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Multistage Solvent Extraction for High Yield Oil and Phorbol Esters Removal from Thai Toxic *Jatropha curcas* Meal

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Abstract

In this study, we investigate the possibility for the production of high yield oil and phorbol esters removed from Thai toxic *Jatropha curcas* meal. Optimum oil recovery by hexane extraction to obtain high oil yield was accomplished in 3 stages of batch extraction, following which the de-oiled meal was further determined for the optimum conditions for removal of phorbol esters (PEs) by aqueous ethanol extraction from the first to the third stage of batch extraction with the aim of yielding detoxified de-oiled meal product for use as a raw material in animal feed. The optimum conditions for oil extraction was 3-stage extraction with each stage operated at 1:3 (w/v) of toxic meal to hexane at 40 °C for 30 min. This condition gave 100 % de-oiling efficiency compared with the Soxhlet extraction method. The optimum condition for PEs removal from the de-oiled meal involved 2-stage extraction with each stage operated at 1:3 (w/v) of de-oiled meal to aqueous ethanol at 50 °C for 30 min. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) in the multiple reaction monitoring mode, was used to confirm the PEs residue in the detoxified de-oiled meal. The confirmation showed that the 2 stages of PEs extraction could remove 100 % of the PEs from the de-oiled meal. The results from our study can provide the basis for the efficient commercial production of both *J. curcas* oil and detoxified de-oiled meal.

Keywords: *Jatropha curcas* oil, phorbol esters, toxic meal, animal feed, removal

Introduction

J. curcas is a drought-resistant plant which belongs to the family Euphorbiaceae. It has been widely cultivated in Central and South America, Southeast Asia, India and Africa [1]. The increasing interest in biofuel has been an incentive to study the potential of *J. curcas* oil as a raw material for biodiesel production. The ability of *J. curcas* to produce high oil yields and grow on marginal land, combined with opportunities for rural development in developing nations, have led to enormous interest and an increase in the planned production area of this crop [2]. With biodiesel as the main product from *J. curcas* seeds, the main by-product after oil recovery by mechanical pressing or solvent extraction is de-oiled press cake or de-oiled meal. This meal is rich in protein, but it cannot be used as food or feed since it contains toxic or anti-nutritional components such as PEs, trypsin inhibitors, lectin and phytate. Additional value might be obtained from de-oiled meal if it can be applied as a protein source for animal feeds [3].

PEs are naturally occurring compounds which are widely distributed in Euphorbiaceae plant species. They are tetracyclic diterpenoids and esters of tigliane diterpenes. The structures of 6 phorbol esters have been determined using NMR spectroscopy [4]. The main PEs structure in *J. curcas* is shown in **Figure 1**.

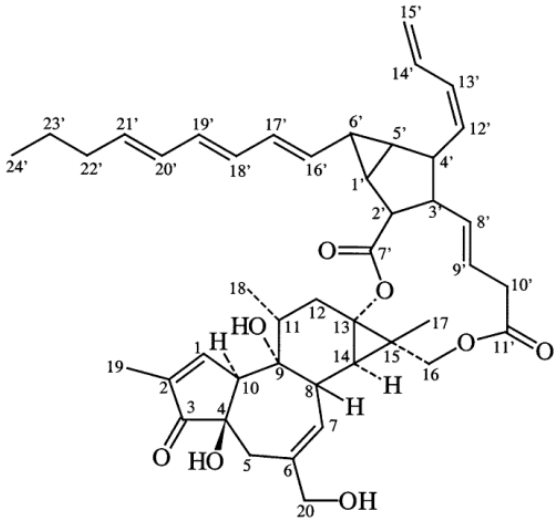

Figure 1 Chemical structure of the main phorbol esters (PEs).

PEs have been found to be responsible for skin irritation and the promotion of tumors since they activate protein kinase C (PKC) [5,6]. The oil and the isolated toxic PEs fractions showed haemolytic activity, disrupting red blood cells in rabbits and caused a severe irritant reaction followed by necrosis in mice [7]. PEs are the major impediment to the wide commercial use of *J. curcas* meal. Various methods such as water leaching, alcohol leaching, autoclaving, acid and alkali treatments have been adopted to remove or destroy PEs from de-oiled meal to make it safe for consumption [8]. The use of 80 - 90 % aqueous ethanol or methanol holds promise for detoxification of meal and the detoxified meal could be used to feed animals [9]. The determination of PEs content is usually done by high performance liquid chromatography with an ultraviolet detector (HPLC-UV) according to the method of Hass and Mittelbach [10]. The PEs confirmation in *J. curcas* oil by ESI-MS/MS showed its parent ion had a molecular mass of 711 and its daughter ions had a molecular mass of 693, 383, 311 and 293, respectively [11]. Vogg *et al*. [12] developed a liquid chromatography-tandem mass spectrometry (LC-MS/MS) technique to detect tumor-promoting diterpene esters of tigliane within plant extracts. The results showed a characteristic precursor ion with a molecular mass of 311 with one of its fragmentation products at a molecular mass of 293. In addition, Punsuvon *et al*. [13] used LC-MS/MS in the multiple reactions monitoring mode for PEs residue confirmation in sweet potatoes grown using *J. curcas* seed cake as a fertilizer.

PEs are natural occurring hydrophobic structures. During mechanical or solvent extraction, the majority of PEs present in the seeds dissolve in the oil fraction [14]. Thus, oil bears more PEs than cake or meal. If most of the oil is extracted from the meal, the de-oiled meal will contain a lower amount of oil and a lower concentration of PEs. This hypothesis is supported by the work of Makkar *et al*. [15] who found that completely de-oiled kernel seed meal (free of shell) contained a PEs content ranging from 0.5 to 1.0 mg/g. Solvent extraction is commonly used for 95 - 98 % of oil recovery from *J. curcas* seeds, where a non-polar solvent such as hexane is used as the standard solvent for the process [16]. In addition, the low concentration of PEs residue in the de-oiled meal can be easily detoxified by leaching with aqueous ethanol as mentioned above. Consequently, solvent extraction and aqueous ethanol leaching methods were selected for our study.

The objective of this research was to evaluate the optimum conditions for obtaining a high yield of toxic oil recovery from toxic meal by multistage solvent extraction and PEs removal from toxic de-oiled meal by multistage aqueous ethanol leaching for the simultaneous production of both products. This process will enhance the value and sustainability of *J. curcas* as a renewable energy source for the biofuel industry. Additionally, liquid chromatography-tandem mass spectrometry (LC-MS/MS) in the multiple

reactions monitoring (MRM) mode was studied to confirm PEs residues in de-oiled meal before and after the detoxification process were sufficiently low for the meal to be safe for use as animal feed.

Materials and methods

Raw material and chemicals

Thirty kgs of Thai *J. curcas* seeds were obtained from Green Energy Group Co., Ltd (Thailand). The seeds were manually deshelled to obtain white kernels. The kernels were further ground with a household grinder to make ground kernel (40 mesh). The ground kernel in this study is referred to as toxic meal. Phorbol-12-myristate-13 acetate (TPA) was obtained from Sigma Chemical Co., Ltd. (St. Louis, MO). All other chemical solvents used were of analytical grade.

Oil extraction from toxic meal

Toxic meal (100 g) was extracted with hexane at a varied ratio of meal to solvent (w/v) from 1:1 to 1:5. The meal (solid) and hexane (solvent) were mixed by magnetic stirring under reflux for 60 min at 40 °C. The hexane layer was then separated off by filtration and evaporated using a rotary vacuum evaporator. The extraction was also carried out at different temperatures (30, 40, 50, 60 and 70 °C) and multistage extraction used in the second and third stage until no further increase in the weight of the extracted oil was detectable. The multistage extraction is shown in **Figure 2**. After every experiment, the amount of extracted oil was weighed and the percentage of de-oiling efficiency was determined using Eq. (1).

$$\text{De} - \text{oiling efficiency } (\%) = \frac{W_a}{W_b} \times 100 \quad (1)$$

where W_a is the weight of the oil extracted using batch extraction, and W_b is the weight of oil extracted using conventional soxhlet extraction with hexane for 6 h.

Phorbol esters extraction from toxic de-oiled meal

Toxic de-oiled meal (50 g) was extracted with aqueous ethanol at 1:3 (w/v) ratio of de-oiled meal to solvent. The de-oiled meal and aqueous ethanol were mixed by magnetic stirring under reflux at different times (15, 30, 45 and 60 min) at 50 °C. The ethanol layer was then separated off by filtration. The extraction was further carried out at different temperatures (40, 50, 60 and 70 °C) and multistage extraction used in the second and third stage until no further decrease in the concentration of PEs was detectable. The multistage extraction is shown in **Figure 3**. The percentage of PEs removed was determined using Eq. (2).

$$\text{PEs removal } (\%) = \frac{C_a}{C_b} \times 100 \quad (2)$$

where C_a is the concentration of PEs in detoxified de-oiled meal and C_b was the concentration of PEs in toxic de-oiled meal.

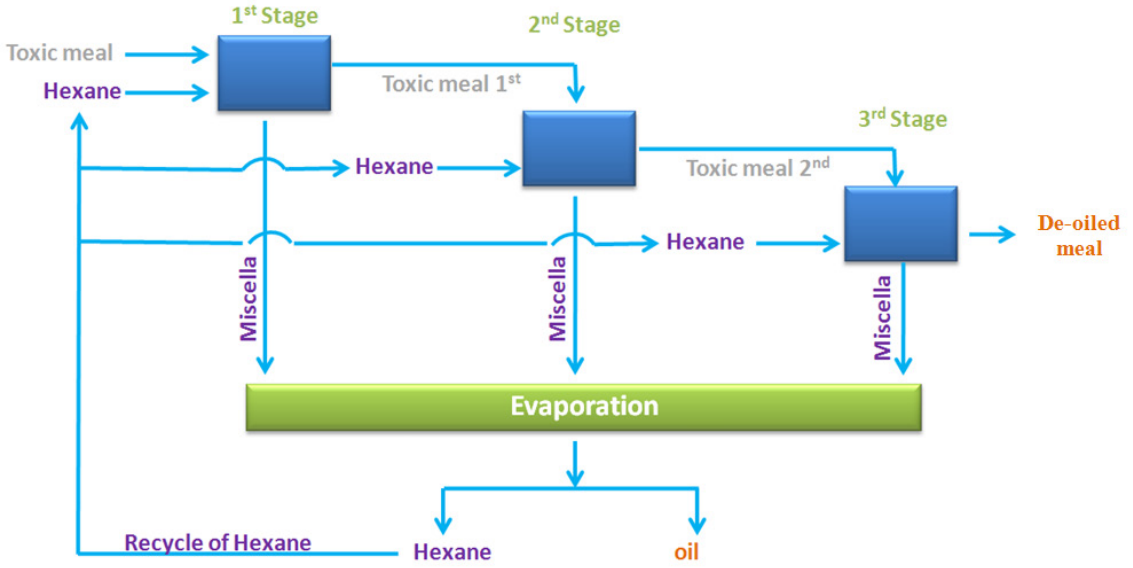

Figure 2 Schematic procedure of the oil extraction.

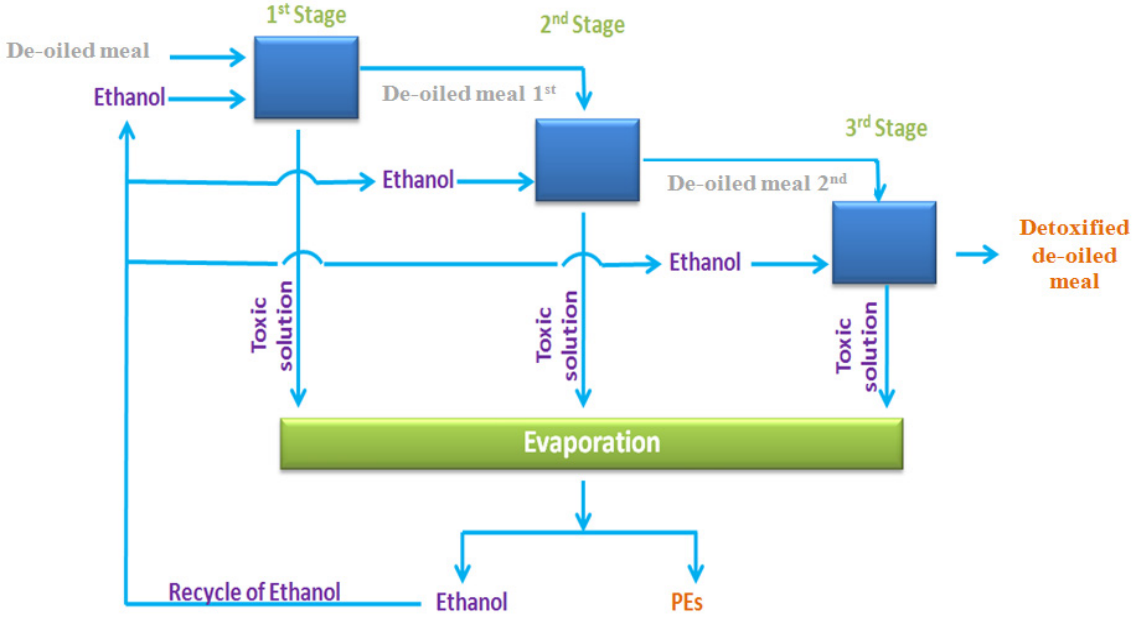

Figure 3 Schematic procedure of the PEs extraction.

Determination of PEs content by high performance liquid chromatograph

PEs were extracted from 10 g of each sample (toxic meal and de-oiled meal, detoxified de-oiled meal) after inactivation of trypsin inhibitor by heat treatment. Two hundred milliliters of methanol was used as a solvent for soxhlet extraction for 4 h. After extraction, methanol was further evaporated on a vacuum rotary evaporator until 10 ml of solution was obtained. A portion of the solution was used to determine the PEs concentration by HPLC with an ultraviolet detector (HPLC-UV: KNAUER, Smartline, Germany). The PEs concentration was determined according to Punsuvon *et al*. [17]. An aliquot was loaded onto a HPLC-UV reverse phase C_{18} Lichrophere 100, 5 μm (250×4 mm. ID. from Merck, Darmstadt, Germany) column. The column was protected with a head column containing the same material. The separation was performed at room temperature and the flow rate was 1 ml/min using an isocratic elution of 1:4 (v/v) deionized water mixed with acetonitrile as the mobile phase. A group of PEs peaks were detected at 280 nm and appeared between 8 - 12 min in the chromatogram. The results were expressed as equivalent to an external standard (phorbol-12-myristate-13-acetate).

Characterization of oil

The physicochemical properties (acid value, saponification value, iodine value, density at 15 °C and viscosity at 40 °C) of the oil were determined by using ASTM methods and the AOCS official method (AOCS cd 3a-64, ASTM D1959-67, AOCS 1c-85, ASTM D941, ASTM D445).

Confirmation of PEs residue in detoxified de-oiled meal by LC-MS/MS

One portion of solution from the soxhlet extraction of de-oiled and detoxified de-oiled meal were analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS, Agilent, USA) for PEs residue confirmation. The conditions used were: confirmation of PEs, LC conditions: chromatographic separation of PEs was performed on a C_{18} water Atlantis (5 μm, 2.7×50 mm). The Isocratic program was used with a mobile phase, consisting of solvent (50 mm. ammonium acetate + acetonitrile, 9:1 by vol.). The flow rate was 0.2 ml/min, 40 μl of injection value. MS/MS condition: the MS/MS was performed on a Micromass Qualttro Ultima triple-quadrupole spectrometer equipped with an ESI source. The parameters used for the mass spectrometry under ESI^+ mode were as follows; capillary voltage 3.00 kV, cone voltage 50 V, source block temperature 120 °C, cone gas 53 l/h, desolvation temperature 350 °C, desolvation gas 593 l/h.

Data analysis

All of the foregoing tests were conducted in duplicate. Variations among the runs were negligible, and the average of the values for each test is reported.

Results and discussion

The oil content of the kernel meal from soxhlet extraction was 52.5 %. It was within the range of oil content of *J. curcas* seed kernel 40 - 60 % which was reported by Makkar *et al*. [18]. The percentage of de-oiling efficiency for various times in single stage extraction of toxic meal into hexane (1:3, w/v), temperature (40 °C), and stirring rate (1,000 rpm) is presented in **Figure 4**.

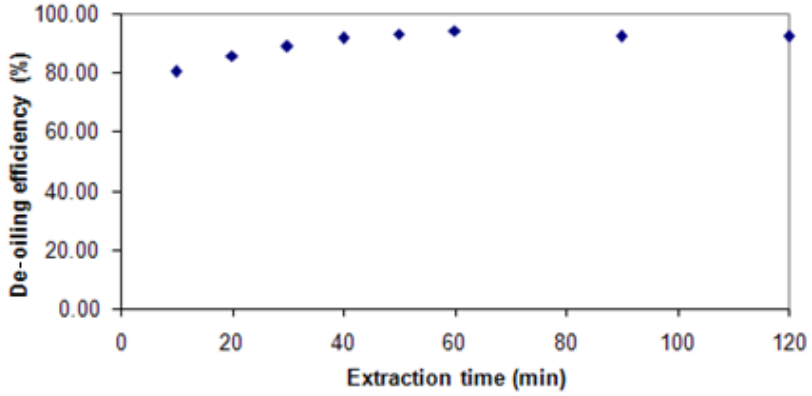

Figure 4 De-oiling efficiency of single-stage extraction at various times.

From **Figure 4**, the percentage of de-oiling efficiency rapidly increased at the first 10 min, slightly increased from 10 to 60 min and then remained constant till 120 min of extraction. The extraction result showed that increasing the extraction time over 60 min did not improve the de-oiling efficiency. So, the optimum extraction time for single-stage extraction was 60 min. At 60 min of extraction time, the de-oiling efficiency was 94 %. The influence of toxic meal to hexane ratios by varying the ratio from 1:1 to 1:5 at 40 °C, and 1,000 rpm stirring rate for 60 min is shown in **Figure 5**.

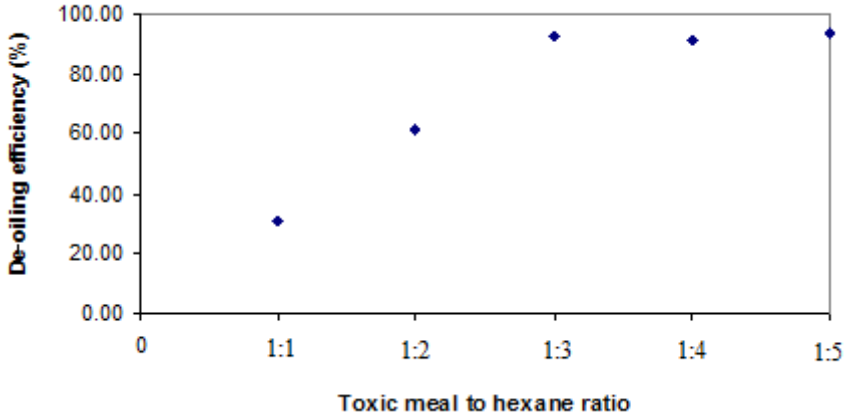

Figure 5 De-oiling efficiency of single-stage extraction at various toxic meal to hexane ratios.

From the result, de-oiling efficiency increased with increasing toxic meal to hexane ratio until the ratio reached around 1:3, after which it was constant. The results show that increasing toxic meal to hexane ratio after 1:3 ratio does not affect the percentage of de-oiling efficiency and this ratio was selected as an optimum condition. At a ratio of 1:3, de-oiling efficiency for single-stage extraction reached around 94 %. Multistage extraction tests using hexane were also conducted for increasing de-oiling efficiency using 60 min of extraction time. As described in the experimental procedures, the solvent used for the second and the third-stage were the same amount as that used for the first-stage extraction. The ratio of toxic meal to hexane was 1:3 at 40 °C with a 1,000 rpm of stirring rate for 20 min used at each stage of extraction. The cumulative de-oiling efficiency is shown in **Figure 6**.

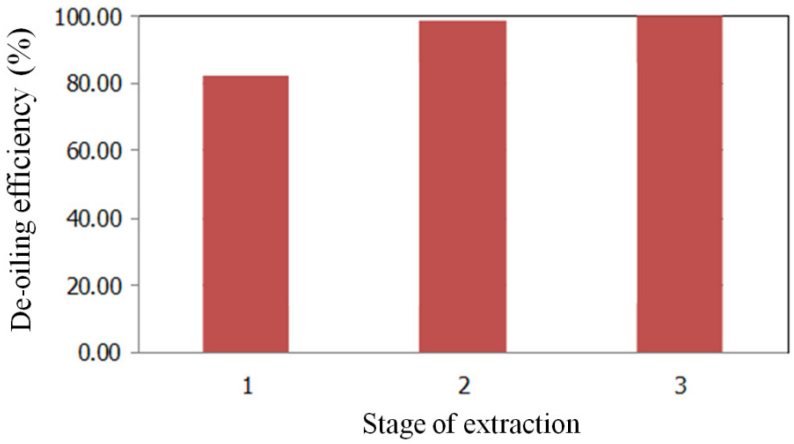

Figure 6 De-oiling efficiency of the multistage extraction using hexane; the 3 bars represent the single-stage, 2-stage and 3-stage extraction.

The de-oiling efficiency is the sum of efficiencies of the current stage plus the former stages. For instance, the de-oiling efficiency for the third stage was the total efficiency from all 3 stages. Given a target efficiency of nearly 100 %, it can be seen that if 3-stage extraction was adopted, the first-stage efficiency was 82.32 %. If 2-stage extraction was used, the de-oiling efficiency was 98.62 %; however, 3-stage extraction provided 100 % efficiency. The results indicate that multistage extraction, with increasing hexane to toxic meal ratio, had the greatest influence on oil extraction. The 3-stage of batch extraction can extract oil from toxic meal as efficiently as with a soxhlet extractor but in shorter time (60 min) and higher hexane to toxic meal ratio (9:1). A 3-stage extraction will likely have higher costs in capital investment, operation, and solvent recovery. However, the cost may be offset by the high yield oil and low content of PEs in the obtained de-oiled meal, if the de-oiled meal is to be detoxified for animal feed.

Quality of extracted oil product

The physicochemical properties of the oil are given in **Table 1**. The oil extract was golden yellow and exhibited good physicochemical properties for use as a biodiesel feedstock in terms of saponification value, iodine value, density and viscosity when compared with the works of Albar *et al*. [19] and Adebowate *et al*. [20]. The acid value was high (8.13 mg KOH/g) because *J. curcas* seeds were kept for a long time before study. The PEs content was 3.07 mg/g and in between the range 2 - 8 mg/g from the work of Devappa [14] who studied PEs concentration in the oil from different genotype. The reason for the PEs dissolving well in oil is their hydrophobic structure. In addition, precautions must be taken when handling the oil to avoid skin contact due to the presence of toxic PEs in the oil.

Table 1 Physiochemical properties of the *J. curcas* oil from extraction

Properties	Acid value (mg KOH/g[1])	Saponification value (mg KOH/g[1])	Iodine value (g I_2/100 g[1])	Density at 15 °C (g/cm^3)	Viscosity at 40 °C (cSt)	PEs* Content (mg/g[2])
Experimental value	8.13	180.74	92.07	0.90	31.67	3.07

*PEs extraction from toxic de-oiled meal
[1]means gram of Oil
[2]means gram of *J.* meal

The de-oiled meal was further studied for PEs removing in a single-stage extraction at various times. The conditions for extraction were a 1:3 (w/v) ratio of de-oiled meal to aqueous ethanol at 50 °C and 1,000 rpm of stirring rate. The result is shown in **Figure 7**.

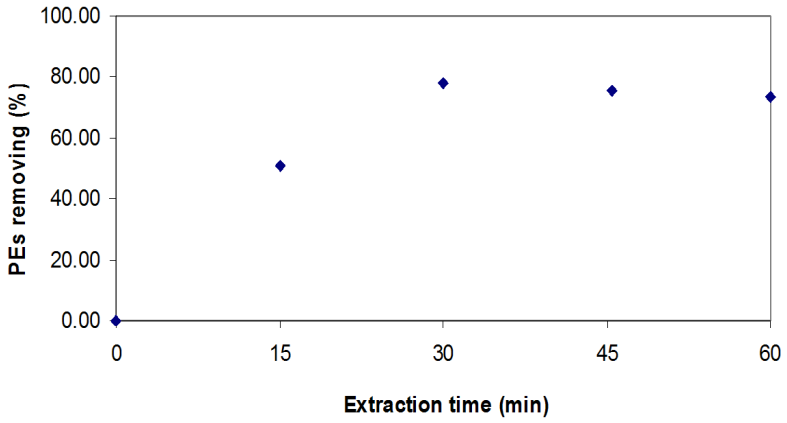

Figure 7 %PEs removed in a single-stage extraction using aqueous ethanol.

The percentage of PEs removed rapidly increased with increasing time to 30 min, after that the % PEs removed slightly decreased from 30 to 60 min of extraction. The % PEs removed showed that increasing the extraction time over 30 min did not result in improvement. So, 30 min of extraction was selected by single-stage extraction. At 30 min, the % PEs removed with single-stage extraction was 77.72 %. Given a target PEs removal of nearby 100 % multistage extraction tests using aqueous ethanol were also conducted. The amount of aqueous ethanol used for the second and the third stages were the same as that used for the first-stage extraction. The ratio of de-oiled meal to aqueous ethanol was 1:3 (w/v), 50 °C, 1,000 rpm stirring rate and 30 min of time for each stage of extraction. The cumulative % PEs removed is shown in **Figure 8**.

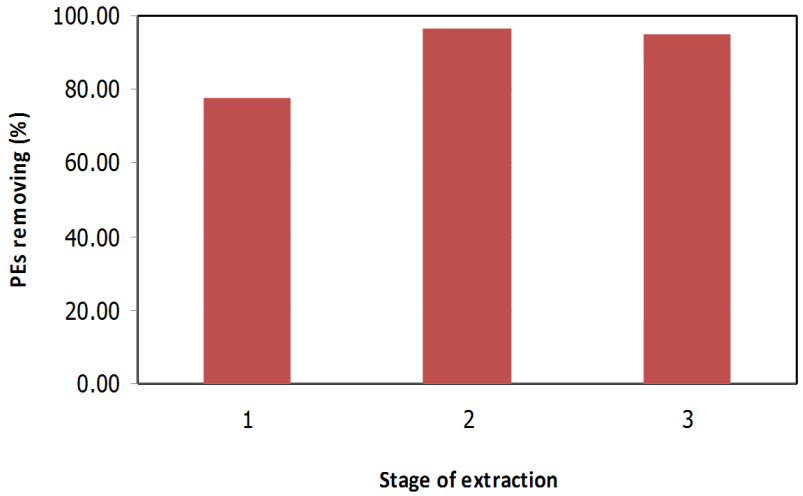

Figure 8 % PEs removed in a multistage extraction using aqueous ethanol; the 3 bars represent the single-stage, 2-stage and 3-stage extraction.

The de-oiling efficiency is the sum of the efficiencies for the current stage plus any former stages. The single stage removed 77.72 % of PEs. If 2-stage and 3-stage extraction were used, 96.52 and 95.15 %, respectively, of the PEs were removed. The results indicated that multistage extraction with an increasing aqueous ethanol to de-oiled meal ratio and extraction time, had the greatest influence on the removal of PEs. The 2 stages of batch extraction extracted nearly 100 % of the PEs and so the 2-stage option was selected as the optimum number of stages. Our results on the removal of PEs were better than those of Xiao *et al*. [7], who detoxified toxic meal by hydrolysis with an enzyme (cellulase + pectinase) for 1 h followed by washing the residue meal with 65 % ethanol for 1 h, in terms of time and the enzyme cost. It can be concluded that this 2-stage extraction of PEs is a promising way to detoxify toxic meal.

Quality of de-oiled and detoxified de-oiled meal products

The 2 samples of de-oiled and detoxified de-oiled meal were analyzed by HPLC-UV. The results of the PEs chromatogram are presented in **Figure 9** and show 4 PEs peaks (**Figure 9a**) and a group of small peaks (**Figure 9b**) at a retention time of between 8 and 12 min for both chromatograms. The PEs contents were calculated by comparison with the TPA standard. The PEs residue concentrations were 0.6555 and 0.0228 mg/g in the de-oiled meal and detoxified de-oiled meal, respectively. The concentration in the detoxified de-oiled meal was less than in the nontoxic Mexican variety (0.11 mg/g) which is recommended as safe for animal feed [8].

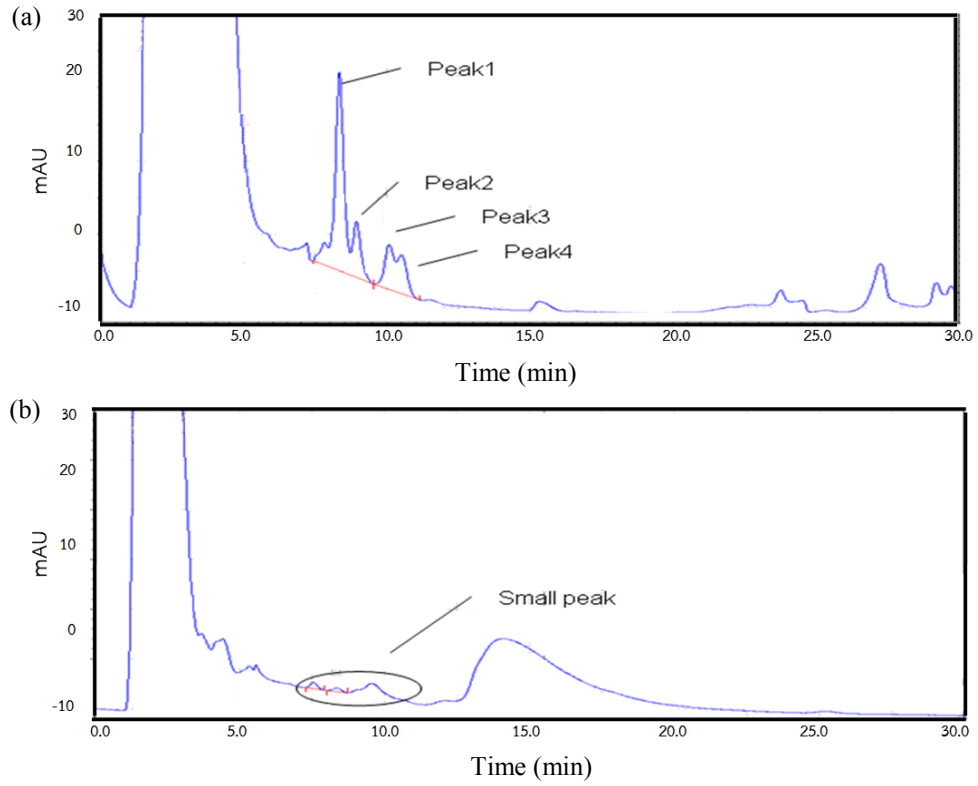

Figure 9 Chromatogram of PEs in (a) de-oiled meal and (b) detoxified de-oiled meal.

From **Figure 9a**, 4 peaks seen at a retention time between 8 and 12 min are the PEs residue peaks for de-oiled meal, but the group of small peaks at the same retention time for the de-oiled meal (**Figure 9b**) are less clear and it was not certain that they represented PEs residue peaks, so we had to seek confirmation using LC-MS/MS with a multiple reaction monitoring technique. In our previous work [13], we developed a technique to confirm PEs residues by using LC-MS/MS with multiple reaction monitoring (MRM) indicating that the ionization of parent molecule PEs had a mass of 711 as a precursor and product ions with masses of 311 and 293, respectively. The results of our work indicated that the PEs were fragmented by eliminating their esters groups (C_{13} and C_{16} in **Figure 1**) and the alcohol group (C_{20} in **Figure 1**) to a tigliane type structure (molecular formula = $C_{20}H_{23}O_{23}$) resulting in a precursor molecule with a molecular mass of 311. After this, the skeleton was further fragmented by losing H_2O (molecular mass of H_2O = 18) to produce an ion with a molecular mass of 293.

The result of fragmentation in both chromatograms revealed one peak of ionization at the same time (2.46 min), where one ionization peak represented the parent molecule with a mass of 711 ionized to a precursor ion with a mass of 311 and another ionization peak ionized from the precursor ion produced an ion with a mass of 293. Thus, this characteristic pattern indicated PEs residues in the de-oiled meal. The chromatogram of the ionization peaks of PEs in the de-oiled meal is shown in **Figure 10**. For PEs confirmation of the detoxified de-oiled meal, both chromatograms showed no ionization peak at the corresponding time (2.47 min) as shown in both ionization peaks of the PEs in the de-oiled meal. This suggested that the detoxified de-oiled meal did not contain toxic PEs and that the 2 stages of aqueous ethanol extraction had been effective in removing 100 % of the PEs, although the PEs concentration removed as calculated from the HPLC-UV procedure was not quite 100 % (96.52 %).

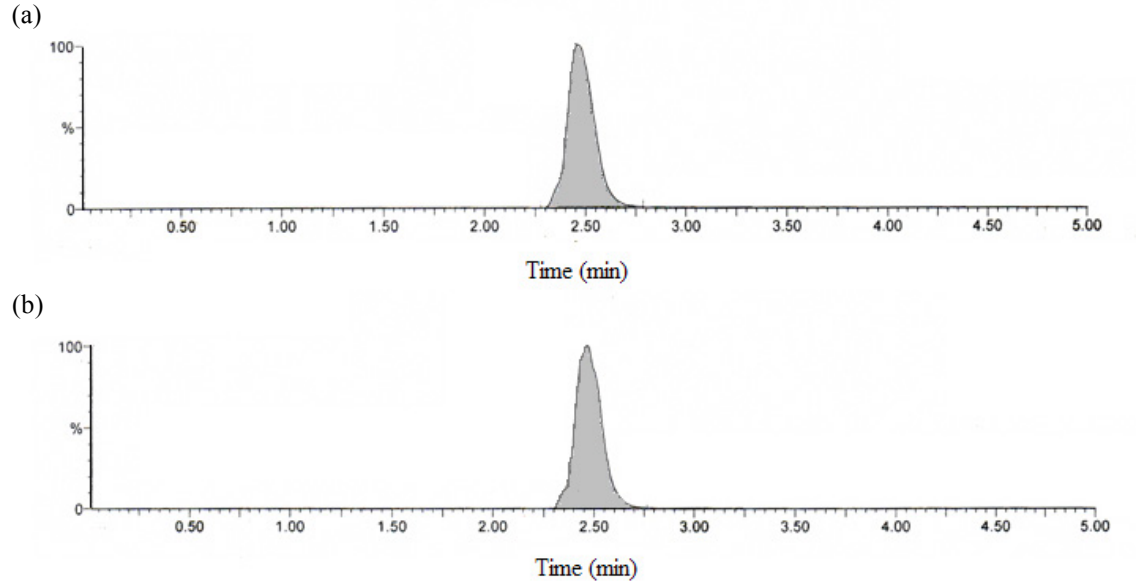

Figure 10 Chromatogram of ionization peaks of PEs (a) ionize from 711 to 311 (b) ionize from 311 to 293 obtained from LC-MS/MS.

Conclusions

The study showed that it is possible to produce high yields of oil and detoxifed de-oiled meal in one factory. On the basis of the results of this study, it can be concluded that the 3-stage batch extraction of toxic meal using hexane resulted in 100 % de-oiling efficiency when compared with Soxhlet extraction. The detoxified de-oiled meal was obtained after 2 stages of batch extraction by aqueous ethanol which removed 100 % of the PEs content. The PEs left in the detoxified de-oiled meal were confirmed by LC-MS/MS in a multiple reaction monitoring mode and indicated that the detoxified de-oiled meal was not contaminated with toxic PEs compounds and that the meal was safe for animal feed.

Acknowledgements

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