. Several limitations to this pilot study need to be acknowledged. Although water is suggested to be a good solvent for extracting PKC phenolics, it is not suitable for extracting less polar phenolics. Besides, it might be cost-prohibitive due to difficulties in isolating phenolics from water and high cost incurred for water removal. Therefore, it may be possible to further optimise the extraction process by using different combinations of solvents, sample to solvent volume ratio and coupling of extraction steps. Moreover, antioxidant activity was not addressed in this study and it should be assessed as compared to other available antioxidant benchmarks and polyphenol extracts.

doi: 10.7243/2052-6237-3-2

Competing interests
The authors declare that they have no competing interests.

Authors' contributions

Authors' contributions	SFK	JI	CKWO	KIN	NH	HMY
Research concept and design	✓	✓	--	--	--	--
Collection and/or assembly of data	✓	--	--	✓	✓	--
Data analysis and interpretation	✓	--	--	✓	--	--
Writing the article	✓	--	✓	--	--	--
Critical revision of the article	--	✓	✓	--	--	✓
Final approval of article	--	✓	--	--	--	✓
Statistical analysis	--	✓	--	--	--	--

Acknowledgement
The authors are grateful to the palm kernel crushing plant (KCP, Carey Island) for providing palm kernel cake. They would also like to thank their colleagues at Sime Darby Technology Centre for their supports, especially: Dr Teh Huey Fang for editorial advise.

Publication history
Editor: Ruanbao Zhou, South Dakota State University, USA.
Received: 02-Feb-2015 Final Revised: 26-May-2015
Accepted: 08-Jun-2015 Published: 16-Jun-2015

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Citation:
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