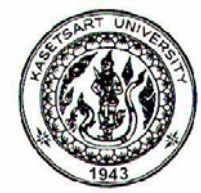

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THESIS

ELIMINATION OF PHORBOL ESTERS IN SEED OIL AND PRESS CAKE OF *JATROPHA CURCAS* L.

RAYAKORN NOKKAEW

A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
Master of Science (Chemistry)
Graduate School, Kasetsart University
2008

Rayakorn Nokkaew 2008: Elimination of Phorbol Esters in Seed Oil and Press Cake of *Jatropha Curcas* L.. Master of Science (Chemistry), Major Field: Chemistry, Department of Chemistry. Thesis Advisor: Associate Professor Vittaya Punsuvon, Ph.D. 158 pages.

Phorbol esters are the main toxic in seed oil and press cake of *Jatropha curcas* L.. The biological effects include tumor promotion and inflammation. However, *J. curcas* is an alternative plant whose the seed oil can be used for biodiesel or edible oil and the press cake is used for animal feed and fertilizer after phorbol esters are eliminated. The objective of this experiment is to investigate the optimum condition of the most suitable adsorbent including adsorption time, amount of adsorbent, temperature and stirring rate. The objective also is to investigate the optimum condition for removal of phorbol esters from press cake including ratio of press cake to water, stirring time, concentration of base solution, temperature, stirring rate and followed with soaking for one night in 95% ethanol. The results show that the optimum adsorption condition from *J.* seed oil is 3.2% (w/v) bentonite 200, 15 min adsorption time at room temperature with 100 rpm stirring rate. The phorbol esters can be removed up to 98% after one-time adsorption and 99% after two-time adsorption. In addition, Freundlich and Langmuir isotherms are fit well for adsorption of phorbol esters from *J.* seed oil by bentonite 200. The results also show that the press cake is better washed with potassium hydroxide solution than sodium hydroxide solution. The optimum washing condition is 3% (w/w) potassium hydroxide solution, 1:5 ratio of press cake to water, 45 min stirring time at room temperature with 200 rpm stirring rate, followed by soaking of press cake for one night in 95% ethanol. This condition can remove phorbol esters from *J.* press cake down to the concentration level which is suitable for an animal feed.

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Student's signature Thesis Advisor's signature

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LIST OF ABBREVIATIONS

BET	=	Brunauer-Emmett-Teller
BT200	=	bentonite200
cSt	=	centistokes
DHPB	=	12-Deoxy-16-hydroxyphorbol-4'-[12', 14'-butadienyl]-6'-[16', 18', 20'-nonatrienyl]-bicyclo[3.1.0]hexane-(13-*0*)-2'-[carboxylate]-(16-*0*)-3'-[8'-butenoic-10']ate
DMSO	=	dimethyl sulfoxide
ESI	=	electrospray negative
KAPI	=	Kasetsart Agricultural and Agro-Industrial Product Improvement Institute
FA	=	fatty acid
FAME	=	fatty acid methyl esters
FAO	=	Food and Agriculture Organization
FT-IR	=	fourier transform infrared spectrometer
GC	=	gas chromatography
HPLC	=	high performance liquid chromatogram
IS	=	internal standard
J. curcas L.	=	*Jatropha curcas* Linn.
LC-MS	=	liquid chromatography and mass spectroscopy
LD_{50}	=	lethal dose 50
mg	=	milligram
nm	=	nanometer
PDA	=	photo diode array spectrophotometers
PEs	=	phorbol esters
RT	=	retention time
TLC	=	thin layer chromatography
TISTR	=	Thailand Institute of Scientific and Technological Research
TPA	=	phorbol-12-myristate 13-acetate or 12-*O*-tetradecanoylphorbol-13- acetate

ELIMINATION OF PHORBOL ESTERS IN SEED OIL AND PRESS CAKE OF *JATROPHA CURCAS* L.

INTRODUCTION

Nowadays, the demand and supply gap of vegetable oil has been widening all over the world because of the oil price is increased. Globally, the usage of friendly environmentally fuels is encouraged. The energy extracted from biomass and tree based materials are perhaps the oldest source of renewable energy. Biomass can be generated from various sources, such as edible and non-edible seed oils, algae and bacteria, forest residues, waste from food and processing, kitchen wastes, etc. The most important biofuels generated from biomass are biodiesel and bioethanol.

Thailand is not rich in petroleum reserves and crude oil, petroleum products must be imported to meet growing energy needs. These fuel and products are usually high prices. The seeking alternative energy is urgently needed for biodiesel production. Plant species which can be processed to provide a diesel fuel substitute have captured the interest of Thai scientists. Most of these plant species are such as palm, coconut, soy bean, sunflower, *Jatropha curcas* L. (Saboodum), etc. Ministry of Thai Energy has a policy on renewable energy strategy in the year 2004 that the use of renewable energy in Thailand will increase about 8% of the total energy or 6,540,000 tons within the year 2011 which biodiesel is the one purpose of renewable energy.

Thai government has a policy to support *J. curcas* L. plantation for farmers mainly for renewable energy. *J. curcas* L. is a drought-resistant shrub. It is a member of Euphobiaceae family which is cultivated in Central and South America, South-east Asia, India and Africa. This plant came to Thailand about 200 years ago by Portuguese. Seed oil of *J. curcas* L. is used for soap making and lighting for lamps. The plants grow quickly, survive in poor stony soil and resist to drought. The height of the plant is 2-7 meters and the lifetime is about 50 years. In Thailand the name Saboodam is usually used for *J. curcas* L. The plant can be used in many ways, such

as to prevent erosion, reclaim land, grown as a live fence, etc. The seed kernels contain 40-60% oil (Makkar *et al*., 1997) in which its fatty acid composition is similar to the oil used for human nutrition (Gübitz *et al*. 1998). A total of 19-27% crude protein can be obtained from press cake (Makkar *et al*., 1997) which can be a protein source for animal feed. The kernels also contain a number of several toxic and antinutritional compounds. These compounds are trypsin inhibitors, lectins, saponins, phytate and phorbol esters which might cause or at least aggravate the adverse effects in the long term contact, except phorbol esters affect on the short term contact (Makkar *et al*., 1997).

Phorbol esters are toxic substances that found in plant species of Euphobiaceae and Thymelaceae families. Their structures are based on tetracyclic carbon skeleton known as tigliane. They are known to cause a wide range of biological effects including tumor promotion, cell proliferation, activation of blood plateles and inflammation (Aitken, 1986). These effects are closely related to the structure of several compounds. Therefore, detoxification of these phorbol esters from the seed oil is required, even when it is industrially used because of the possibility to direct contact of persons with the seed oil, including detoxification from the press cake before utilization as animal feed. Many experiments eliminate phorbol esters in seed oil by the extraction with ethanol (Gross *et al*., 1997). This experiment is difficult to apply for industrial scale because of the immense solvent consumption. Experiment on traditional oil refining process that examines the effects on the phorbol esters content from *J. curcas* oil was performed by Hass (Hass, 2000). It showe that deacidification step and bleaching step could reduce the content of phorbol esters up to 55%. In addition, phorbol esters are heat stable and can withstand roasting temperature as high as 160°C for 30 min (Makkar and Becker, 1997). Aregheore *et al*. (2003) experimented detoxification of phorbol esters in press cake using heat and chemical treatments. The results show that phorbol esters are left in the amount of 0.09 mg/g when *J. curcas* press cake is treated by washing with 92% methanol for four times. Another experiment is done using the mixture of sodium hydroxide and sodium hypochlorite for washing and then followed by washing with distilled water for four times or washing with 92% methanol for two times. After that, followed by

heating at 121°C for 30 min. The results show that phorbol esters could completely be removed from press cake.

In this experiment, the adsorption process of phorbol esters from *J.* seed oil and press cake is examined. In seed oil, bleaching steps in refining of edible oil process can be replaced by the adsorption process. The chemical treatment is employed in detoxification of press cake of *J. curcas* L.. The experiment uses potassium hydroxide solution in comparison with sodium hydroxide solution because the waste from NaOH washing can increase the salt in environment and result in problem for agriculture. In the opposite way, waste from KOH washing can be used as fertilizer for plants. The experiment is expected to eliminate phorbol esters from seed oil and press cake to the level that is safe for human and animals.

The main objects of this experiment are:

1. To study the optimum condition for the elimination of phorbol esters in *Jatropha* seed oil by the most suitable adsorbent.

2. To study the adsorption of *Jatropha* seed oil by Freundlich and Langmuir isotherm models.

3. To study the optimum condition for the elimination of phorbol esters in *Jatropha* press cake by the most suitable base solution.

LITERATURE REVIEW

1. *Jatropha Curcas* Linn.

1.1 Botanical description

Jatropha curcas L., as known as 'physic nut, purging nut, big purging nut, American purging nut, black vomit nut, saboodum, etc.', is a member of the Euphobiaceae familily. It is a tropical plant which can reach a height of 2-7 meters. It is cultivated mainly as a hedge in many Latin America, Asia and African countries. It can be grown in low and high rainfall areas either in the farms as a commercial crop or on the boundaries as a hedge to protect fields from grazing animals and to prevent erosion.

1.2 Utilization of various parts of *Jatropha curcas* L.

All parts of *J. curcas* L. have been used in traditional medicine and for various purposes. The oil has been used as a purgative, to treat skin diseases and to soothe pain such as rheumatism. Decoction of the leaves has been used against coughs or as antiseptics after birth, and the branches as chewing sticks (Heller, 1996). Various extracts from *Jatropha* seeds and leaves show molluscicidal, insecticidal and fungicidal properties (Nwosu and Okafor, 1995; Liu *et al*., 1997; Solsoloy *et al*., 1997). The utilization of various parts of *J. curcas* L. is reviewed in Figure 1 (Gübitz *et al*., 1999).

Jatropha curcas L.

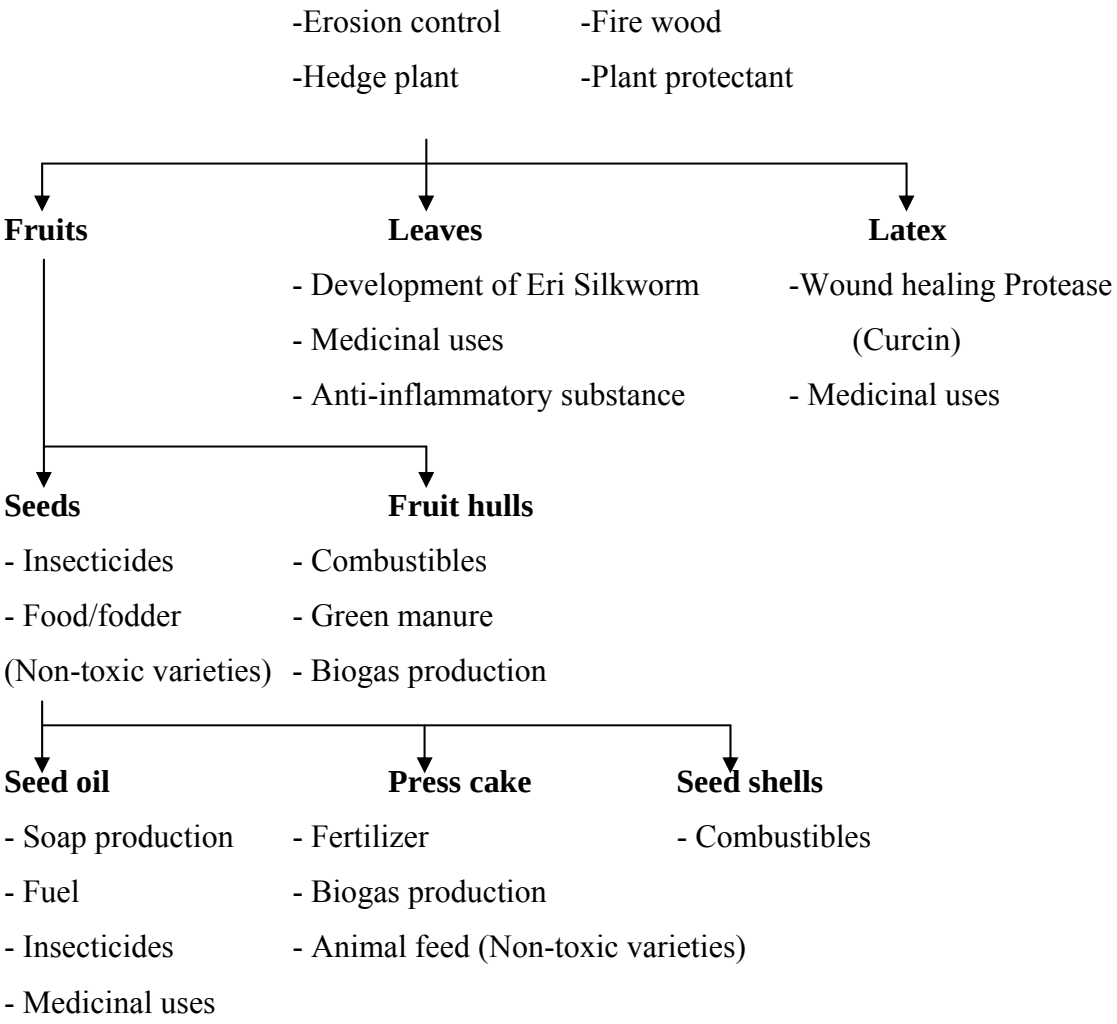


Figure 1 Exploitation of *Jatropha curcas* L.
Source: Gübitz *et al*. (1999)

1.3 Chemical and physical properties of *Jatropha curcas* L.

The seed kernels, which seem to be the part of the plant with the highest potential for utilization, contain 40-60% oil (Makkar *et al*., 1997) with a fatty acid composition similar to oils used for human nutrition (Gübitz *et al*., 1999). A total of 19-27% crude protein found in press cake (Makkar *et al*., 1997) could be used as a crude protein source with a content of essential amino acids even higher (except

lysine) than the Food and Agriculture Organization (FAO) reference protein. The physical properties of *Jatropha* seed oil are 0.915 of specific gravity, 34.55 cSt of viscosity at 40°C, 1.0-2.0% of free fatty acid, 0.99 mg KOH/g oil of acid value (Pisamay and Lalita , 2006; Pijit *et al*., 1987).

1.4 Toxicology of the *Jatropha curcas* L.

At present, *Jatropha* press cake cannot be used as an animal feed because of the presence of several antinutrients and toxic substances. If the *Jatropha* press cake is detoxified, it has a high potential to be a competition in the feed market.

The kernels also contain a number of several toxic or antinutritional compounds. Typsin inhibitors, lectins, saponins and phytate might cause or at least aggravate the adverse effects that they are long term toxic. The short term toxicity of the kernel is ascribed mainly to the phorbol esters content (Makkar *et al*., 1997). Phorbol esters constitute a major single antinutritive component of *Jatropha* meals which is not heat labile and can have adverse effects on bioavailability of minerals, whereas other antinutritional factors like trypsin inhibitors and lectins can be destroyed by heat treatments.

The *Jatropha* seeds are toxic to humans and animals. Numerous feeding experiments with different animal species have demonstrated the toxicity of the seeds as well as of the oil and the press cake. Raw or cooked seeds kill rats within 2-3 days, whereas oil (raw or cooked) cause death within 6-8 days and roasted and cooked seeds after 14-16 days (Liberalino *et al*., 1988). The minimal lethal dose of *Jatropha* seeds for sheep, goat and calf have been determined to be 7.4, 1.5 and 3.0 g of seeds/kg of body weight, respectively. They are killed in 9, 12 and 12 days, respectively (Felke, 1913; Böhme, 1988). The *Jatropha* oil from Indian variety is fed to rats and later show the severe inflammation. A LD_{50} of 6 mg/kg body weight is determined (Gandhi *et al*., 1995).

2. Phorbol Esters

Phorbol esters have been identified as the major toxic principal in *J. curcas* L. (Makkar and Becker, 1997). Phorbol esters were first isolated in 1934 as the hydrolysis product of *Croton tiglium* oil and its structure was determined in 1967. Later, phorbol esters analogues are found in several members of the plant family Euphorbiaceae and *J. curcas* L. is also the plant in family Euphorbiaceae. Phorbol esters in *Jatropha* kernels content at least four different types which can cause theshort term toxicity (Makkar *et al*., 1999). The main chemical structure of phorbol esters in *Jatropha* kernel is 12-Deoxy-16-hydroxyphorbol-4'-[12',14'-butadienyl]-6'-[16',18',20'-nonatrienyl]-bicyclo[3.1.0]hexane-(13-*0*)-2'-[carboxylate]-(16-*0*)-3'-[8'-butenoic-10']ate (DHPB) as shown in Figure 2.

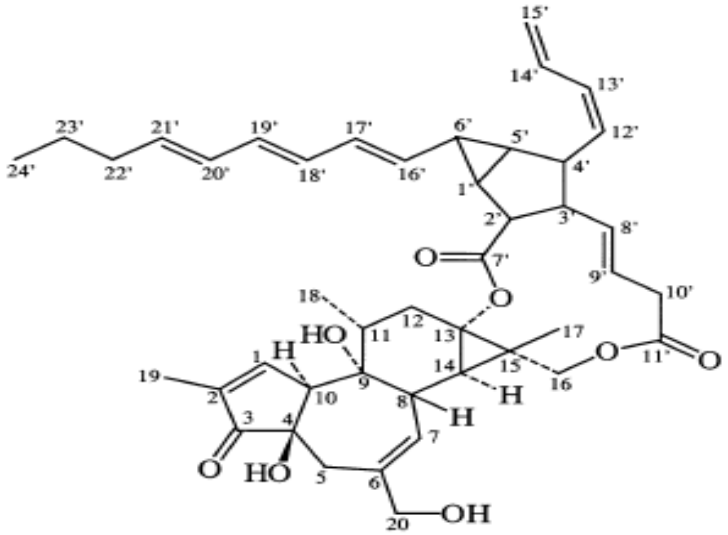


Figure 2 Structure of 12-Deoxy-16-hydroxyphorbol-4'-[12', 14'-butadienyl]-6'-[16', 18', 20'-nonatrienyl]- bicyclo [3.1.0]hexane-(13-*0*)-2'-[carboxylate]-(16-*0*)-3'-[8'-butenoic-10']ate; (DHPB).

Source: Hass and Mittelbach (2000)

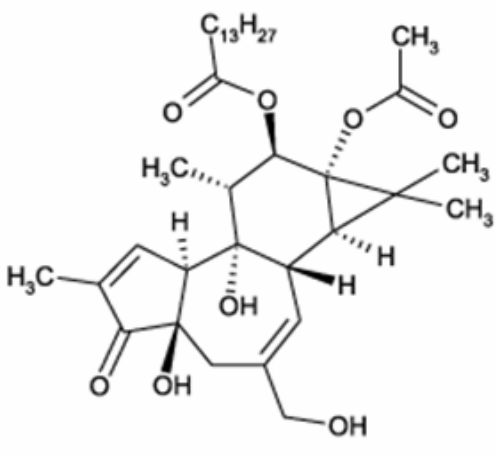


Figure 3 Structure of phorbol-12-myristate 13-acetate or 12-*O*-tradecanoylphorbol13-acetate; (TPA).

Figure 3 shows the chemical structure of phorbol-12-myristate 13-acetate (TPA). It is the phorbol esters standard found in the commercial market and used as phorbol esters standard in this experiment. Mitsuru *et al*. (1988) reports the tumors-promoting activity of DHPB. It is weaker than TPA because the application of 2.5 μg of TPA induces tumors nearly 100% in mice within 12 weeks. DHPB results in 46.7% incidence of tumors within 30 weeks. The weaker activity of DHPB might be explained by the structural difference between DHPB and TPA. They contain (a) the alcohol moiety that is 12-deoxy-16-hydroxy phorbol of DHPB and TPA, (b) the acid moieties that is the unsaturated acid of DHPB and saturatured acid of TPA. DHPB alone does not produce significant tumors.

2.1 Definition of phorbol esters

The fundamental substance of phorbol esters is the alcohol moiety, of this family of compounds is tigliane, a tetracyclic diterpene. Hydroxylation of this fundamental substance in various positions and connection to various acid moieties by ester bonding characterize the large number of compounds termed as phorbol esters (Evans, 1986), as shown in Figure 4.

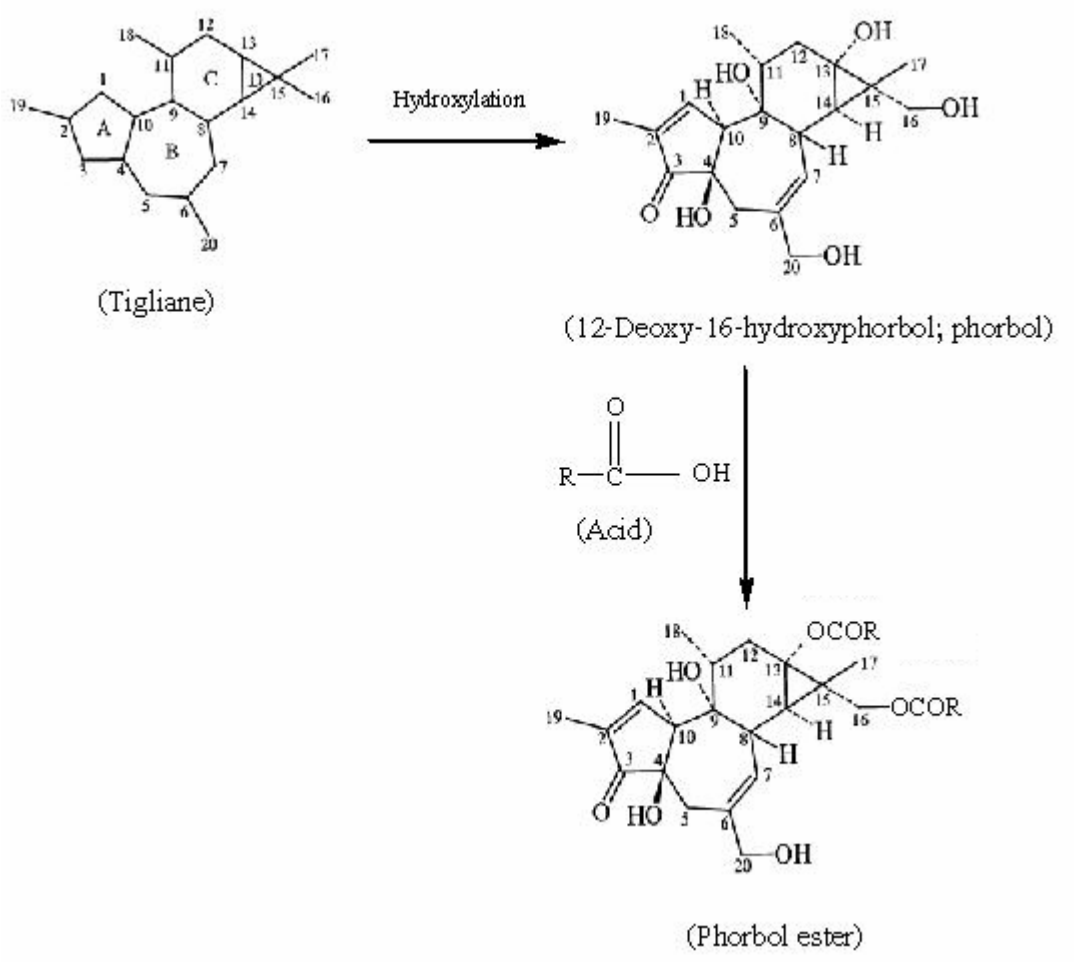


Figure 4 Occurring of phorbol esters.

Phorbol esters in *Jatropha* seed oil have six forms that are the isomers of chemical structures (Hass *et al*., 2002). They have a main structure of 12-deoxy-16-hydroxyphobol (Figure 5(1)) and also contain different side chains R_1 and R_2 to form six different isomers of phorbol esters (Figures 5(2-7)). Figures 5(4) and 5(5) are actually epimer and could not be separated by chromatography technique. All of these phorbol esters structures are named as DHPB.

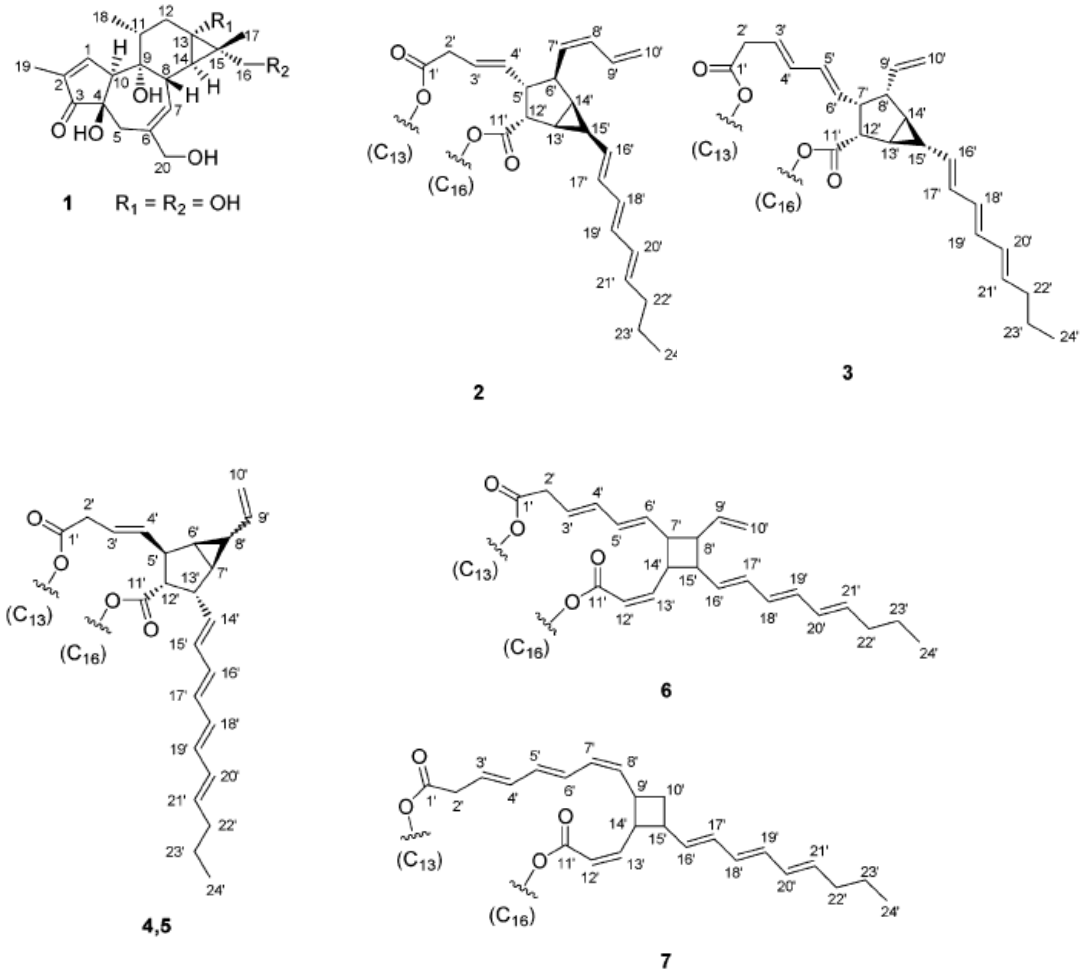


Figure 5 Different chemical structures of phorbol esters named DHPB.
Source: Hass *et al*. (2002)

2.2 Physical and chemical properties of phorbol esters

2.2.1 Description

Phorbol esters are isolated as white crystals or powders. When isolated from volatile organic solvents (ether, methylene dichloride) during fractionation of oil, they form brittle foams which change to amorphous which are soften at temperature below 100˚C. Phorbol-12-myristate 13-acetate (TPA) is like phorbol, strongly retains solvent molecules which it forms addition compounds. The same probably applies to other phorbol esters as well. They are soluble in water and

polar organic solvents. Anhydrous phorbol (crystallized from water) has a melting point of 250-251˚C. Phorbol crystallized from ethanol and methanol retains solvent molecules tenaciously and these "alcohol phorbols" have sharp melting points in the region of 230-240˚C.

2.2.2 Stability

Phorbol esters are very sensitive to acid, alkali, elevated temperatures, light and atmospheric oxygen. Solid TPA appears to be stable when stored in the dark at -20˚C. It shows slow decomposition at 4˚C within 3 monts in the dark and more extensive decomposition at 25˚C in diffuse daylight within 3 moths. The solution of TPA in dimethyl sulfoxide may be kept at -20˚C in the dark for 6 months. Solution of TPA in ethanol may be kept in the dark under nitrogen at -4˚C in the dark for 5 months. At -4˚C there are only traces of decomposition, while at 25˚C (in acetone, ethyl acetate or methylene chloride) autoxidation is extensive. The main products have been identified and consist mainly of oxidation products at the double bonds (Schimdt and Hecker, 1975; Jacobson *et al*., 1975; Ohuchi and Levine, 1978).

2.2.3 Chemical reactivity

Hecker and Schmidt (1974) reviewe phorbol esters and its esters. Phorbol esters reduce Fehling's and Tollen regents, and form esters and ethers. The C_5 carbonyl group shows weak activity in the reaction with carbonyl agents but is reduced by sodium borohydride. The double bonds are subjected to reduction and to autoxidation. The primary alcohol group at C_{20} is oxidized to the aldehyde with MnO_2 or CrO_3.

2.3 Biological of phorbol esters

The phorbols themselves do not induce tumors but promote tumor growth following exposure to a subcarcinogenic dose of a carcinogen. They are rapidly absorbed through the skin and probably the intestinal tract. They may cause severe

irritation of tissues (skin, eyes, mucous membranes and lungs) and induce sensitivity. Laboratory operations should be conducted in a fume hood and glove. If phorbol esters contact skin, wash with soap and cold water, avoid washing with solvents.

Highly irritant factors to skin are isolated from the seed oil of four *Jatropha* species (Adolf *et al*., 1984). These irritant factors are determined and that one is new polyunsaturated esters of 12-deoxy-16-hydroxyphorbol . The seed oil of *J. curcas* L. in Thailand is intended to produce in large amounts for the use as a substitute of a biodiesel and an ingredient in commercial printing ink. The irritant factors are tumor promoters, therefore its widely use might result in exposure of a large population to tumor promoters. In 1987 the irritant factors were partially purified from the seed oil of *J. curcas* L. in Thailand (Horiuchi *et al*., 1987). It shows the tumor-promoting activity in 12-Deoxy-16-hydroxyphorbol-4'-[12' ,14'-butadienyl]-6'-[16',18',20'-nonatrienyl]-bicyclo [3.1.0] hexane-(13-*0*)-2'- [carboxylate] -(16-*0*)-3'-[8'-butenoic-10']ate (DHPB) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) when it is experimented on mouse skin. The results showed that DHPB (unsaturated acid) has slightly weaker biological effect than TPA (saturated acid). TPA is widely used as standard phorbol esters in biochemical experiment.

2.4 Experimentation on phorbol esters

Many researches study and try to detoxify the phorbol esters substances in oil or press cake of *J. curcas* L. as follows.

The demand and supply gap of vegetable oil in the world because of the oil price increasing. *J. curcas* L. as an energy crop and *J.* seed oil is produced for biodiesel. In 1996, Foidl *et al*. developed *J. curcas* L.. They study a technical process to produce methyl ester and ethyl ester from seed oil. The results shows that the fuel properties of both esters are followed the standard properties of biodiesel. Shweta *et al*. (2005) illustrate that the combination of sonication and enzyme treatment with a commercial preparation of pH 9 ledds to 97% oil yield within 2 hours.

J. curcas L. has a large number of potential utilizations. The seed weighs about 0.75 g, contains 30-32% protein and 60-66% lipid (Liberalino *et al*., 1988) indicating a good nutritional value. However, the seed or oil is found to be toxic to mice (Adam, 1974), rat (Liberalino *et al*., 1988), calves, sheep and goats (Ahmed and Adam, 1979), human (Mampane *et al*., 1987) and chickens (Samia *et al*., 1992). Hence, it is restricted to use as a food or feed source. The biological effects of phorbol esters are found by Aitken *et al*. in 1986. The biological effects are tumor promotion, cell proliferation, activation of blood platelets, lymphocyte mitogenesis, inflammation, prostaglandin production and stimulation of degranulation in neutrophils. So, phorbol esters substances are interested and in 1988 Mitsuru *et al*. find a new type of phorbol esters which has a macrocyclic dicarboxylic acid diester structure. It is isolated from the seed oil of *J. curcas* L. and its structure is proposed as an intramolecular 13, 16 diester of 12-deoxy-16-hydroxyphorbol-4'-[12',14'-butadienyl] -6'- [16',18',20'-nonatrienyl] -bicyclo [3.1.0] hexane- (13-*0*) -2'-[carb-oxylate] -(16-*0*)-3'- [8'-butenoic-10'] ate (DHPB). The results showe that DHPB is tumor promotion with weaker biochemical activity than12-*o*-tetradecanoylphorbol-13-acetate (TPA). In 1995, Gandhi *et al*. provide data on toxicity of *Jatropha* seed oil which contains phorbol esters. A toxic fraction of the phorbol esters is isolated from the oil and LD_{50} is tested in rats. The acute oral LD_{50} of the oil is 6 mg/kg body weight in rats. Gross *et al*. (1997) suggeste a method for detoxification of oil by extraction phorbol esters using ethanol. This method is in economic effort because of a lot of solvent consumption.

The toxic of phorbol esters substances have different biochemical activities depending on species of *J. curcas* L.. In 1997, Makkar *et al.* evaluated the non-toxic and toxic varieties of *J. curcas* L.. They describe that *Jatropha* meal contains high protein, high energy and low fiber. The amino acids composition of meals from the non-toxic and toxic varieties is also similar. The meal contains significant level of trypsin inhibitor, lectin and phytate. Their levels do not differ much between the non-toxic and toxic varieties. The differences between non-toxic and toxic varieties are the amount of phorbol esters content. They find that the low level of the phorbol esters in the seed of non-toxic varieties from Mexico is about 20-

times higher than that in toxic varieties. The amount of phorbol esters in non-toxic from Mexico is 0.11 mg/g of kernel whilst toxic varieties content about 3.45 mg/g of kernel. Non-toxic varieties from Mexico could be a suitable alternative to toxic *Jatropha* varieties and the press cake could be a good protein source for human as well as livestock. Makkar *et al*. describe the kernels of *J. curcas* L.. They contained a number of several toxic compounds and the toxic contents are "phorbol esters". It is not possible to destroy phorbol esters by heat treatment because they are heat stable and could withstand roasting temperature as high as 160˚C for 38 min. In 1998, Bekker and Makkar describe that phorbol esters are toxic substances found in *Jatropha* seed. They act as a co-carcinogen and have a wide range of adverse biochemical and cellular effects in animal. Common crap is found to be extremely sensitive to these compounds. At a level of 31 ppm in feed, they induce depression in feed intake, growth production of faecal mucus. Use of non-toxic varieties of *J. curcas* L. with phorbol esters are absent in an obvious solution. In 2005, Gonzalez-Guerrico and Kazanietz describe interference with the activity of protein kinase C, effects a number of processes including, phospholipid and protein synthesis, enzyme activities, DNA synthesis, cell differentiation and gene expression. The phorbol esters themselves do not induce tumors but promote tumor growth following exposure to a subcarcinogenic dose of a carcinogen. They could thus be designated as a co-carcinogens.

The biological effects of phorbol esters are necessary to find routes for detoxification of the oil and press cake. In 2000, Hass *et al*. experiment the edible oil processing steps on phorbol esters detoxification. They find that deacidification step and bleaching step are efficient for phorbol esters removal by 55% whereas degumming step and odor removal step are not effective on phorbol esters removing. In the same year, Rug and Ruppel (2000) also find phorbol esters to be an effective biopesticide against diverse fresh-water snails. Extracts from *J. curcas* L. are found to be toxic against snails transmitting *Schistosoma mansoni* and S. *haematobium*. When compared with aqueous extract, methanol extract shows the highest toxicity against all organisms that are tested with values 25 ppm for cercariae and the snail *Biomphalaria glabrata* and 1 ppm for the snails *Bulinus truncates* and B. *natalensis*.

Attenuation of cercariae leading to reduced infectivity in mice could be achieved in concentration below those exporting acute toxicity. *Jatropha* oil or methanol extract of *Jatropha* oil containing phorbol esters has also been shown to have strong insecticidal effects against *Busseola fusca* and *Sesamia calamistis* larvae (Mengual, 1997) and pesticidal effects against *Sitophilus zeamays* and *Callosobruchus chinesis* and deterred their oviposition on sprayed corn and mungbeans seeds (Solsoloy and Solsoloy, 1997). In 2003, Aregheore *et al*. experiment the detoxification of phorbol esters and lectin from the *Jatropha* press cake using heat and chemical substances. They find that lectin is destroyed at 121°C along with 30 min, however, it does not affect on phorbol esters removing. Washing press cake with 4% (w/w) sodium hydroxide solution and then followed with 92% methanol for 2 times or with only water for 4 times could eliminate phorbol esters.

3. Adsorption

Deacidification and bleaching steps of the traditional refinery oil process can reduce phorbol esters content in seed oil of *J. curcas* L. up to 55% (Wilhelm *et al*., 2000). This research is interested to select the method of phorbol esters elimination in seed oil of *J. curcas* L.. The bleaching agent can adsorb color of oil and may also adsorb phorbol esters. Therefore, the adsorption process is selected a method to eliminate phorbol esters from seed oil.

3.1 General Definition of adsorption

Adsorption is a term used to describe the process which materials are accumulated on the interface between two phases. These phases can be liquid-liquid, liquid-solid, gas-liquid and gas-solid phase. In this experiment, the adsorb substance is called adsorbent, whereas the substance being adsorbed is called adsorbate.

3.2 Kinetics

Two important properties in an adsorption system using adsorbent include the adsorptive capacity of a given amount of adsorbent for particular solute and the adsorption rate out of solution.

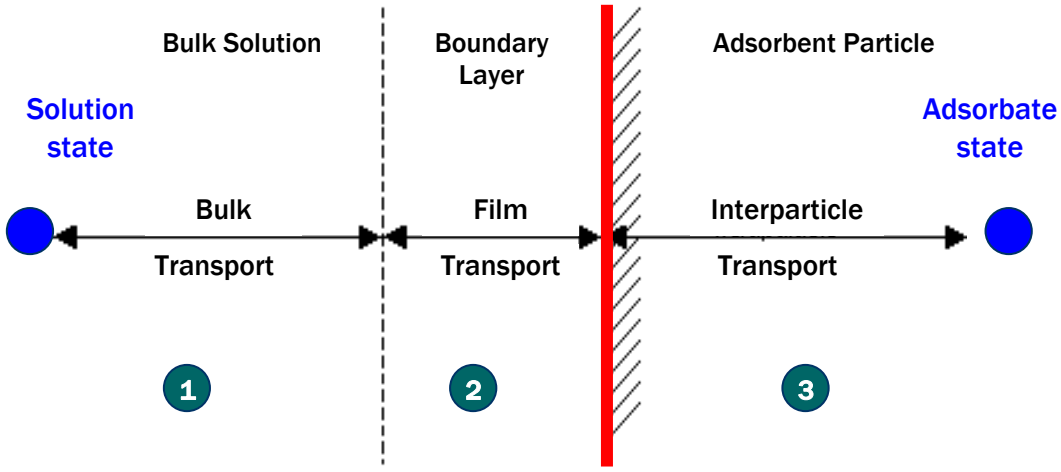


Figure 6 Adsorption process on a porous adsorbent proceeds in three consecutive steps; (1) Bulk transport, (2) Film transport and (3) Interparticle transport.
Source: Nipon and Kanita (2007)

The adsorption process on a porous adsorbent usually proceeds in three consecutive steps; (1) Transport of solute from the bulk of the solution to the outer surface of the film surrounding the particle-bulk transport, (2) Transport of solute within the thin-film transport, (3) Transport in the interior of the particle-intraparticle transport, as shown in Figure 6.

3.3 The theory of adsorption

Adsorption of a material occurs at the surface since it reduces the imbalance of attractive forces, and therefore, the surface frees energy of the heterogeneous system. Two phenomena are observed; physical adsorption and

chemical adsorption. Nevertheless, physical adsorption (physisorption) differs from chemical adsorption (chemisorption) according to the parameters listed in Table 1.

Table 1 Parameters of physical and chemical adsorptions.

Parameter	Physical adsorption	Chemical adsorption
Heat of adsorption	Low	High
Specificity	Not site specific	Highly site specific
Nature of adsorbed phase	Monolayer or multilayer, no dissociation of adsorbed species	Monolayer only
Temperature range	Only significant at low temperatures	Possible over wide range of temperature

Physical adsorption

Physical adsorption involves the balancing of weak attractive force, for example, van der waals force, between the solid surface and adsorbate, with the repulsive force associated with close contact. The process is always exothermic and the energy is given out on adsorption (McCash, 2001). Physical adsorption is non-specific and any atom or molecules can adsorb on any surfaces of adsorbents under appropriate experimental conditions. The high amounts of physical adsorption are favored when the surface is at low temperature.

Chemical adsorption

Chemical adsorption is characterized mainly by a strong chemical bond which forms between the adsorbent surface and the adsorbate. Chemical adsorption involves chemical bonding by the exchange of electrons between the adsorbing molecule and surface of adsorbent. The heat of chemical adsorption is generally larger than physical adsorption. The heat is found that chemical adsorption layers with large heats of adsorption tend to be very stable at high temperature. The bond formed may be ionic, covalent, or mixture of the two.

3.4 Adsorption isotherms

The adsorption isotherm is the equilibrium relationship between the concentration in the fluid phase and the concentration in the adsorbent particles at a given temperature. The concentration of the adsorbate on the adsorbent is given as mass adsorbed per unit mass of the original adsorbent.

3.5 Adsorption equilibrium

Adsorption from aqueous solution involves concentration of the solute on the solid surface. The adsorption equilibrium is an equilibrium state in which equal amounts of solute eventually are being adsorbed and desorbed simultaneously; in other words, the rate of adsorption equals desorption. Therefore, at equilibrium, no change can be observed in the concentration of the solute on the solid surface or in the bulk solution as shown in Figure 7.

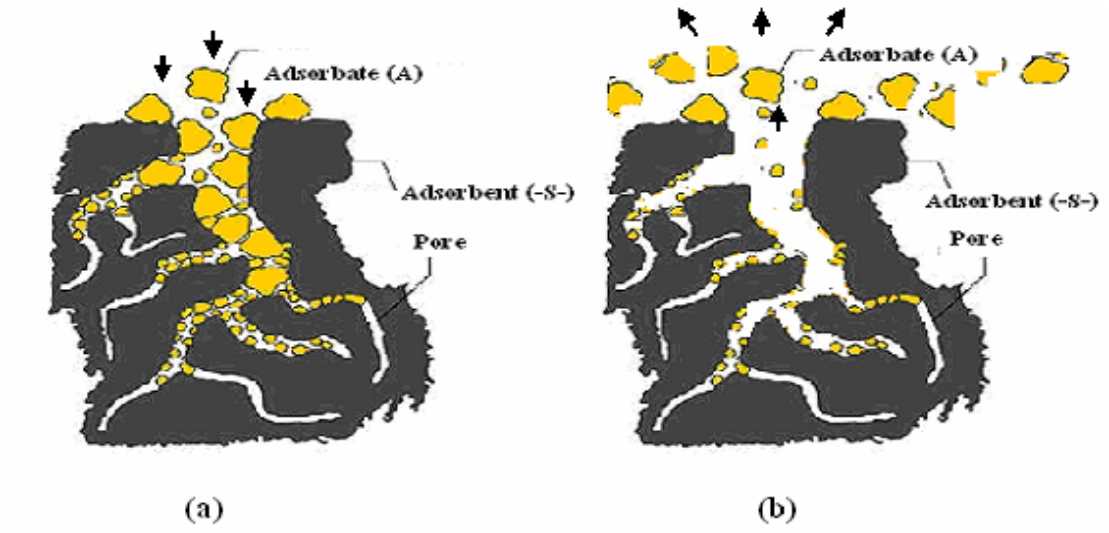


Figure 7 Two characterizations of adsorbate on adsorbent; adsorption (a) and desorption (b).

3.4.1 Adsorption process

$$A + -S- \xrightarrow{k_1} A\text{-}S-$$

$$r_1 = k_1 [C] (1-q) \qquad \text{---------- (1)}$$

Where

A = molecules of adsorbate in solution, it has initial concentration (C_o; mg/L, mol/L)

-S- = molecules of adsorbent

q = fraction of the site in adsorption or adsorption capacity

$(1-q)$ = fraction of the site to be vacant

r_1 = rate of adsorption

k_1 = adsorption rate content

C = adsorbate concentration in solution after adsorption (mg/L, mol/L)

3.4.2 Desorption process

$$A\text{-}S- \xrightarrow{k_2} A + -S-$$

$$r_2 = k_2 q \qquad \text{---------- (2)}$$

Where

r_2 = rate of desorption

k_2 = constant of desorption

At equilibrium the amount of molecules in the adsorbed state is constant.

$$r_1 = r_2$$

$$k_1 [C] (1-q) = k_2 q$$

$$\frac{q}{(1-q)} = \frac{k_1}{k_2}[C] = K[C]$$

$$q = \frac{K[C]}{1+K[C]} \qquad \text{---------- (3)}$$

Where

K = adsorption equilibrium constant

$$A + \text{-S-} \rightleftharpoons \text{A-S-}$$

At equilibrium state

$$K = \frac{q}{C} \qquad \text{---------- (4)}$$

Or, quantity of adsorbate on surface of adsorbent = Quantity of adsorbate desorbs from adsorbent

$$qW = V(C_o - C) \qquad \text{---------- (5)}$$

Where

q = loading of adsorbate on adsorbent (mol/kg, mol/g, mg/kg)

W = mass of adsorbent present (g, kg)

V = volume of solution used in the batch (cm^3, L)

C = adsorbate concentration in solution after adsorption (mol/L, mg/L)

C_o = initial adsorbate concentration in solution (mol/L, mg/L)

This experiment used adsorption technique to adsorb phorbol esters from *J.* seed oil. In bioseparations, the Linear, Freundlich and Langmuir are used to fit adsorption isotherms. Freundlich and Langmuir isotherms are very popular models. The equations are commonly used to describe the experiment adsorption data.

Freundlich adsorption isotherm

This isotherm is an empirical expression and no physical basis. The equilibrium relationship proposed by Freundlich is only valid when the adsorption is a purely physical process without any change in the configuration of the molecule in the adsorbed state. The equation equilibrium of this model is as follows:

$$q = KC^{1/n} \qquad \text{---------- (6)}$$

Where K and $1/n$ = constants characteristic of the system

The constants in the model can be obtained by linearization in logarithmic from

$$\log q = \log K + \frac{1}{n}\log C \qquad \text{---------- (7)}$$

Plotting log q versus log C should give a straight line with

$$\frac{1}{n} = \text{slope}, \qquad \log K = \text{intercept}$$

See Figure 8.

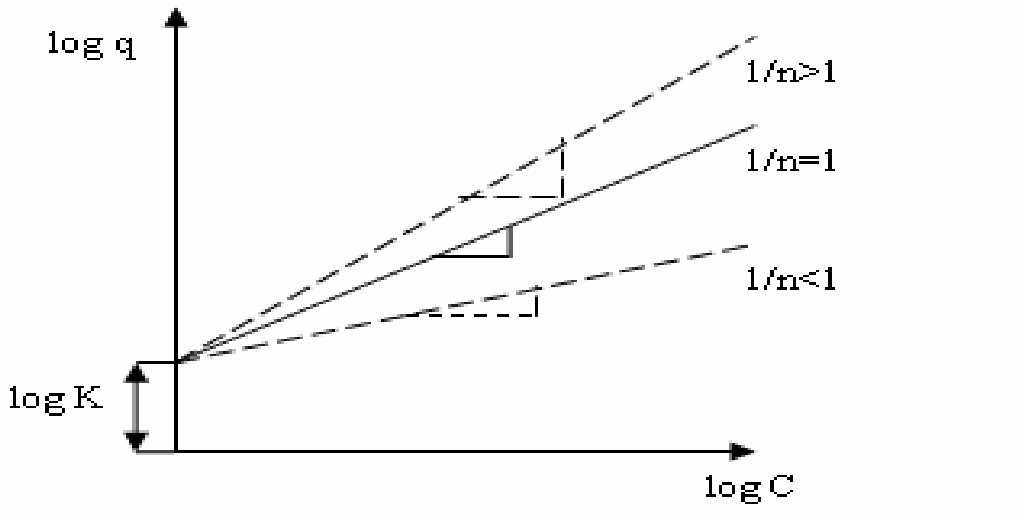


Figure 8 Freundlich isotherm of linearization in logarithmic.

If

$\frac{1}{n}$ = 1, adsorption isotherm of linearization, equilibrium adsorption

$\frac{1}{n}$ < 1, adsorbent has limited surface for adsorption, unfavorable adsorption

$\frac{1}{n}$ > 1, adsorbent has large surface for adsorption, favorable adsorption

Langmuir adsorption isotherm

Langmuir isotherm is the model for an adsorption isotherm and describes ideal chemical adsorption. It is based on four hypotheses:

1. The surface of adsorbent is uniform that is all adsorption sites are equal.
2. Adsorbed molecules do not interact.
3. All adsorption occurs through the same mechanism.
4. At the maximum adsorption, only a monolayer is formed. Molecules of adsorbate do not deposit on other molecules.

From equilibrium equation (3) it is assumed that the maximum adsorption is 1, so the Langmuir equation uses equation (3) multiply the maximum loading of adsorbate on adsorbent (q_m), the Langmuir adsorption isotherm is expressed as:

$$q = \frac{q_m KC}{1 + KC} \qquad \text{---------- (8)}$$

Dividing equation (7) to equation 8:

$$\frac{1}{q} = \frac{1}{q_m} + \frac{1}{Kq_m C} \qquad \text{---------- (9)}$$

Plotting $\frac{1}{q}$ versus $\frac{1}{C}$ should give a straight line, where

$$\frac{1}{Kq_m} = \text{slope}, \quad \frac{1}{q_m} = \text{intercept}$$

See Figure 9.

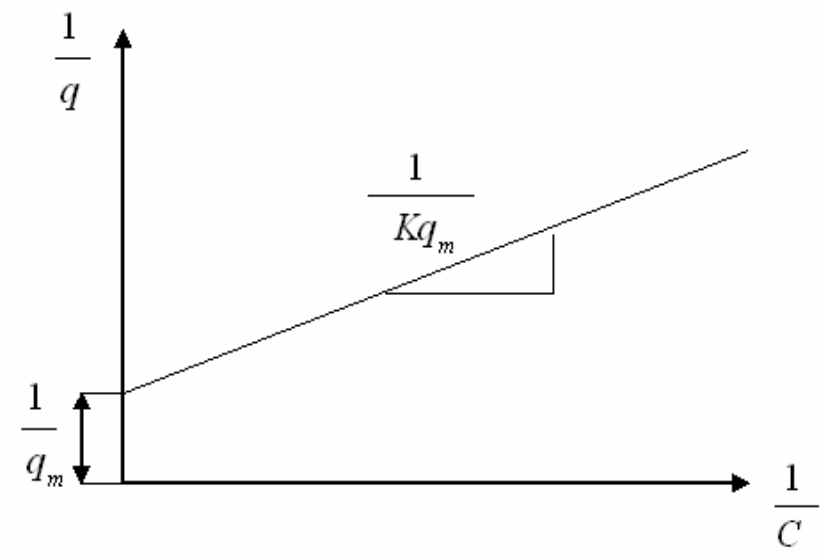


Figure 9 Langmuir isotherm of linearization.

4. High Performance Liquid Chromatography

The naturally occurring phorbol esters are unstable and are subsection to oxidation, hydrolysis, transesterification and epimerization during isolation procedures. Due to their oxygen sensitivity, the isolation must be conducted in oxygen free conditions: solvent must be degassed and extraction should be conducted under continuous flow of nitrogen or argon. The isolation protocols involve derivatization of the functional groups in phorbol esters, mainly acylation or esterification of hydroxy group by chemical reagents. The derivatized phorbols are then separated using different high performance liquid chromatography (HPLC). The crystallization is reported to induce loss of material due to oxidation and/or

epimerization of the phorbol nucleus. The purity of isolated phorbols can be deduced using thin layer chromatography (TLC), gas-liquid chromatography (GLC), mass spectrometry (MS), or HPLC with electrospray ionization tandem mass spectrometry (HPLC/MS/MS). Normal-phase HPLC using photodiode array detection system has been used by Dimitrijevic *et al*. (1999) for isolation of different tigliane and daphnane esters. The diterpene esters are transformed to their quasimolecular ions, which are analyzed to obtain the full-scan mass spectrum of the compound using HPLC/MS/MS. In this experiment, the phorbol esters have been isolated using HPLC (Makkar and Becker, 1997).

HPLC has its original in classical column chromatography although in both theory and practice, it is similar to gas chromatography (GC). In column chromatography the sample introduced into mobile phase which flows through a column of relatively coarse particles of a stationary phase, usually is silica or alumina, under the influence of gravity. Flow rates are of the order of 0.1 cm^3 min^{-1} which result in extremely lengthy separation times and quite inadequate efficiencies and separations of multicomponent mixtures. The poor performance is largely due to very slow mass transfer between stationary phase and mobile phase and poor packing characteristics leading to a large multiple path effect. It is recognized that much higher efficiencies and hence better resolution could be achieved through the use of smaller particle of stationary phase and that rapid separation would require higher flow rate necessitating the pumping of the mobile phase through the column under pressure. The means of meeting two basic requirements are developed during the 1960s together with suitable pumps, injection system and low dead-volume detectors and the new technique, which is now at least as extensively use as GC, became known as "*high performance liquid chromatography* (HPLC)" or simple "*liquid chromatography* (LC)". The mobile phase is typically pumped at pressure to about 3000 psi (200 bar) and flow rates of 1-5 cm^3 min^{-1} can be achieved through 10-25 cm columns packed with particles as small as 3 μm in diameter. At it best, HPLC is comparable to GC for speed, efficiency and resolution and it is inherently more versatile. It is not limited to volatile and thermally stable samples and the choice of stationary phase includes solid adsorbents, chemical modified adsorbents, ion-

exchange and exclusion materials thus allowing all four sorption mechanisms to be exploited. A much wider choice of mobile phases than in GC facilitates a very considerable variation in the selectivity of the separation process.

4.1 Principle

Separation of mixtures in microgram to gram quantities by passage of the sample pass a column containing a stationary solid by means of a pressurized flow of a liquid mobile phase; components migrate through the column at different rates due to different relative affinities for the stationary and mobile phase base on adsorption, size or change.

4.2 Partition systems

In a partition system the stationary phase is a liquid coated onto a solid support. Silica gel, diatomaceous earth and cellulose powder are the most frequently used. Conditions closely resemble those of counter-current distribution so that in the absence of adsorption by the solid support, solutes move to the system at rates determined by their relative solubility in the stationary and mobile phases. Partition isotherms usually have a longer linear range than adsorption isotherms, so tailing or fronting of elution peak is not a particular problem, except at high concentration.

4.2.1 Stationary and mobile phase

There is a very wide choice of pairs of liquids to act as stationary and mobile phases. It is not necessary for them to be totally immiscible, but a low mutual solubility is desirable. As already described, the rate of movement of a solute is determined by its distribution ratio defined as

$$D = \frac{C_{stationary\ phase}}{C_{mobile\ phase}} \qquad \text{---------- (10)}$$

Where $C_{stationary\ phase}$ is concentration of solute in stationary phase and $C_{mobile\ phase}$ is concentration of solute in mobile phase. The larger the value of *D*, the slower will be the progress of the solute through the system and the components of a mixture will therefore reach the end of column or the edge of a surface in order of increasing volume of *D*. In column methods, a solute is characterized by the volume of mobile phase required to move it from one end of the column to the other. Known as the *retention volume*, V_R, it is defined as the volume passing through the column between putting the sample on the top of the column and the emergency of the solute at the bottom. It is given by the equation

$$V_R = V_M + kV_M \qquad \text{---------- (11)}$$

Where V_M is the volume of mobile phase in the column, *k* is the retention factor which is directly proportional to *D* but takes account to volume of each phase. Sometimes *k* is used to characterize a solute rather than V_R.

If $k = 0$, then $V_R = V_M$ and the solute is eluted without being retarded or retained by stationary phase. Large volumes of *k*, which reflect large volume of *D*, result is very large retention volumes and hence long retention times. At a constant rate of flow of mobile phase *F*, V_R is related to the retention time, t_R, by the equation

$$V_R = Ft_R \qquad \text{---------- (12)}$$

If the flow of mobile phase is monitored by a detector and recorder system, such as used in gas and HPLC, then t_R can be used as measure of V_R.

4.2.2 Chromatographic performance

The ideal chromatographic process is one in which the components of a mixture from narrow bands which are completely resolved from one

another in as short a time as possible. The performance of a particular chromatographic system can be assessed in the following ways.

Efficiency and resolution

The width of band or peak is measure of the efficiency of the process whilst resolution is assessed by the ability to resolve the peaks of components with similar t_R values. Efficiency, N, for column separation is related to retention time and peak width measured in terms of the standard deviation, assuming an ideally Gaussian-shaped peak. In practice it is easier to measure baseline width or the width as one half of the peak height, so N is generally calculated using one of the alternative formulae:

$$N = \left(\frac{t_R}{W_B} \right)^2 \qquad \text{---------- (13)}$$

$$N = 5.54 \left(\frac{t_R}{W_{h/2}} \right)^2 \qquad \text{---------- (14)}$$

Where W_B is the baseline peak width and $W_{h/2}$ is the peak width measured as half of the peak height. Valid comparisons of efficiencies can be made only if the same formula is used throughout, as the computed values of N using each of the above formulae differ considerably.

The parameter N is universally referred to as the plate number, but an alternative means of quoting efficiency is in terms of a plate height, H. Plate number and plate height are inversely related by the equation.

$$N = L/H \qquad \text{---------- (15)}$$

Where L is the length of the column. Values of N may be many thousands for column having high efficiencies, the corresponding values of H being less than 1 mm.

Resolution, R_s, is measured from a chromatogram by relating the peak-to-peak separation to the average peak width. This is expressed by the equation.

$$R_s = 2\Delta t_R / (W_1 + W_2) \qquad \text{---------- (16)}$$

The t_R is the separation of the peak maximum and W_1 and W_2 are the respective peak widths (Figure 10). Because of the Gaussian profile of the peak, a 100% separation is never attainable but an R_s value of 1.5 or more indicates cross-contamination of 0.1 % or less and is known as baseline resolution.

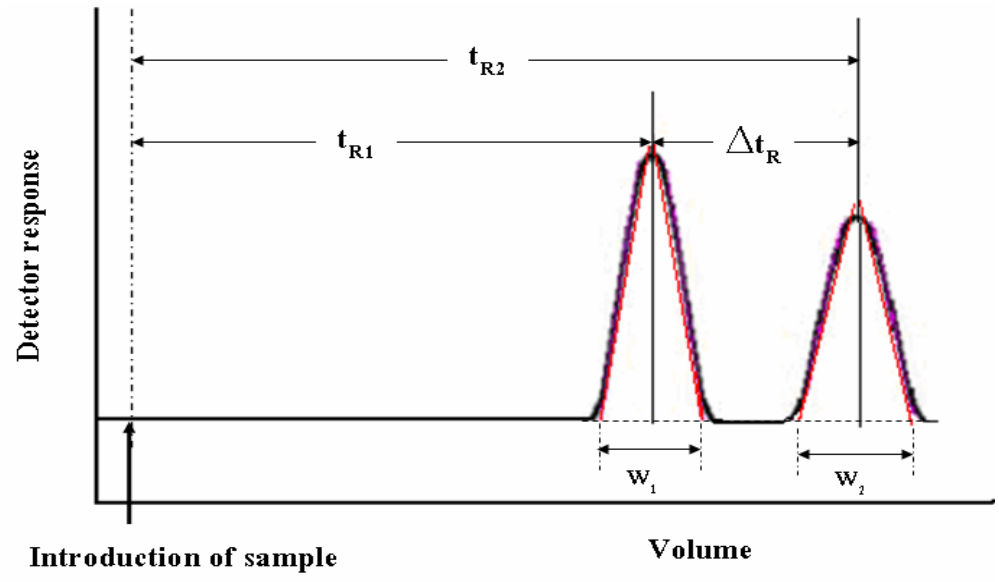


Figure 10 Resolution of adjacent peaks.

Source: Fified and Kealey (2000)

Peak asymmetry (peak skew)

Few chromatographic peaks are perfect (symmetrical), most exhibiting a degree of symmetry due to tailing or fronting. It is common practice to quantify the degree symmetry, or skewness, using one of number formulae that incorporate the front and rear half-width of the peak as defined in Figure 11. A perpendicular is down from the peak maximum to the baseline and the horizontal distance from this perpendicular to the front and rear edged of the peak (A and B) is measured a specified height above the baseline, usually 5% or 10% of the peak height, the peak *symmetry factor*, A_s, is defined as B/A and *tailing factor*, T_f are exactly 1. Volumes of 0.9 (fronting) to 1.2 (tailing) are considered acceptable, whereas outside this range improvement in the chromatography should be sought. Comparison should be based on the same formula, although differences between A_s and T_f for the same peak are small when the values are close to 1. Some laboratories use A_s whilst others prefer T_f.

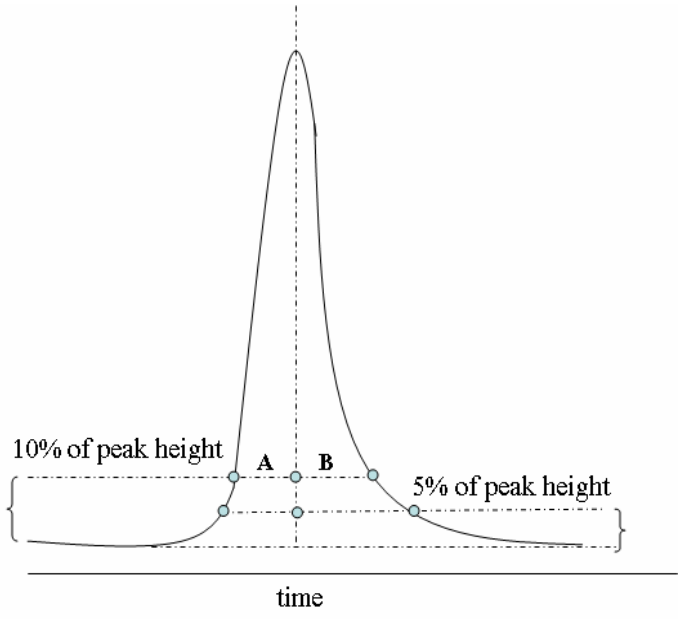


Figure 11 Determining peak-asymmetry and peak-tailing factor. Peak asymmetry = *B/A* and peak tailing factor = *(A+B)/2A.*

Source: Fified and Kealey (2000)

4.3 Applications

HPLC is used largely for the separation of non-volatile substances including ionic and polymeric samples, complementary to GC.

4.4 Disadvantages

Column performance is very sensitive to setting of the packed bed or the accumulation of strongly adsorbed materials or particulate matter at the top, universal detection system is not available.

A schematic diagram of HPLC is shown in Figure 12 and details of the components are discussed below.

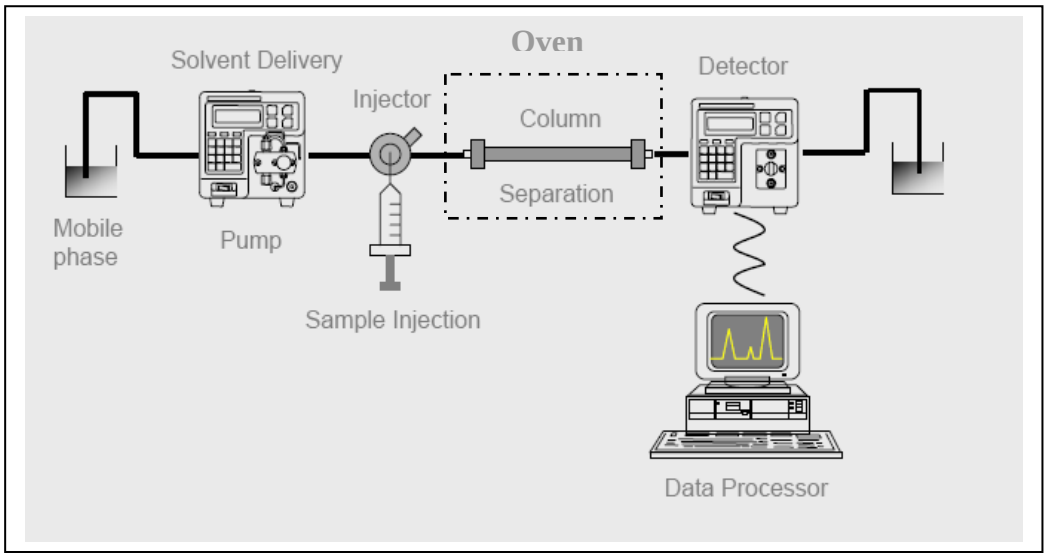


Figure 12 Schematic diagram of an HPLC instrument.

The main components of an HPLC system are a high-pressure pump, a column and an injector system as well as a detector. The system works as follows, eluent is filtered and pumped through a chromatographic column, the sample is loaded and injected onto the column and the effluent is monitored using a detector and recorded as peaks.

4.5 Detectors

The ideal HPLC detector should have the same characteristics as those required for GC detectors that are rapid and reproducible response to solutes, a wide range of linear high sensitivity and stability of operation. No truly universally HPLC detector has yet been developed but two most widely application types are those based on the adsorption of UV or visible radiation by the solute species and those which monitor refractive index difference between solutes dissolved in the mobile phase and the pure mobile phase. Other detectors which are more selective in their response rely on such solute properties such as fluorescence, electrical conductivity, diffusion currents (amperometric), radioactivity, etc. This experiment used UV-Vis and photo diode array detectors (PDA) as discussed below.

UV/visible photometers and dispersive spectrophotometers

These detectors respond to UV/visible absorbing species in the range 190-800 nm and their respond is linear with concentration, obeying the Beer-Lambert law. They are not appreciably flow or temperature sensitive, have a wide linear range but variable sensitive.

Photometers are designed to operate at one or more fixed wavelengths only, whereas spectrophotometers facilitate monitoring at any wavelength within the operating range of the instrument. Both types of detector employ low-volume (10 μm or less) flow-through cells fitted with quartz windows. Careful design of the cell, which should be of minimal volume to reduce band-spreading and maximal path length for high sensitive, is necessary to reduce undesirable refraction effects at the cell wall as solutes pass through.

Although many substances absorb appreciably at 254 nm or one of the other fixed wavelengths available with a photometer, a much more versatile system is based on a spectrophotometer fitted with a grating monochrometer and light source such as a deuterium lamp for the UV region and a tungsten-halogen lamp for the visible

region. They have double-beam optics, stable low-noise electronics and are often microprocessor controlled. Some can be programmed to select a sequence of optimum monitoring wavelengths during or between chromatographic runs and the recording of a complete UV spectrum after stopping the flow with a selected peak in the detector cell is a feature of other designs. Another development is a rapid scanning capability that allows a complete spectrum to be recorded in a fraction of a second without the need to stop the flow, therefore rivaling diode array detector. Like the latter, full computer control and high resolution color graphics enable chromatogram to be displayed in 3D and other format and peak purity assessed. Sensitivity and resolution are better than some diode array detectors.

Photometers are more sensitive than spectrophotometers, are cheaper and more robust and are well suited to routine work where monitoring at 254 nm or some other fixed wavelength is acceptable. Spectrophotometers, however, allow 'tuning' to the most favorable wavelength either to maximize sensitivities for a particular solute or to 'detune' the respond to other solutes. By allowing monitoring down to 190 nm, weakly absorbing or saturated compounds can be detected.

Diode array spectrophotometers

These can device more spectral information than photometers or conventional dispersive spectrophotometers but are much more expensive and generally less sensitive. However, they enable sets of complete UV or UV and visible spectra of all the samples components to be recorded as they elute from the column. The stored spectra information can be processes in several ways by the microcomputer and displayed using sophisticated color graphic software packages. The most usual is a 3D chromatogram of time/absorbance/wavelength. This can be rotated on the screen to allow examination or the otherwise hidden regions behind major peaks or viewed from directory above and shown as an absorbance contour map to provide useful information on peak purity. The comparison of spectra selected from any points on the time axis by over-laying them in various colors in an alternative assessment of peak purity and spectra can be matched with a laboratory of

standards for identification purposes. Some software packages also include the facility for calculating peak purity factors from absorbance ratios at two or more wavelength for difference time slices of an eluting peak, values close to indicating a high degree of purity.

Mass spectrometric detectors

The combination of LC and mass spectrometry would seem to be an ideal merger of separation and detection. Today, the most popular approaches use a low-flow-rate atmospheric pressure ionization technique. The HPLC system is often a nanoscale capillary LC system with flow rate in the μL/min rang. Alternatively, some interfaces allow flow rates as high as 1 to 2 mL/min, which are typical of conventional HPLC conditions. The most common ionization sources are electrospray ionization and atmospheric pressure chemical ionization. The combination of LC and mass spectrometry gives high selectivity because unresolved peaks can be isolated by monitoring only a selected mass. The LC/MS technique can provide fingerprinting of a particular elute instead of relying on retention time as in conventional HPLC. The combination can give molecular mass, structural information and accurate qualitative analysis.

For some complex mixtures, the combination of LC and MS does not provide enough resolution. In recent year it has become feasible to couple two or more mass analyzers to form tandem mass spectrometers. When combined with LC, the tandem mass spectrometry is called an LC/MS/MS instrument. Tandem mass spectrometers are usually triple quadrupole systems or quadrupole ion-trap spectrometers. To attain higher resolution than can be achieved with quadrupole, the final mass analyzer in a tandem MS system can be a time-of-flight mass spectrometer. Sector mass spectrometers can also be combined to give tandem systems. Ion cyclotron resonance and ion-trap mass spectrometers can be operated in such a way as to provide not only two states of mass analysis but *n* stages. Such MS systems provide the analysis steps sequentially within a single mass analyzer. These have been combined with LC systems in LC/MS instruments. LC/MS systems are

invariably computer controlled. With these instruments, both real-time and computer-reconstructed chromatograms and spectra of the eluted peaks can be obtained.

MATERIALS AND METHODS

Materials

1. *Jatropha curcas* seed oil from KU Biodiesel Project, Kasetsart University
2. *Jatropha curcas* press cake from KU Biodiesel Project, Kasetsart University
3. Reagents
   - Methanol (Analytical grade, Merck, Germany)
   - Acetronitrile (HPLC grade, Merck, Germany)
   - Hexane (Analytical grade, Merck, Germany)
   - Sodium chloride (Analytical grade, APS, Australia)
   - Sodium hydroxide (Analytical grade, J.T. Baker, USA)
   - Potassium hydroxide (Analytical grade, J.T. Baker, USA)
   - Heptane (Analytical grade, Merck, Germany)
   - Boron trifluoride in methanol (BF_3,14%v/v, Supelco Analytical, USA)
4. Chemical standards
   - 4β, 9α, 12β, 13α, 20-pentahydroxytiglia-1, 6-dien-3-on-12β-myristate-13- acetate (tetradecanoylphorbolacetate, TPA) (Sigma, USA)
   - Mehylheptadecanoate
   - Fatty acid methyl esters mixture (C_8-C_{24}) (Supelco Analytical, USA)
5. Adsorbent agents
   - Activated carbon from Patum Vegetable Oil Co., Ltd., Thailand
   - Bentonite 150 mesh from Patum Vegetable Oil Co., Ltd., Thailand
   - Bentonite 200 mesh from Patum Vegetable Oil Co., Ltd., Thailand
   - Chitosan (Seafresh chitosan (lab), Thailand)
   - Chitin (Seafresh chitosan (lab), Thailand)

Equipments

1. Balance 4 digit (Percisa, 120A, USA.)
2. Soxhlet Extraction Instrument (Büchi, B811, Switzerland)
3. Gas Chromatography Instrument (Agilent Technique, 6890N, USA.)
4. High Performance Liquid Chromatography with UV detector (Shimadzu, LC-10AC , Japan)
5. High Performance Liquid Chromatography with diode array and mass spectrometry detector (Agilent Technique, USA.)
6. Surface area analysis (Quanta Chrome, Atosorpb-1)
7. Platfrom Shaker (Inonva 2100, Japan)
8. Centrifugation (Mermle, Z323, Germany)
9. Autoclaving (Dectra, USA.)
10. Rota evaporator (BÜCHI, R114, Switzerland)
11. Overhead Stirrer (Ingenieurbüro, CAT R17, Germany)
12. Hot air oven (Binder, German)
13. Fourier transform infrared spectrophotometer (Perkin Elmer System 2000, USA)
14. Kjeldakl-digestion and distillation system (C. Gerhardt GmbH & Co. KG, VAP30, Germany)
15. Water bath (Memmert, WB14, Germany)

Methods

1. Elimination of phorbol esters from seed oil by adsorption process

1.1 Selection of the most suitable adsorbent

About 25 ml of seed oil were mixed with 0.8 g of each adsorbent (activated carbon, bentonite150, bentonite200, chitin and chitosan) into a 250 ml Erlenmeyer flask. Adsorption was experimented at room temperature for 45 min of stirring time and 200 rpm of stirring rate. After that adsorbent and seed oil were separated by filtration with filter paper No.1. Extracted phorbol esters from 10 g of the seed oil with methanol. Content of phorbol esters was analyzed by HPLC. The best adsorbent was selected from maximum adsorbed phorbol esters from seed oil.

1.2 Optimization of the one-time adsorption

About 25 ml of seed oil were mixed with the most suitable adsorbent from experiment 1.1 in a 250 ml Erlenmeyer flask. The experiments were continued in order to find the optimum conditions of adsorption in terms of the following factors:

a. Amount of adsorbent: 0.4, 0.6, 0.8, 1.0, 1.2, 1.4 and 2.0 g.
b. Stirring time: 15, 30, 45, 60, 120 and 180 min.
c. Temperature: 32, 45, 65, 85 and 120˚C.
d. Stirring rate: 0, 100, 150, 200, 250 and 300 rpm.

After each experiment, the adsorbent and seed oil were separated by filtration with filter paper no.1. Phorbol esters substance was extracted from the seed oil and the amount of phorbol esters was analyzed by HPLC.

1.3 Optimization of two-time adsorption

About 25 ml of seed oil from the one-time adsorption were mixed with the most suitable adsorbent from experiment 1.1 in a 250 ml Erlenmeyer flask. The experiments were continued in order to find the optimum conditions of adsorption in terms of the following factors:

a. Amount of adsorbent: 0.2, 0.4, 0.6, 0.8 and 1.0 g.
b. Stirring time: 0, 15, 30 and 45 min.

After each experiment, the adsorbent and seed oil were separated by filtration with filter paper No.1. Phorbol esters substance was extracted from the seed oil and amount of phorbol esters was analyzed by HPLC.

2. Removal of phorbol esters from press cake by the base solution

2.1 Treatment of the base solution

About 20 g of press cake were mixed with 200 ml of 2% (w/w) base solution (sodium hydroxide and potassium hydroxide in distilled water) in a 400 ml beaker. The experiments were carried out at room temperature by stirring at 200 rpm for 60 min. After that, press cake and solution were separated by filtration through filter cloth and the press cake was washed with water until the pH of the waste water was 7. Then, the press cake was dried in oven at 105˚C for 8 hours. The phorbol esters substance was extracted from dried press cake with methanol by soxhlet extraction.

2.2 Optimization of washing condition

About 20 g of press cake were mixed with the best chemical treatment from experiment 2.1 in a 400 ml beaker. The experiments were carried out at various factors affecting the washing condition:

a. The concentration of base solution: 1, 2, 3, 4 and 5% (w/w).

b. The ratio of press cake and base solution from 2.2(a): 1:5, 1:8, 1:10 and 1:12 (w/v).

c. Washing time: 15, 30, 45, 60, 120, 180, 300 and 420 min.

d. Temperature: 32, 40, 60, 70 and 120˚C.

e. Stirring rate: 0, 100, 200, 300 and 400 rpm.

After each experiment, press cake and solution were separated by filtration through filter cloth. After that, the press cake was dried in oven at 105˚C for 8 hours. The phorbol esters substance was extracted from dried press cake with methanol by soxhlet extraction instrument.

The press cake from the optimum washing was soaked in 95% ethanol for 1 night. After that, press cake and solution were separated by filtration through filter cloth. Then, the press cake was dried in oven at 105˚C for 8 hours. The phorbol esters substance was extracted from dried press cake with methanol by soxhlet extraction instrument.

3. Analytical methods

3.1 Phorbol esters extraction

3.1.1 Phorbol esters extraction in *Jatropha* seed oil

Phorbol esters in seed oil were extracted from 10 g of seed oil with 10 ml of methanol for 4 times using funnel separation. The combined extracts were centrifuged to separate the extracts from the oil residue at 3000 rpm for 15 min. Then, the extracts were concentrated with rotaevaporator at 45˚C and 200 mmHg. After that, the concentrated extracts were transferred into a 25 ml volumetric flask and filled up to 25 ml with methanol. The extracts were stored at -20˚C for HPLC analysis.

Figure 13 Phorbol esters extraction from *Jatropha* seed oil with funnel separation.

3.1.2 Phorbol esters extraction from solid sample

Solid samples (such as press cake, kernel, leaves, seed, fruit hull, etc.) were milled and dried at 105˚C in oven for 8 hours. The dried solid sample was weighed around 6.0 g and extracted with 125 ml methanol by soxhlet apparatus. The extraction program was settled at 30 cycles for 4 hours. Then the extracts were concentrated with rotaevaporator at 45˚C and 200 mmHg. After that, the concentrate extracts were transferred into a 25 ml volumetric flask and filled up to 25 ml with methanol. The extracts were stored at -20˚C for HPLC analysis.

Figure 14 Phorbol esters extraction from solid samples (i.e. kernels, bark, fruit bark, etc.) with soxhlet apparatus.

3.2 Analysis of phorbol esters content

3.2.1 Preparation of sample

About 1.5 ml of the extracts were filtered through 0.45 µl membrane prior to the measurement of the phorbol esters by HPLC. The operation condition was 1 ml/min flow rate, 35˚C thermal control column, 280 nm UV detector and 20 µl samples were injected. The mobile phase was acetronitrile and deionized water (80:20, v/v) with isocratic mode.

3.2.2 Calibration curve of phorbol esters standard

The standard tetradecanoylphorbolacetate (TPA) was dissolved in methanol. TPA standard concentrations were prepared at 10, 20, 30, 40 and 50 ppm, respectively. After that, phorbol esters content in the form of TPA was measured as previously described in 3.2.1. Area peak and phorbol esters concentration were plot on y-axis and x-axis, respectively. The calibration curve was a straight line that passed through the origin point. The external standard technique was used to quantify phorbol esters content according to the standard curve.

3.2.3 Analysis of phorbol esters spectrum

About 1.5 ml of the preparated sample were filtrated through 0.45 µl membrane. The HPLC operation condition was 0.4 ml/min of flow rate, 25˚C of thermal control column, 282 nm of wave length of PDA detector and a total of 20 µl of total samples were injected. The mobile phase was acetronitrile and deionized water (80:20, v/v) with isocratic mode. The operation MS detector condition was 45 psig of nebulizer gas pressure, 325˚C of nitrogen gas temperature, 3000 V of capillary voltage, 100 V for fragmentation and used eletrospray negative (ESI) of ionization mode.

3.3 Fatty acid composition analysis

3.3.1 Preparation of sample

About 40 mg oil and 5 ml of 0.5 M sodium hydroxide in methanol were refluxed at 90˚C for 30 min. Then, 3 ml of boron trifluoride (BF_3, 14% v/v) in methanol were added and refluxed continuously at 90˚C for 2 min. After cooling to room temperature, 3 ml of saturated sodium chloride and 10 ml of heptane were added followed by shaking for 5 min and allowed the mixture to separate. The upper layer was analyzed for fatty acids content by GC.

About 1 μl of fatty acid methyl esters (FAME) was injected into GC of splitless mode. The condition of GC analysis was 11.49 psi head pressure and 2.5 ml/min splitless vent flow rate. The injector temperature was set at 250˚C. Concentration of FA was calculated from the standard curve. Methylheptadecanoate was used as an internal standard (IS) for quantification of fatty acids content.

Figure 15 Derivatization of the *Jatropha* seed oil for fatty acids analysis.

3.3.2 Calibration curve of FA standard

The standard (fatty acid methyl esters mixture, C_8 – C_{24}) was dissolved in heptane. FA standard concentrations were prepared at 625, 1250, 2500, 5000 and 10000 ppm, respectively. FA concentration was measured and followed the measurement procedure of FA method. Area peak and FA concentration were plotted on y-axis and x-axis, respectively. The calibration curve was a straight line which passed through the origin point.

3.4 Analysis of adsorbent surface area

The surface area of all adsorbents was estimated by the Brunauer-Emmett-Teller (BET) method. The 1.00-0.02 g samples were pretreated at 300˚C under vacuum. This method was analyzed by Thailand Institute of Scientific and Technological Research (TISTR).

3.5 FT-IR analysis of adsorbent

The adsorbent samples before and after phorbol esters adsorption were analyzed by FT-IR (Fourier transform infrared spectrophotometer). FT-IR spectra of phorbol esters on adsorbent surface were collected by taking 32 scan at 2 cm^{-1} resolution. The spectra were scan from 4000 to 400 cm^{-1} and all samples were taken in potassium bromide (KBr).

3.6 Analysis of protein

The protein in press cake was analyzed by the Kjeldahl method. The 0.5-1.0 g samples were used. This technique is based on the conversion of the bound nitrogen to ammonia which is then separated by distillation and determined by titration. This method was analyzed by Kasetsart Agricultural and Agro-Industrial Product Improvement Institute (KAPI).

3.7 Analysis of amino acids

Amino acid analysis in press cake was a process to determine the quantities of each individual amino acid in a protein. There are four steps in amino acid analysis that are hydrolysis, derivatization, separation of derivatized amino acids and data interpretation. This method was analyzed by Central Instrument Facility of Mahidol University.

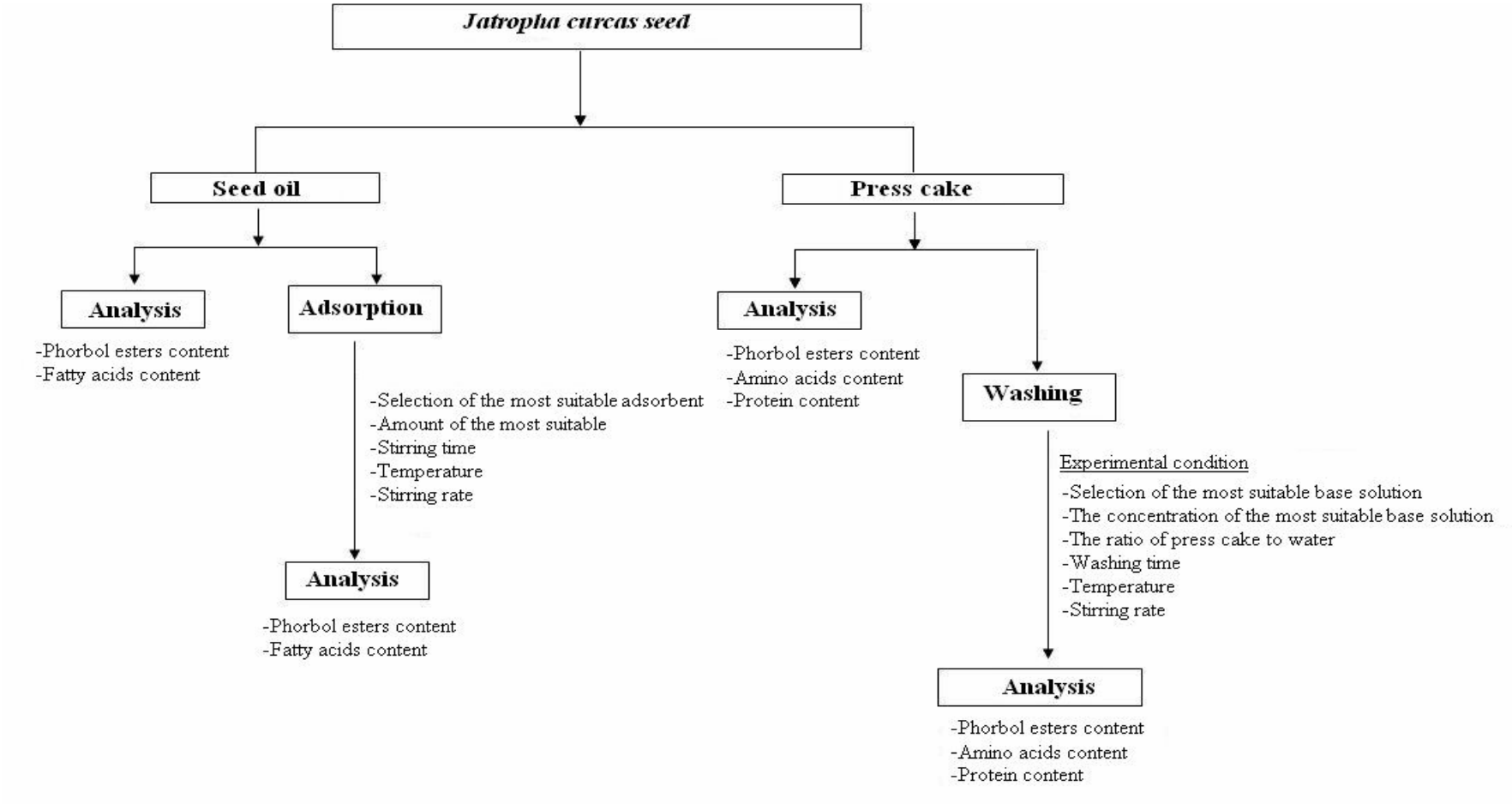


Figure 16 Summary diagram of experiment concept.

RESULTS AND DISCUSSION

1. Raw Material

The seed oil and press cake were presented in Figure 17. The seed oil was pressed from *Jatropha* seed by screw press and then the seed oil and the press cake were separated. After that, the seed oil was filtered through filter paper No.1. Phorbol esters content in seed oil and press cake analyzed by HPLC were approximately 3-6 mg/g and 1-2 mg/g, respectively. However, phorbol esters content of seed oil and press cake depended on the region culture of *J. curcas* L.. For example, phorbol esters content of *Jatropha* varieties from Mexico was about 0.11 mg/g while phorbol esters content of *Jatropha* varieties from Thailand contained about 3-6 mg/g.

(a) **(b)**

Figure 17 The materials of *Jatropha* press cake from screw press (a) and *Jatropha* seed oil (b).

The adsorbents used in this study were activated carbon, bentonite 150, bentonite 200, chitin and chitosan as presented in Figure 18. Activated carbon was a

general term covering carbon material mostly derived from charcoal. Bentonite was special clay and usually formed from weathering of volcanic ash. Bentonite 150 and bentonite 200 are the same material with different particle sizes. The number '150' and '200' behind the word 'bentonite' present the mesh bentonite particle size in mesh. Chitin and chitosan are co-polymers of carbohydrates and included the derivative of Nitrogen-Glucose combination cation molecules. Chitin is a natural organic compound which is insoluble in water and general organic solvents but dissolved in concentrate organic acids. Chitosan can dissolve in various organic acids and form gel, granule and fiber and is used in surface coating. We can find the hard-shelled of shellfish which have many profits for plants, animals and humanity like these.

Figure 18 The adsorbents of Activated carbon (a), Bentonite 150 (b), Bentonite 200 (c), Chitin (d) and Chitosan (e).

The physical properties of adsorbents

The physical properties of 5 adsorbents in this experiment were presented in Table 2. Among all adsorbents, the activated carbon has the highest surface area and is strong alkaline which the activated carbon has 923.80 m^2/g and pH equal of 9.84, respectively. Particle sizes of bentonite 150 and 200 were 150 and 200 mesh, respectively. Bentonite 150 and 200 are strong acidic condition which pH of 3.05 and 2.50, respectively. Chitin and chitosan have large particle sizes with 40 and 60 mesh, respectively, indicating that they contain low surface area and are neutral. However, the surface area of chitosan could not be detected because the temperature of surface area test was 300°C where the chitosan cannot stand for.

Table 2 The physical properties of 5 adsorbents.

Types of adsorbent	Particle size (mesh)	Surface area (m^2/g)	Pore volume (cc/g)	Pore size (°A)	pH
Activated carbon	150	923.80	0.4818	60.1060	9.84
Bentonite 150	150	190.40	0.0885	101.1500	3.05
Bentonite 200	200	327.30	0.1488	101.4000	2.50
Chitin	40	1.13	$5.856E^{-4}$	83.8200	5.60
Chitosan	60	-	-	-	7.90

Figure 19 showed the *Jatropha* seed oil after the adsorption experiment. It demonstrated that all adsorbents improved the clarity of *Jatropha* seed oil. However, bentonite 150 and 200 showed the best adsorption capability as indicated by the clearest of *Jatropha* seed oil after adsorption, followed by activated carbon, chitin and chitosan. The highest adsorption capability of bentonite could be because bentonite is usually applied as a bleaching agent in a traditional edible oil refining.

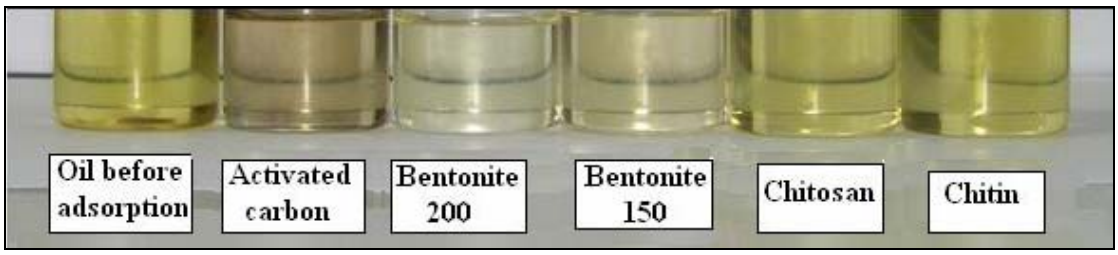


Figure 19 Comparison of *Jatropha* seed oil before and after adsorption with adsorbents.

2. Phorbol Esters Chromatogram

Analysis of phorbol esters by an isocratic mixture of 80% acetronitrile and 20% deionized water showed the retention time of phorbol esters about 8-12 min ast referred with the method of Wink *et al*. (1997). Within 8-12 min, the phorbol esters chromatogram contained 5 peaks and therefore the total area of the 5 phorbol esters peaks were used for quantification (Figure 21). Although the phorbol esters found in *Jatropha* seed was DHPB (12-Deoxy-16-hydroxyphorbol-4'-[12',14'-butadienyl]-6'-[16',18',20'-nonatrienyl]-bicyclo[3.1.0] hexane-(13-*0*)-2'-[carboxylate]-(16-*0*)-3'-[8'-butenoic-10']ate, Hass and Mittelbach, 2000) but it is not commercially available. TPA was used as external standard for quantification of phorbol esters according to Wink *et al*. (1997). However, this could lead to far higher values than when using DHPB in this experiment, only the relative decrease of phorbol esters was interesting thus this difference was neglected (Gläser, 1991).

The chromatograms of standard phorbol esters (phorbol-12-myristate 13-acetate, TPA), phorbol esters obtained from *J. curcas* oil and *Jatropha* wood were presented in Figures 20, 21 and 22, respectively.

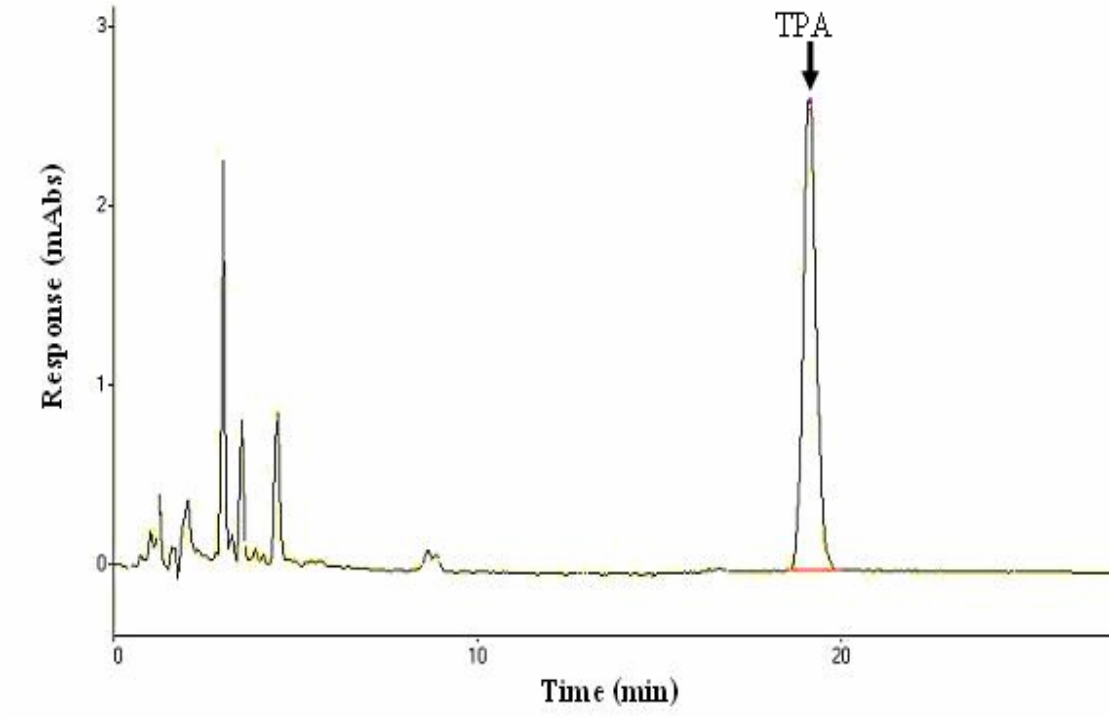


Figure 20 Chromatogram of phorbol-12-myristate-13-acetate (TPA) standard.

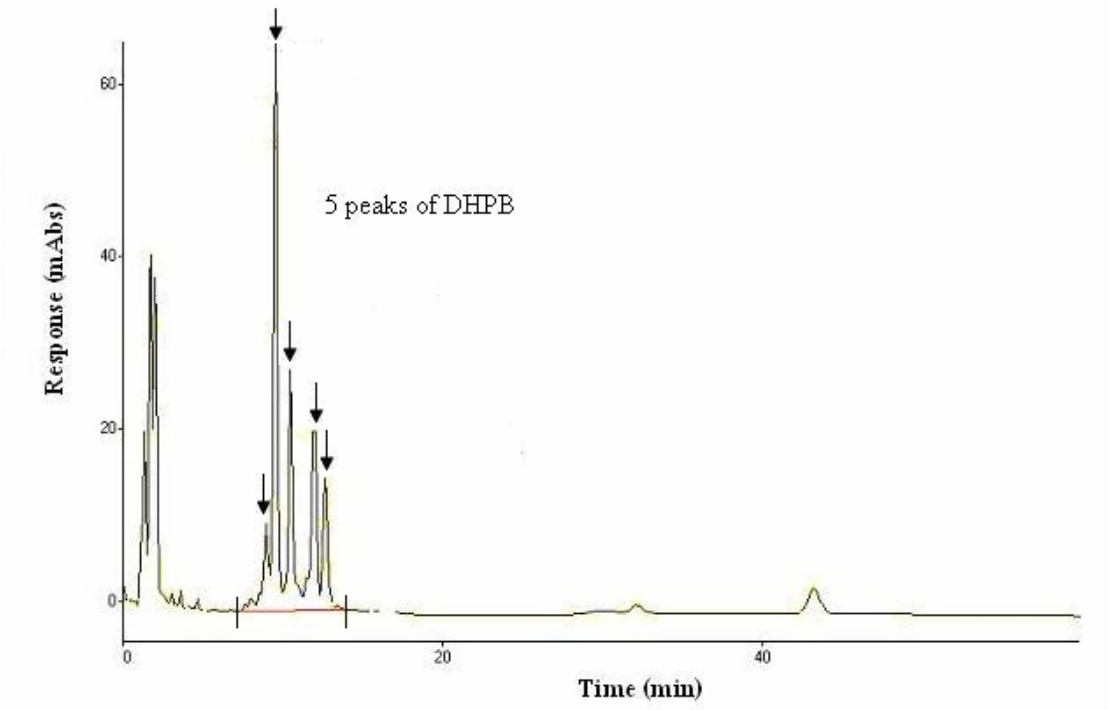


Figure 21 Chromatogram of phorbol esters (DHPB) in *Jatropha* seed oil.

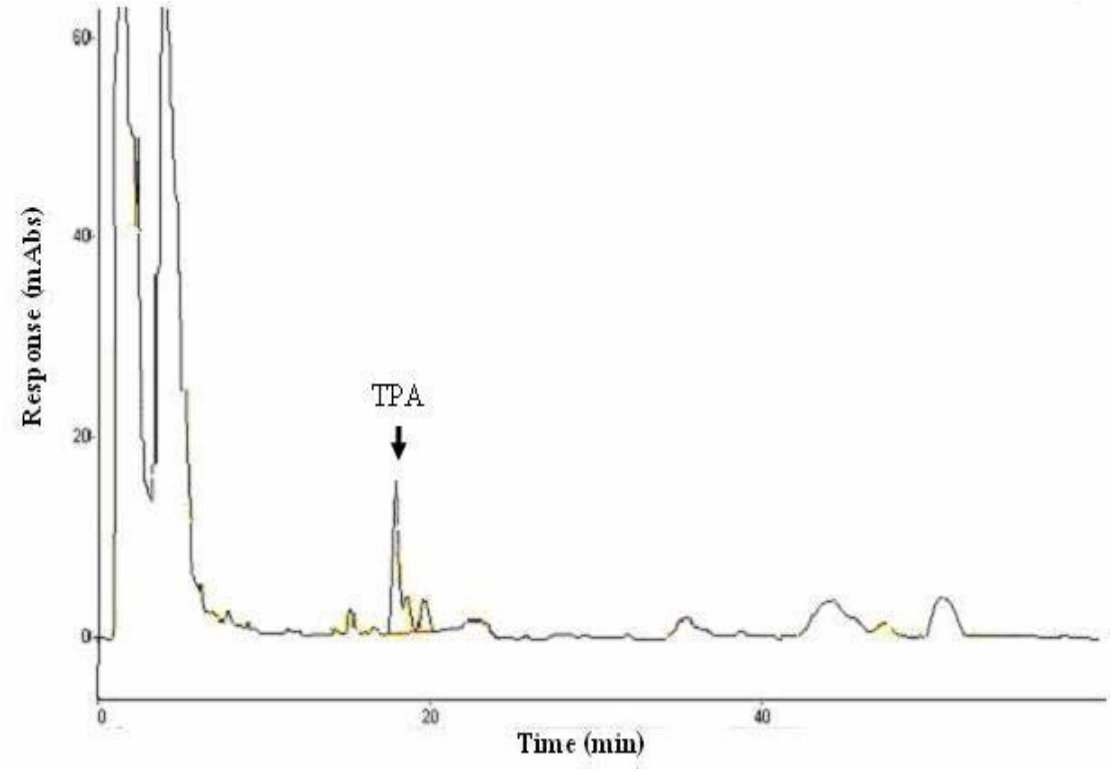


Figure 22 Chromatogram of phorbol esters (TPA) in *Jatropha* wood.

The chromatograms in Figures 20 and 22 from standard TPA and *Jatropha* wood showed the same retention time about 18-21 min indicating that, the detected phorbol esters should be TPA. Figure 21 demonstrated a five peak group during retention time 8-12 min indicating that the phorbol esters in *Jatropha* seed oil was not in the same form as standard and wood TPA. The chromatogram of phorbol esters in *Jatropha* seed oil was consistent with the result by Hass *et al.* (2002) who illustrated that the phorbol esters in *Jatropha* seed oil might be in form of other phorbol esters types. They had main structure of 12-deoxy-16-hydroxyphobol and also contained different side chains to form 6 different phorbol esters. However, the calculation of phorbol esters in this experiment was done based on the commercially available standard TPA according to Hass *et al.* (2000). The phorbol esters content in *Jatropha* seed oil was approximately 4.51 mg/g by weight based on standard TPA.

3. Analysis of Phorbol Esters Spectrum

The utilization of HPLC separation was greatly enhanced by mass spectrometric detection (MS), which allowed to confidently identify the compounds in *Jatropha* seed oil. *Jatropha* seed oil was extracted with methanol and it called the methanol extracts. Methanol extracts were analyzed by combined HPLC/PDA/MS spectrometry. The chromatogram result shown in Figure 23.

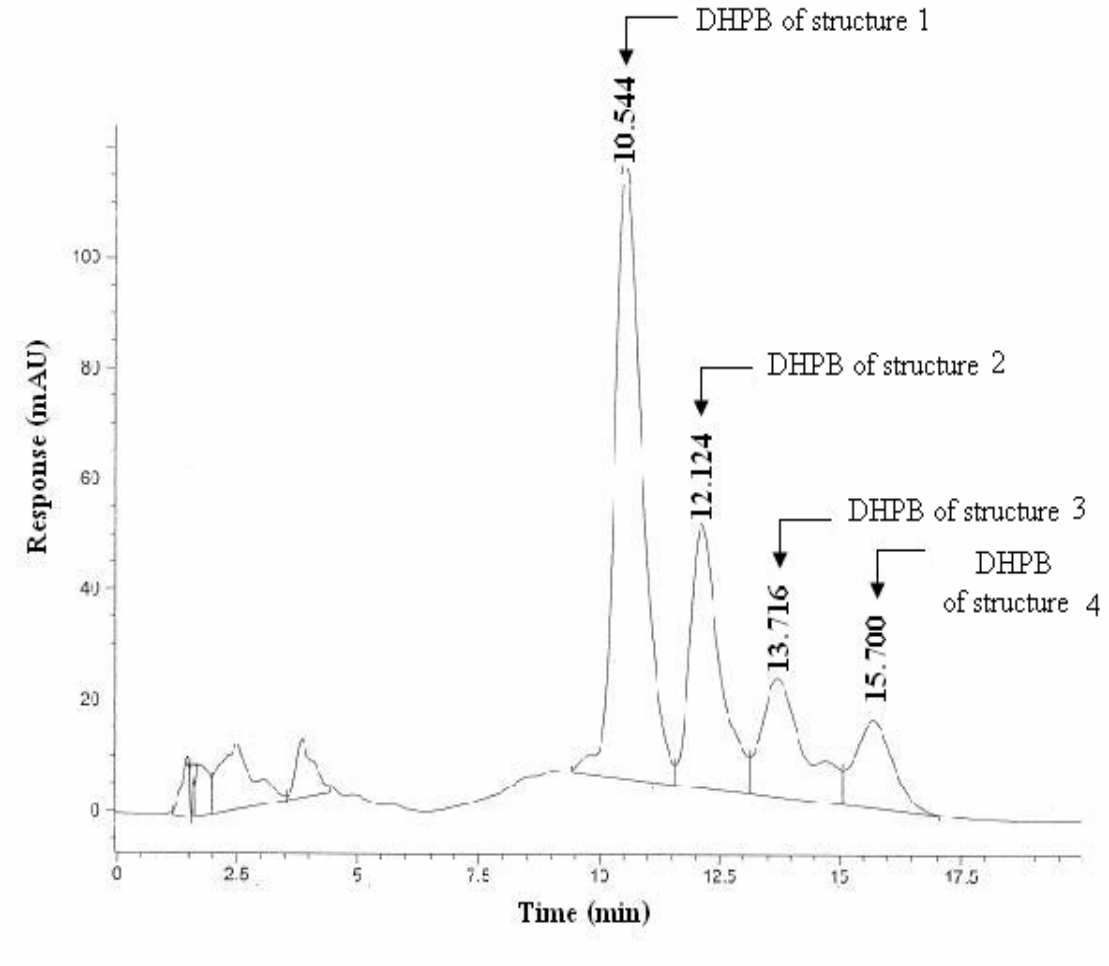


Figure 23 Chromatogram of phorbol esters (DHPB) in *Jatropha* seed oil analyzed with photodiode array detector (PDA).

The combination of HPLC/PDA and tandem mass spectrometry (MS) performed with Ion Trap analyzer permitted to have qualitative data on phorbol esters

(DHPB) derivative. Figure 23 showed DHPB chromatogram of phorbol esters extracts from *Jatropha* seed oil that appeared as a four peak group (detected at 282 nm) during retention time 10-16 min. A four peak group was observed at 10.544, 12.124, 13.716 and 15.700 min, respectively. They were demonstrated with mass spectrometric showing the spectrum of DHPB with molecular weight of 710 corresponding to an intramolecular diester of 12-desoxy-16-hydroxyphorbol as described by Adolf *et al.* (1984) and Hirota *et al*. (1988). This compound was isolated by HPLC/MS as shown in Figures 24, 25, 26 and 27, respectively.

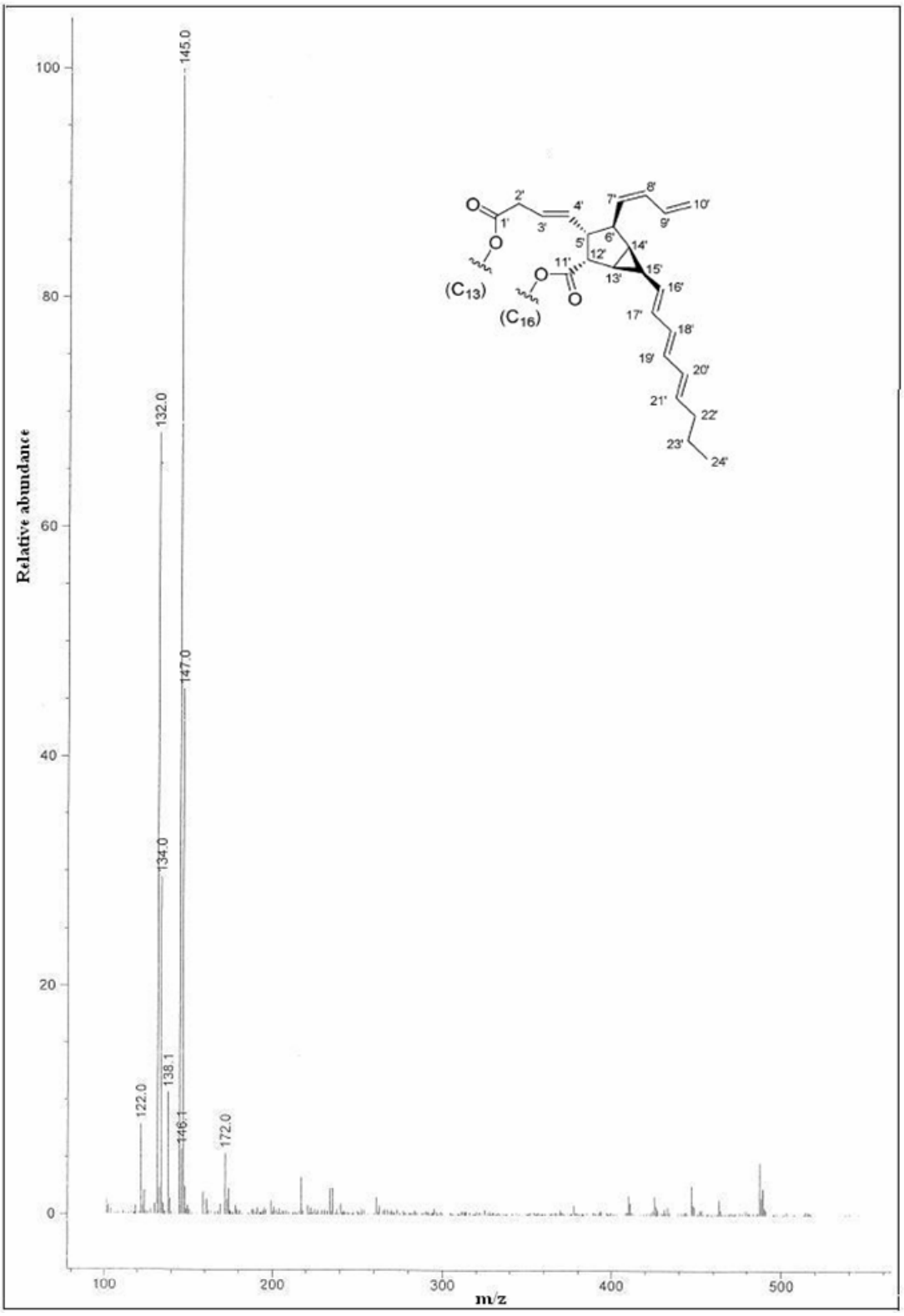


Figure 24 Combination HPLC/PDA/MS of phorbol esters extracts on peak at the retention time of 10.544 min.

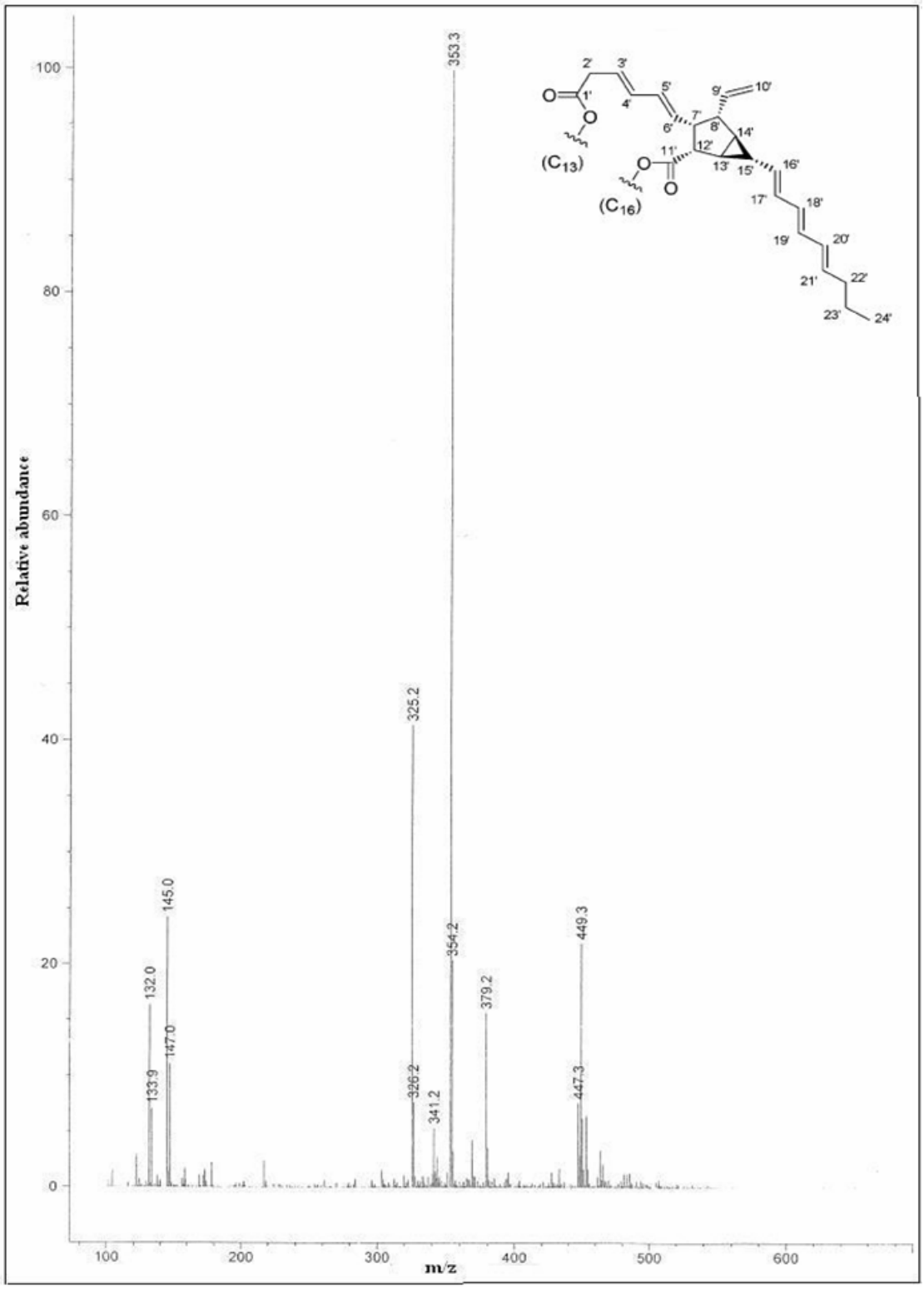


Figure 25 Combination HPLC/PDA/MS of phorbol esters extracts on peak at the retention time of 12.124 min.

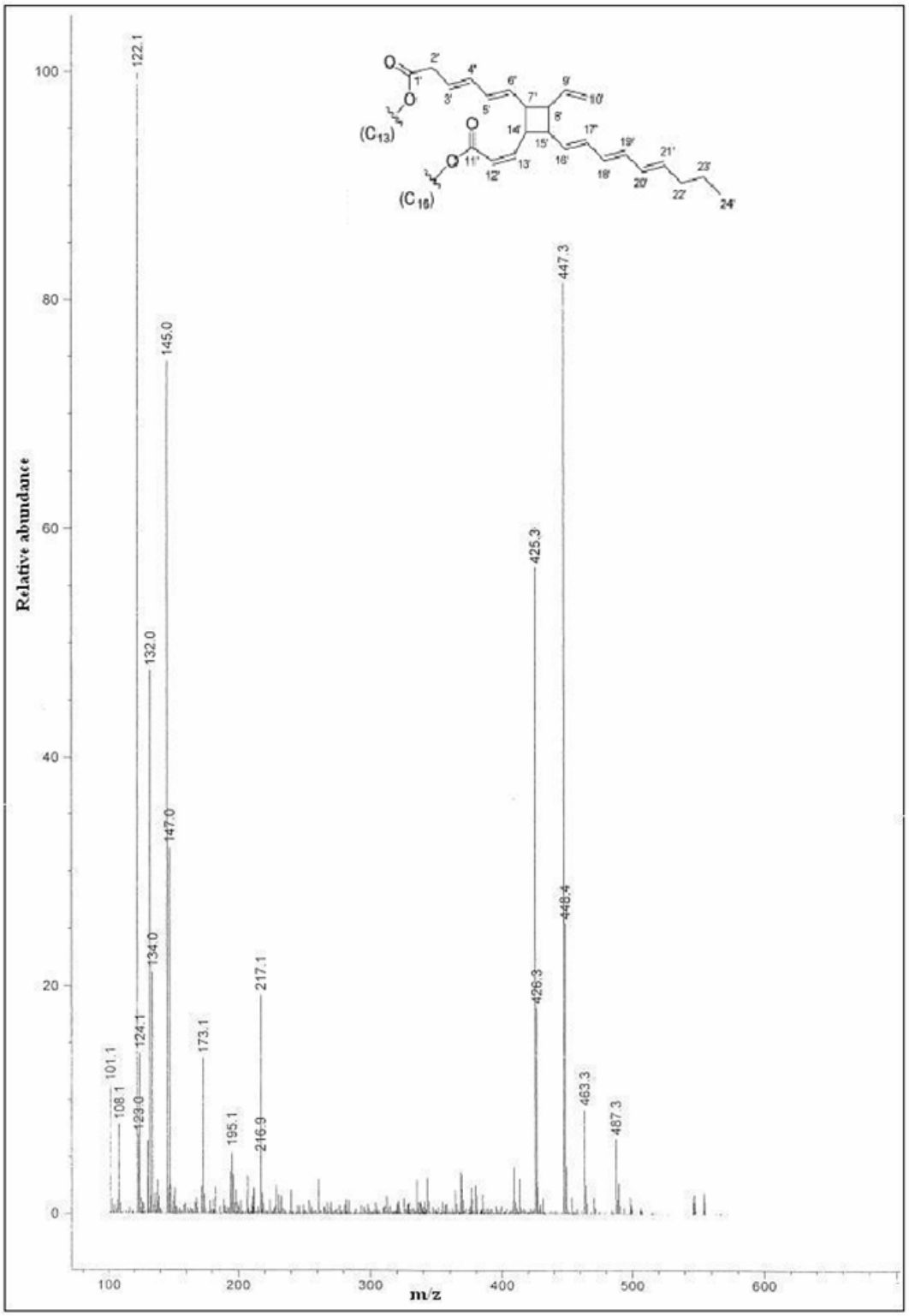


Figure 26 Combination HPLC/PDA/MS of phorbol esters extracts on peak the retention time of 13.716 min.

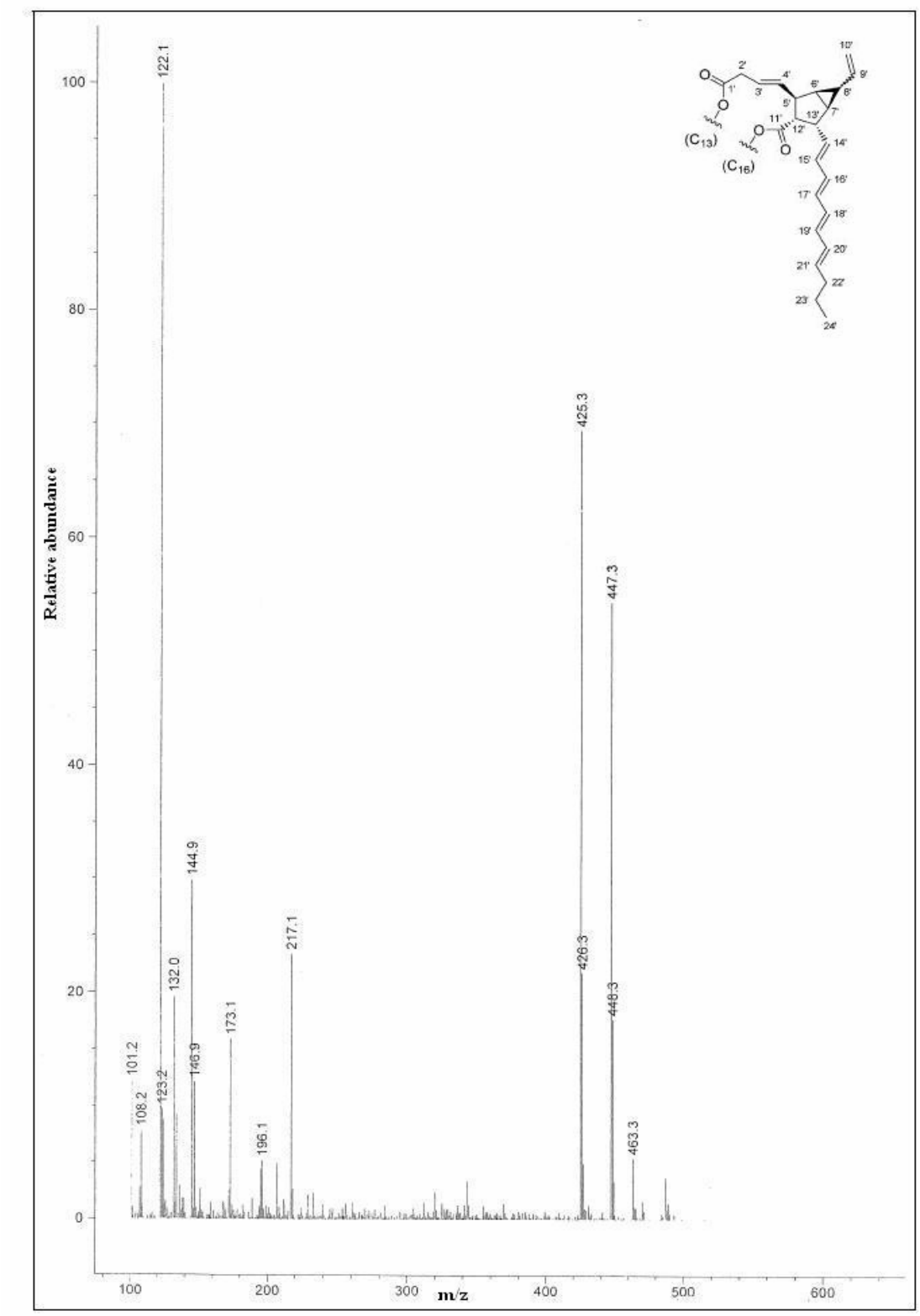


Figure 27 Combination HPLC/PDA/MS of phorbol esters extracts on peak at the retention time of 15.700 min.

Table 3 Mass ion spectrum of phorbol esters extract detected by HPLC/MS.

Retention time (min)	Mass ion at m/z
10.544	122.0, 132.0, 134.0, 138.1, 145, 146.1, 147.0, 172.0
12.124	132.0, 133.9, 145.0, 147.0, 325.2, 326.2, 341.2, 353.3, 354.2, 379.2, 447.3, 449.3
13.716	122.1, 132.0, 134.0, 145.0, 147.0, 173.1, 195.1, 216.9, 217.1, 425.3, 426.3, 447.3, 448.4, 463.3, 487.3
15.700	122.1, 132.0, 144.9, 146.9, 173.1, 196.1, 217.1, 425.3, 426.3, 447.3, 448.3, 463.3

The spectrum in Figures 24, 25, 26 and 27 were summarized in Table 3. The main structure of mass ion at m/z of each spectrum was the same. They were 122.0, 132.0, 145.0 and 147.0. Those spectrums were m/z of tigliane which was the basic structure of occurring phorbol esters. Hass *et al.* (2002) found that 4 peaks of chromatogram in Figure 23 were 4 different structures of DHPB. Those structures of DHPB were illustrated in Figures 24, 25, 26 and 27.

4. Phorbol Esters Content in Different Parts of *Jatropha Curcas* L.

Figure 28 and Table 4 showed parts of *J. curcas* L. used in this experiment and the contents of phorbol esters in those parts analyzed by HPLC. The retention time of phorbol esters based on their types was in 2 ranges at 8-12 min and 18-21 min.

Table 4 Phorbol esters content in different parts of *J. curcas* L..

The parts of *J. curcas* L.	Concentration of Phorbol esters (mg/g)	
	$RT_{8\text{-}12\ min}$	$RT_{18\text{-}21\ min}$
Seed hull	0.3573	-
Kernel	3.6524	-
Oil	4.5084	-
Press cake	1.0326	-
Fruit bark	-	0.7688
Bark	-	2.4449
Wood	-	2.9730

RT = Retention time (min)

The result in Table 4 demonstrated 2 groups of phorbol esters. The first group from seed hull, kernel, oil and press cake were called 'DHPB' with retention time at 8-12 min. DHPB had 6 different chemical structures when analyzed by HPLC/PDA/MS. The second group from fruit bark, bark and wood were not DHPB. They might be TPA because the chromatogram peaks was within 18-21 min which was the same as the retention time of standard TPA. The quantitative content based on TPA of seed hull, kernel, oil, press cake, fruit bark, leave and wood demonstrated 0.0357, 0.3652, 0.4508, 0.1033, 0.0769, 0.2445 and 0.2373 % by weight, respectively. The *Jatropha* seed oil contained highest content of DHPB when compared among the first group whereas the *Jatropha* wood contained the highest content of TPA when compared among the second group.

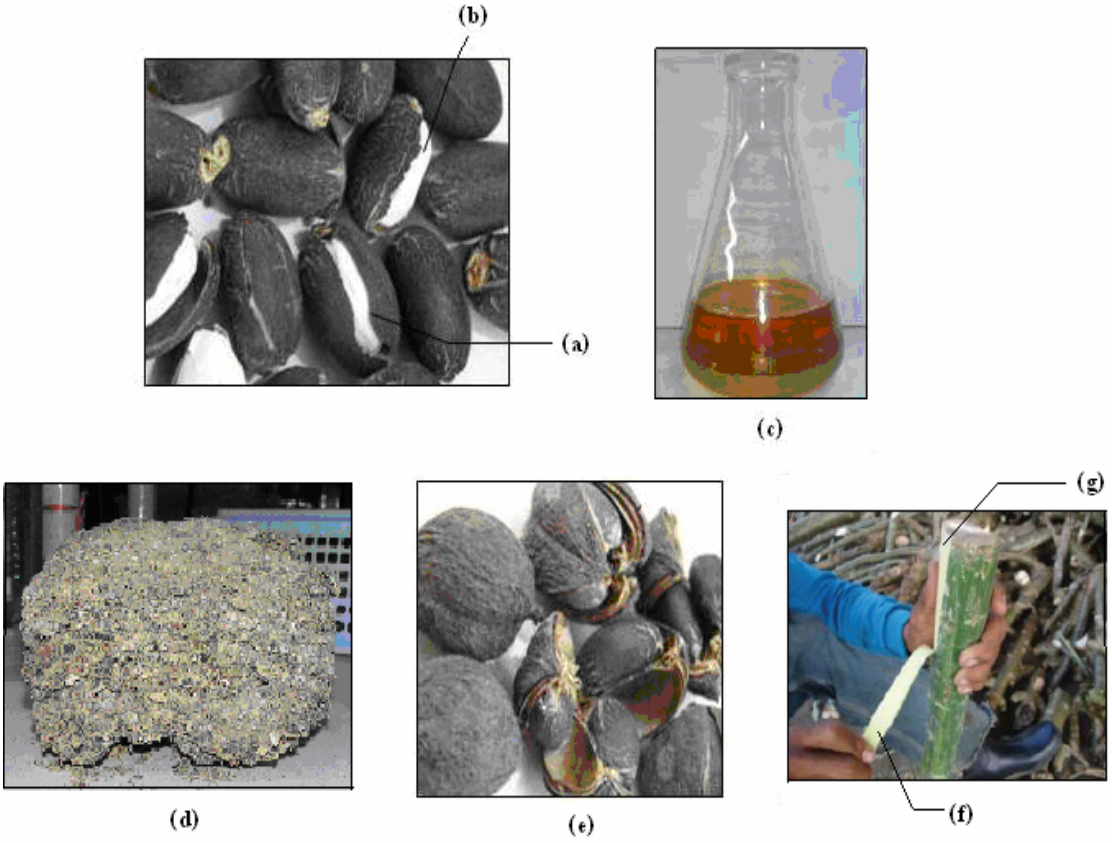


Figure 28 Parts of *J. curcas* L.; Seed hull (a), Kernel (b), Oil (c), Press cake from hydraulic press (d), Fruit bark (e), Bark (f) and Wood (g).

5. Elimination of Phorbol Esters from *Jatropha* Seed Oil

5.1 Selection of the most suitable adsorbent

The phorbol esters adsorption by different adsorbents was presented in Figure 29.

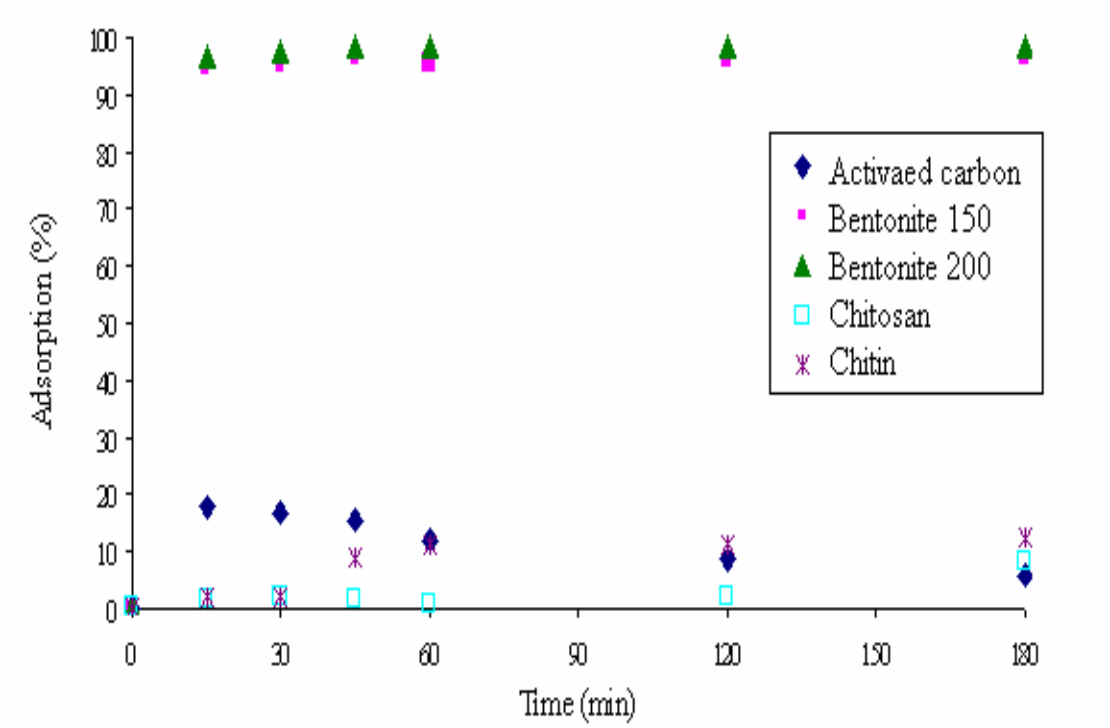


Figure 29 Phorbol esters adsorption capability in *Jatropha* seed oil by 5 adsorbents**.**

All experiments were performed under the same condition which was 3.2% adsorbents (w/w) at room temperature with 200 rpm stirring rate. When increased the stirring time of adsorption, the phorbol esters adsorption was also increased. The results illustrated that the highest % phorbol esters obtained from activated carbon, bentonite 150, bentonite 200, chitosan and chitin were 18.01% (15 min stirring time), 96.09% (45 min stirring time), 98.38% (45 min stirring time), 8.12% (300 min stirring time) and 12.28% (300 min stirring time), respectively.

When considered the physical properties of adsorbents, pH and pore size, the results indicated that pH of adsorbent affected the adsorption capability more than their surface areas and pore sizes. It demonstrated that bentonite 150 and 200 had the adsorption capability more than those of activated carbon, chitin and chitosan. This might be due to stronger acidity of bentonite 150 and 200 (pH 3.05 and 2.50, respectively) compared with the basicity of activated carbon and chitosan. Therefore, it resulted in the hydrolysis reaction simultaneously with the adsorption of phorbol esters. The comparison between bentonite 150 and 200 showed that bentonite 200

had smaller size, stronger acidity and more contact surfaces area and therefore showed higher efficiency for phorbol esters adsorption. As shown that the adsorption capability was higher under the acidic condition. When bentonite 150 and 200 were applied, they contained the same pore size (101°A). Therefore, these results indicated that pH affected of adsorbent the capability more than the surface area and pore size of adsorbents. The phorbol esters adsorption of bentonite 200 reached equilibrium after 15 min and also reached the highest adsorption capability at 96.72%. The removal of phorbol esters from our study was far better than that obtained from Hass *et al.* (2000) which phorbol esters were removed only by 55%.

As a result, bentonite 200 was the most suitable adsorbent for phorbol esters adsorption from the *Jatropha* seed oil and applied for further experiment.

5.2 Optimization of one-time adsorption

The optimum phorbol esters adsorption condition by bentonite 200 (the most suitable adsorbent from previous section) was summarized as follows. The stirring time, amount of bentonite 200, temperature and stirring rate of adsorption were optimized. The results were showed in Figure 30.

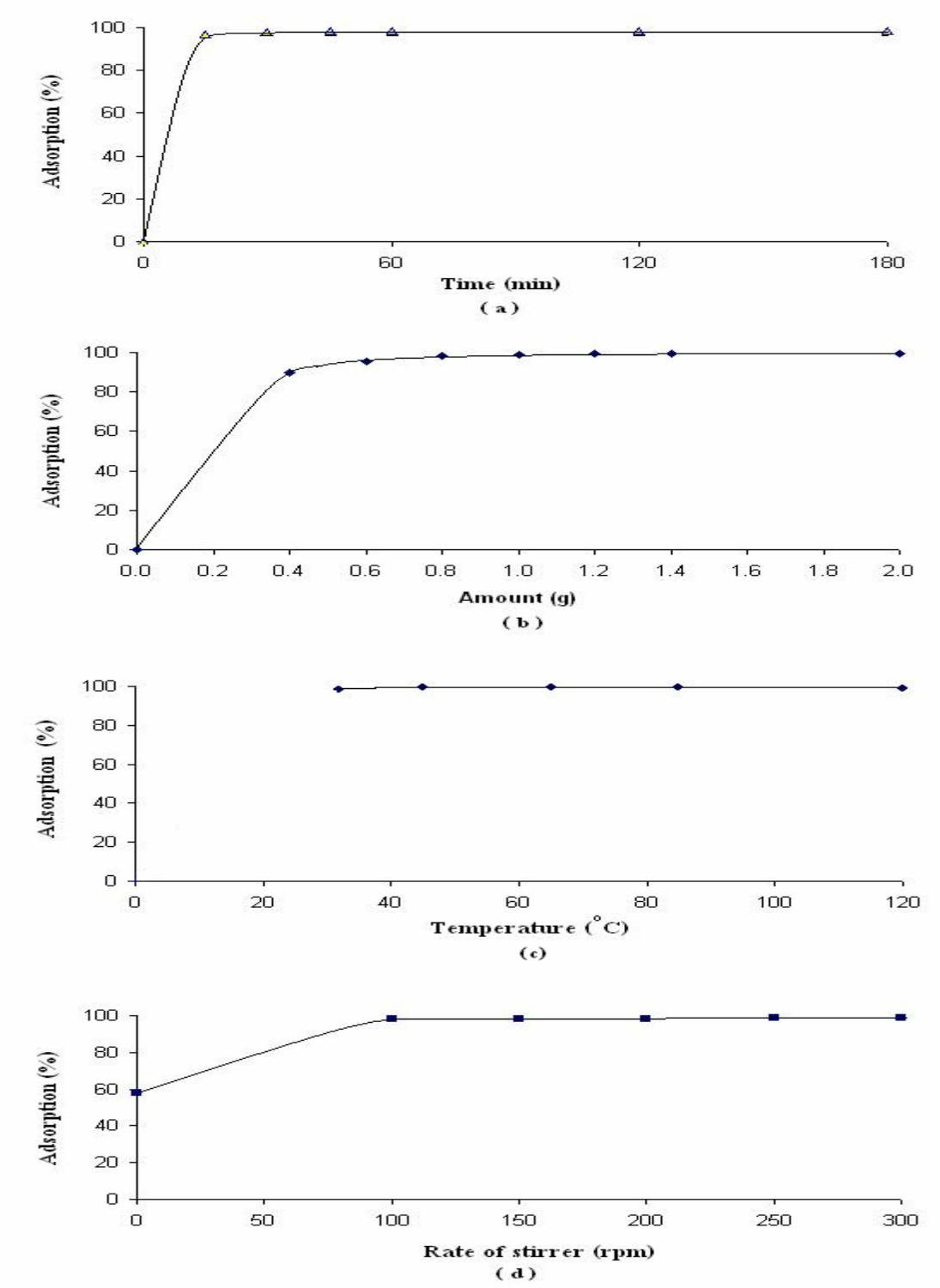


Figure 30 The effect of stirring time (a), amount of bentonite 200 (b), temperature (c) and stirring rate (d) on one-time adsorption of phorbol esters in *Jatropha* seed oil.

Figure 30(a) showed the effect of stirring time on adsorption. The optimum stirring time was 15 min where the phorbol esters adsorption was 96.72%. When increasing the stirring time, %adsorption was also increased until at 45 min where the adsorption became flatten out. In Figure 30 (b), the results indicated an increase in adsorption capability with increasing the bentonite amount with the maximum adsorption at 99.63% when 2.0 g of bentonite 200 were applied. However, the optimum adsorption with 0.8 g bentonite 200 was selected because at the amount of bentonite 200 more than 1.0 g, the adsorption became flattened out. The effect of temperature on adsorption was shown in Figure 30(c). The adsorption capability of all tested temperatures was about 99 % with remaining phorbol esters content as small as 0.11 mg/g (equivalent to that in the non-toxic of *Jatropha* curcas L.). The effect of stirring rate on adsorption capability of bentonite 200 was shown in Figure 30 (d). As the stirring rate increased, the adsorption capability also increased. However, the stirring rate faster than 100 rpm gave constant adsorption about 98%, therefore, 100 rpm was selected as the most optimum stirring rate for adsorption by bentonite 200.

The result also showed that the equilibrium condition of adsorption was obtained under the condition: 3.2% (w/v) bentonite 200 at room temperature, 100 rpm of stirring rate and stirring time for 15 min, illustrating high phorbol esters adsorption efficiency at 96.72%, 98.45%, 98.25% and 98.38%, respectively. Temperature and stirring rate of adsorption almost did not affect the phorbol esters adsorption with bentonite 200, whereas stirring time and amount of bentonite 200 highly affected the adsorption.

5.3 Optimization of the two-time adsorption

Even though one-time adsorption of *Jatropha* seed oil with bentonite 200 showed high efficiency of phorbol esters removal up to 98.44% or 0.0928 mg/g phorbol esters remained in *Jatropha* seed oil, it would be of more advantageous if there was no remaining phorbol esters at all. As a result, the two-time adsorption was experimented when the seed oil after the one-time adsorption was selected to the adsorption again with new bentonite 200 under the new adsorption condition. Stirring

rate and temperature of the two-time adsorption were fixed at 100 rpm and 32˚C, respectively, according to the previous results. The results were demonstrated in Tables 5 and 6.

Table 5 The effect of bentonite 200 amount on the two-time adsorption.

Weight of BT200 (g)	PEs content (mg/g)		Adsorption for one time (%)	Weight of BT200 (g)	PEs content (mg/g)	Adsorption for two-time (%)
	Before adsorption	After one-time adsorption				
0.8	5.9670	0.0928	98.44	0.0	0.0928	98.45
				0.2	0.0213	99.64
				0.4	0.0186	99.689
				0.6	0.0204	99.66
				0.8	0.0215	99.64
				1.0	0.0212	99.64

Table 6 The effect of stirring time on the two-time adsorption.

Weight of BT200 (g)	PEs content (mg/g)		Adsorption for one time (%)	Weight of BT200 (g)	PEs content (mg/g)	Adsorption for two-time (%)
	Before adsorption	After one-time adsorption				
0.8	5.9670	0.0928	98.44	0	0.0928	98.44
				15	0.0216	99.64
				30	0.0213	99.64
				45	0.0174	99.71

PEs = Phorbol esters

BT200 = Bentonite200

Tables 5 and 6 showed the effect of bentonite 200 amount and stirring time on the second time adsorption, respectively. Phorbol esters content in *Jatropha* seed oil was 5.9670 mg/g. After one-time of adsorption, the remaining phorbol esters in *Jatropha* seed oil was 0.0928 mg/g. The results remonstrated that an increase in bentonite 200 amount increased a little of adsorption capability with the remaining phorbol esters content in *Jatropha* seed oil about 0.0213 mg/g or 99.64% of adsorption. Increasing of stirring rate also increased a little of adsorption capability with the remaining phorbol esters content in *Jatropha* seed oil about 0.0216 mg/g or 99.64% of adsorption. Thus, it was concluded that the maximum adsorption efficiency of phorbol esters from the second time was about 99.60% with remaining phorbol esters in the seed oil of approximately 0.02 mg/g.

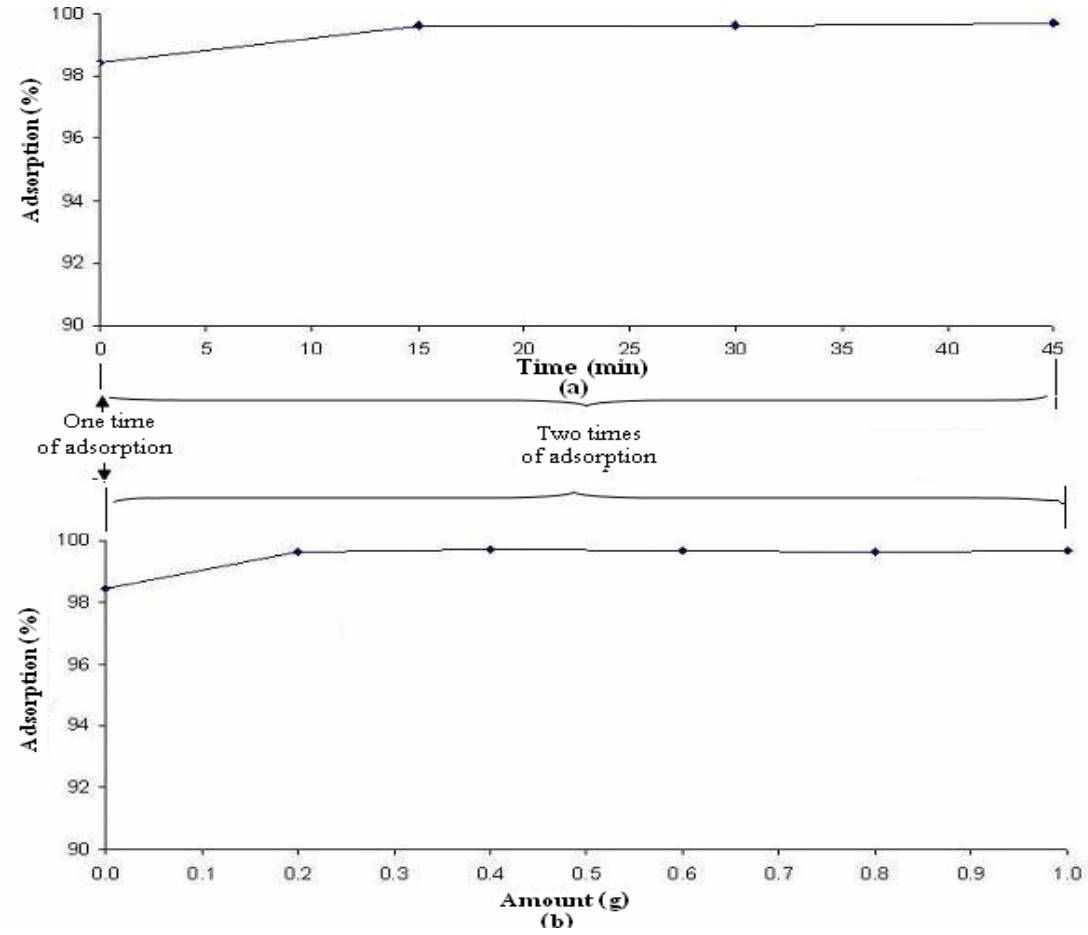
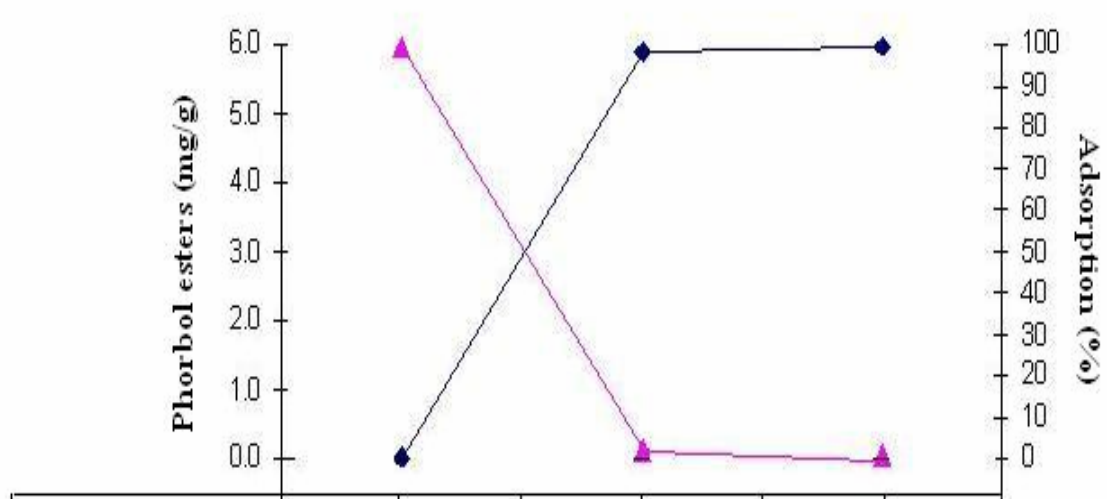


Figure 31 The effect of stirring rate (a) and bentonite 200 amount (b) on the two-time adsorption capability of phorbol esters.

Figure 31 summarized the adsorption efficiency of bentonite 200 on phorbol esters for one and two times. The equilibrium condition was 0.2 g bentonite 200 (0.8%, w/v), 15 min stirring time at 32˚C (room temperature) and 100 rpm stirring rate. The adsorption of phorbol esters was up to 99%.

Figure 32, the comparison between one and two-time adsorption showed the phorbol esters content remained in *Jatropha* seed oil and percentage of adsorption. The two-time adsorption could increase adsorption about 1.20% from the one-time adsorption and the remaining phorbol esters were about 0.0213 mg/g. When increased amount of bentonite 200 more than 0.8% (w/v) and increased stirring time longer than 15 min, it showed almost no effect on the adsorption capability. As a result, bentonite 200 had limite to adsorb phorbol esters in *Jatropha* seed oil in the two-time adsorption.



Time of adsorption	0	1st	2nd
Phorbol esters (mg/g)	5.9670	0.0928	0.0213
Adsorption (%)	0.0000	98.4448	99.6430

Figure 32 One-time and two-time of adsorption capability of phorbol esters in seed oil.

6. Adsorption Isotherm

Adsorption isotherm of phorbol esters from *Jatropha* seed oil by bentonite200 was fitted well by Freundlich and Langmuir as presented in Figures 33 and 34.

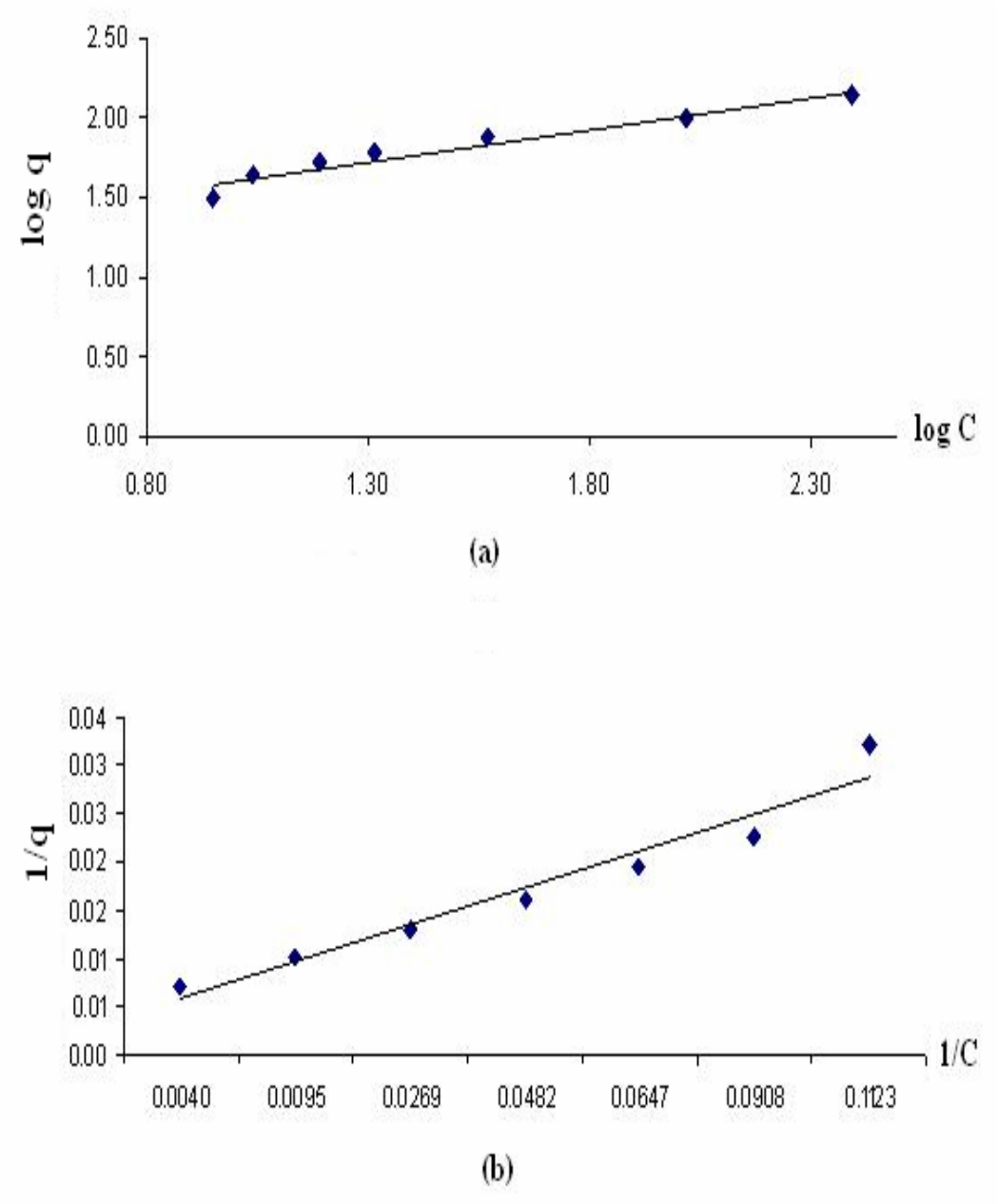


Figure 33 Adsorption isotherms of phorbol esters from *Jatropha* seed oil by bentonite200 (the one-time); Freundlich isotherm (a) and Langmuir isotherm (b).

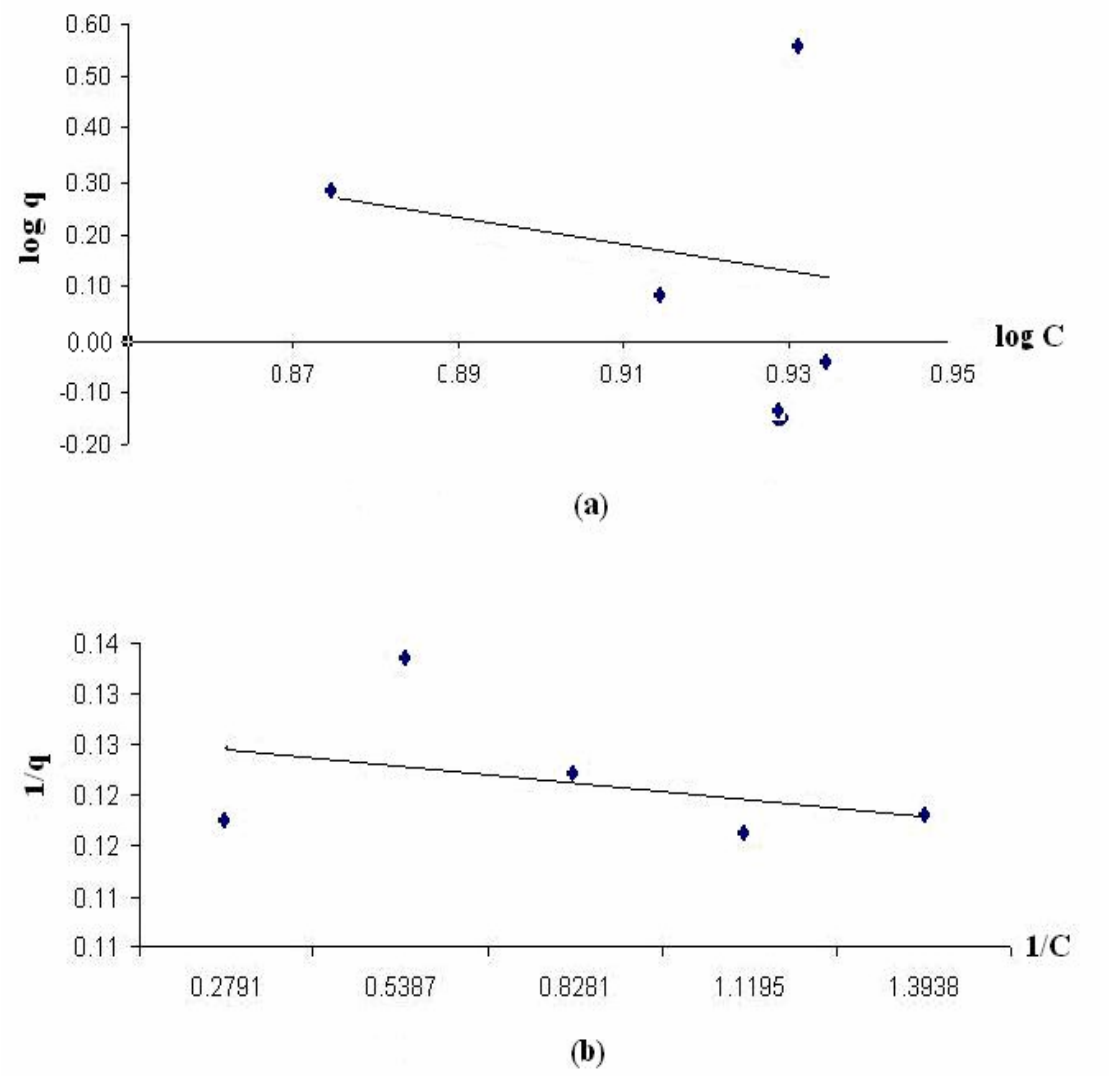


Figure 34 Adsorption isotherms of phorbol esters by bentonite200 (the two-time); Freundlich isotherm (a) and Langmuir isotherm (b).

Table 7 showed the correlation coefficients (R^2) of Freundlich and Langmuir models. The R^2 of Freundlich and Langmuir isotherms for one-time adsorption were 0.9428 and 0.9446, respectively. The high R^2 indicated well fit with the isotherms. The 1/n (constant of Freundlich) was 0.4000 while q_{max} (maximum loading adsorbate on adsorbent of Langmuir) was 500 mg/g of bentonite 200. The high R^2 indicated well fit with the isotherms. As a result, the one-time adsorption was monolayer and multilayer adsorption. The R^2 of Freundlich and Langmuir isotherms for two-time adsorption were 0.0521 and 0.1292, respectively. The low R^2 indicated not fit well

with the isotherms because of the fluctuated information of the experiment. The 1/n (constant of Freundlich) was -2.5451 while q_{max} was 7.9239 mg/g of bentonite 200. Freundlich isotherm was not detectable as the two-time adsorption was not linear and therefore did not fit well with this isotherm. As a result, indicated the phorbol esters adsorption by bentonite 200 from the *Jatropha* seed oil was monolayer and multilayer of adsorption which it was physical adsorption.

Table 7 Freundlich and Langmuir parameters of phorbol esters adsorption by bentonite 200.

Adsorption	Freundlich parameters		Langmuir parameters	
	R^2	1/n	R^2	q_{max} (mg/g)
One-time	0.9428	0.4000	0.9446	500.0000
Two-time	0.0521	-2.5451	0.1292	7.9239

7. Fourier Transform Infrared Spectrophotometer Analysis

The adsorption of phorbol esters by bentonite 200 was from chemical and physical reactions. The acid and alkaline properties of adsorbent affected the adsorption of phorbol esters. Stronger acid property of bentonite 150 and 200 resulted in simultaneous hydrolysis with adsorption reactions of phorbol esters. As a result, this adsorption was chemical adsorption. The Freundlich and Langmuir isotherms fitted well with the experiment, therefore this adsorption was physical.

The chemical adsorption was proven with the functional groups of phorbol esters adsorbed on the bentonite 200 surface in Fourier transform infrared spectrum as shown in Figures 35 and 36.

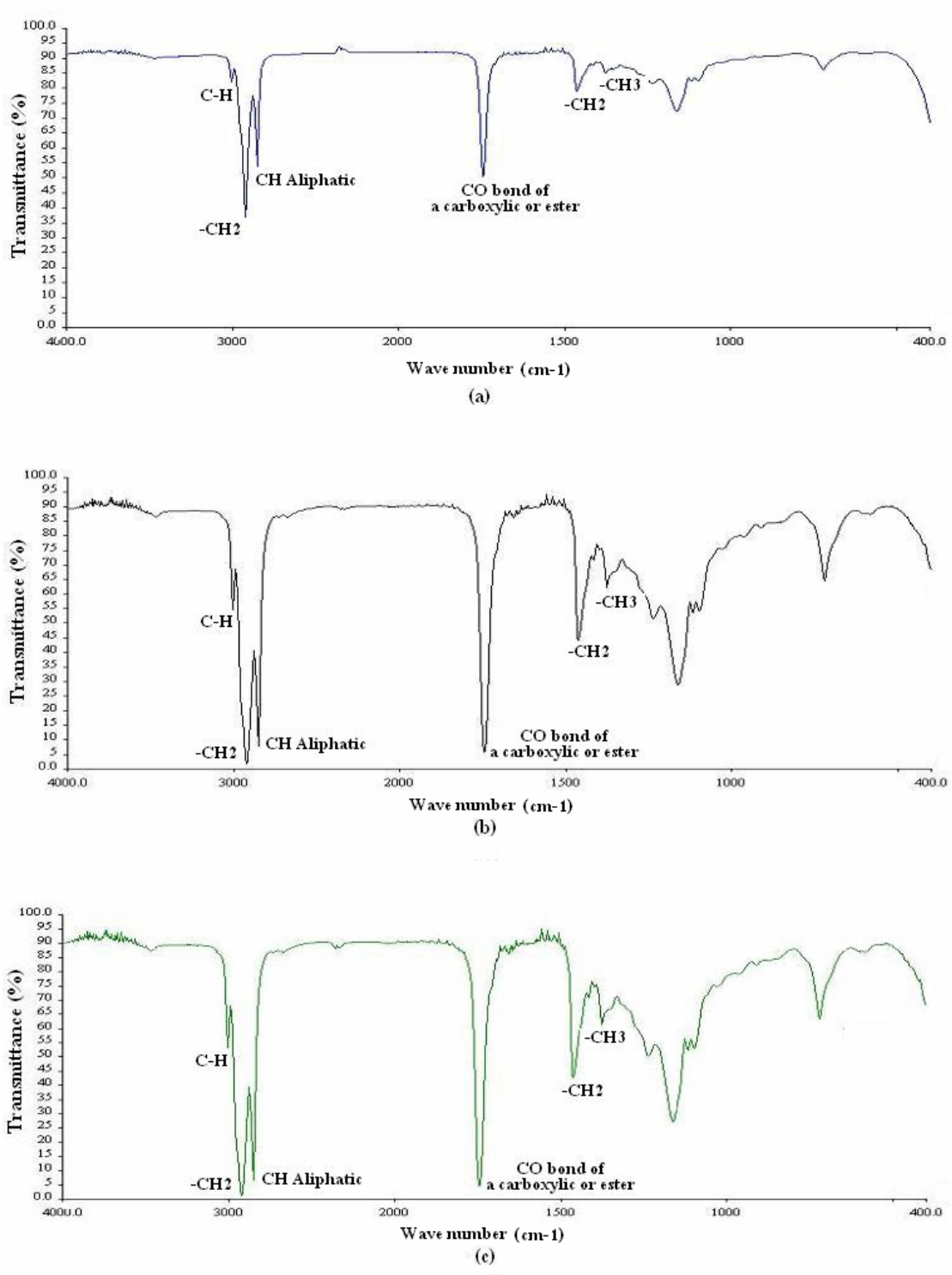


Figure 35 Spectrums of *Jatropha* seed oil detected by Fourier transform infrared spectrophotometer (FT-IR); seed oil (a), one-time adsorption (b) and two-time adsorption (c).

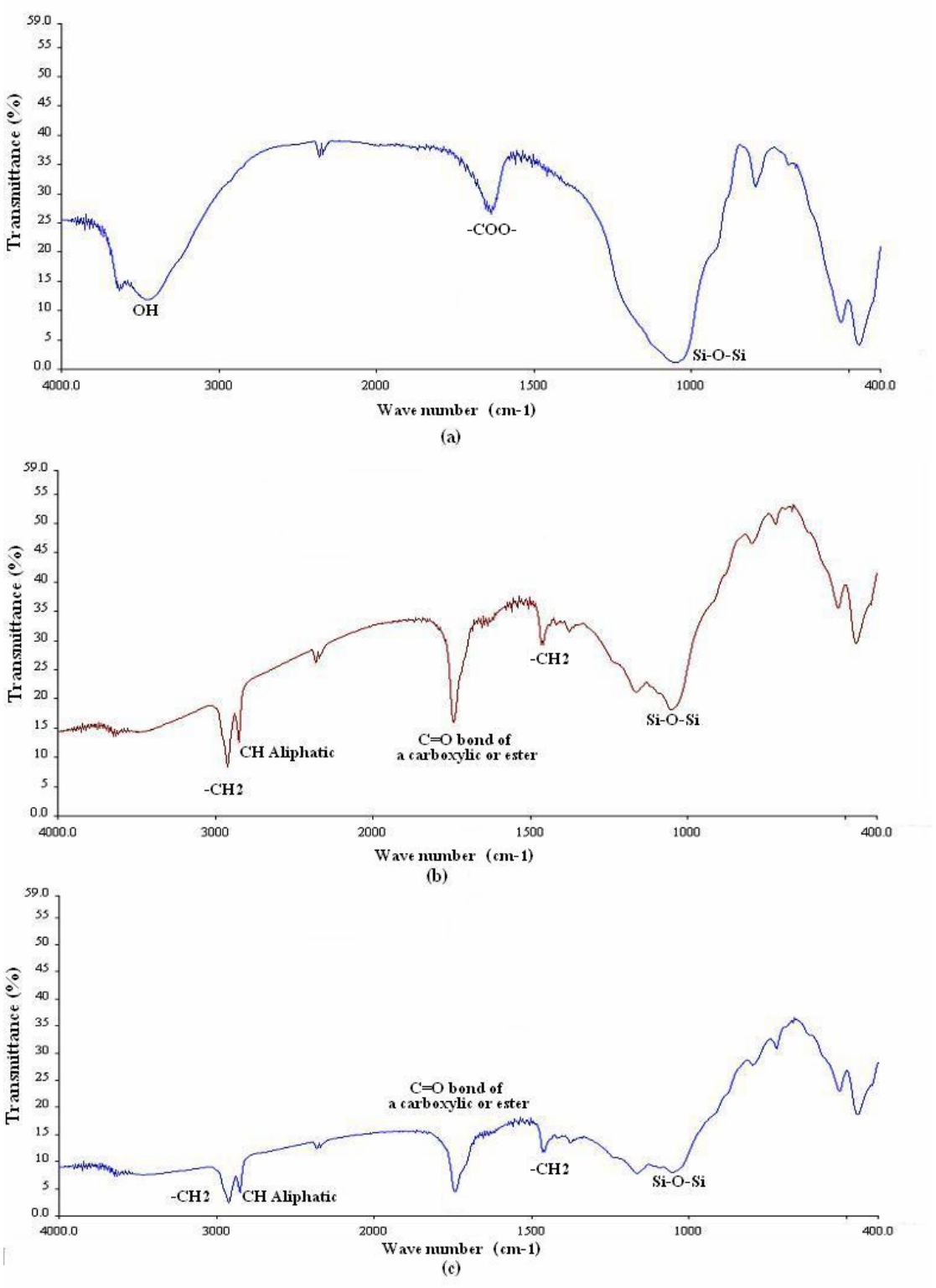


Figure 36 Spectrums of bentonite 200 detected by Fourier transform infrared spectrophotometer (FT-IR); bentonite 200 (a), one-time adsorption (b) and two-time adsorption (c).

Table 8 Wave number and functional groups of *Jatropha* seed oil and bentonite 200 detected by Fourier transform infrared spectrophotometer (FT-IR).

Sample		Wave number (cm^{-1})	Function group
Jatropha seed oil	1. Raw material	3007	C-H (-CH_2-, -CH_3)
	2. One-time adsorption	2925	-CH_2 bond
	3. Two-time adsorption	2854	-CH aliphatic
		1746	C=O bond of ester
		1465	CH_2, CH_3
		1377	CH_3
Bentonite 200	1. Raw material	3448	-OH bond
		1637	-COO-
		1054	Si-O-Si
	2. One-time adsorption	2925	-CH_2 bond
		2854	-CH aliphatic
		1744	C=O bond of ester
		1458	CH_2, CH_3
		1054	Si-O-Si
	3. Two-time adsorption	2925	-CH_2 bond
		2854	-CH aliphatic
		1743	C=O bond of ester
		1466	CH_2, CH_3
		1056	Si-O-Si

Figure 35 presented the spectrum of *Jatropha* seed oil before and after adsorption. The results showed the same spectrum indicating the same functional groups for all experiments. The wave number of spectrums before and after adsorption were 2925, 2854, 1746 and 1465 cm^{-1} equivalent to the functional groups -CH_2 bond, -CH aliphatic, C=O bond carboxylic or ester and CH_2, CH_3, respectively (Table 8). Those functional groups were in the chemical structure of triglyceride in *Jatropha* seed oil.

Figure 36 presented the spectrum of bentonite 200 before and after adsorption. Before adsorption, bentonite 200 was acidic showing the spectrums at 3448, 1637 and 1056 cm^{-1} which were equivalent to –OH, -COO- and Si-O-Si functional groups, respectively. After adsorption, the spectrum was shown at 2925, 2854 and 1746 cm^{-1} relating to the functional group of -CH_2, -CH aliphatic and C=O bond carboxylic or ester, respectively (Table 8) which were in the chemical structure of triglyceride in *Jatropha* seed oil and –COO- functional group was shifted from 1637 cm^{-1} to 1744 cm^{-1}. As a result, this adsorption may be chemical adsorption with bentonite 200 but it could not proved chemical bond in adsorption. Therefore, this adsorption in experiment was physical adsorption and may be chemical adsorption.

8. Fatty Acids in *Jatropha* Seed Oil

The seed kernels contained 40-60% of oil (Makkar *et al.*, 1997) with a fatty acid composition (Gübitz *et al*., 1999) similar to that of oil used for human nutrition (Soy bean, Peanut, Palm oil, etc.). The effect of phorbol esters elimination method on the fatty acid composition of *Jatropha* seed oil was case study. GC was used to determine the fatty acid composition of *Jatropha* seed oil. Peaks associated with the fatty acids were summarized in Figure 37 and Table 9.

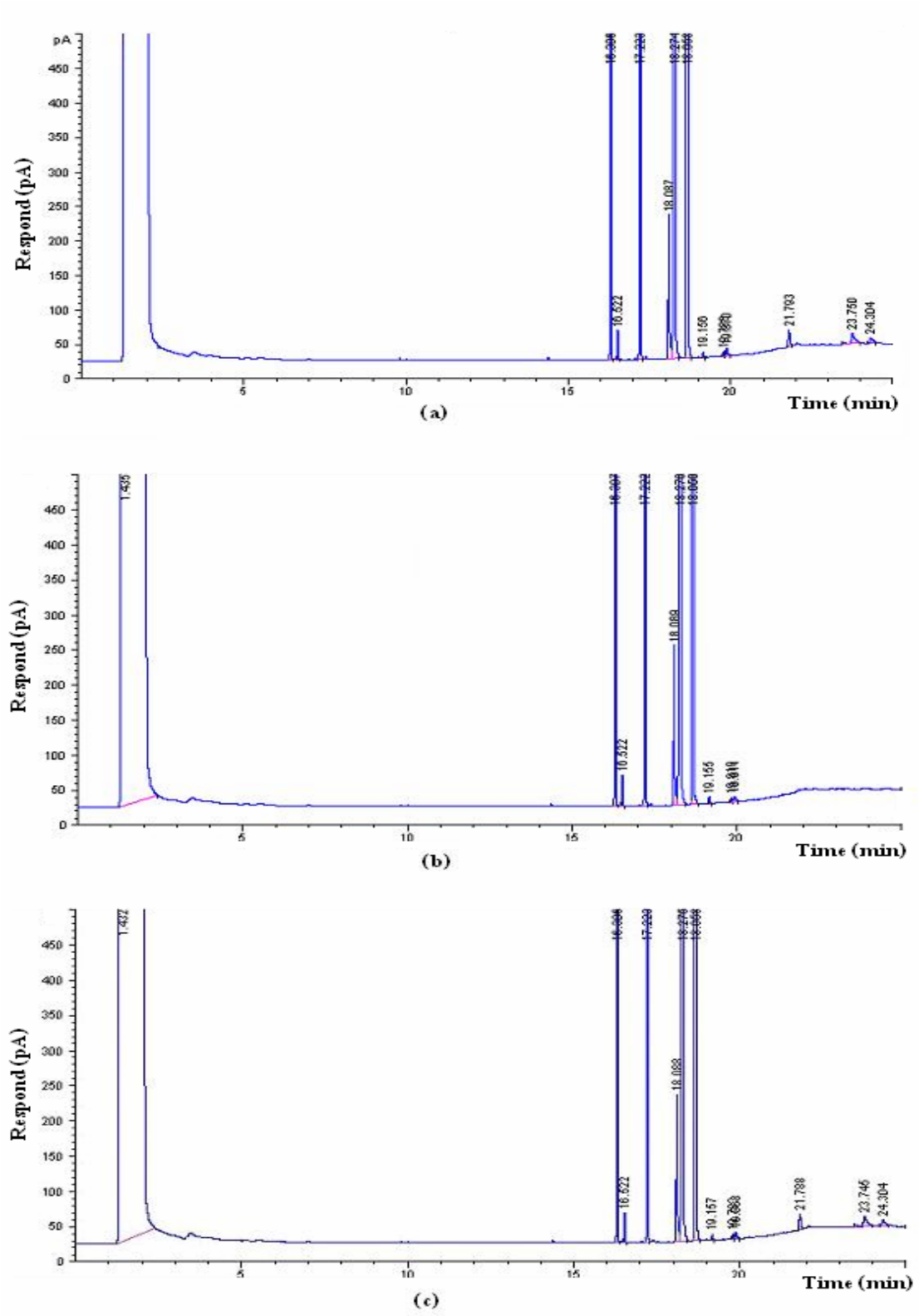


Figure 37 Fatty acid composition chromatograms of *Jatropha* seed oil detected by GC; seed oil (a), one-time adsorption (b) and two-time adsorption (c).

Table 9 Fatty acids in *Jatropha* seed oil before and after adsorption.

Fatty acids	Area of peak (%)					
	Before adsorption		After adsorption			
	RT (min)	Raw *Jatropha* seed oil	RT (min)	One-time adsorption	RT (min)	Two-time adsorption
Sat. fatty acids:						
Myristic acid (C_{14})	14.305	0.0629	14.305	0.0611	14.304	0.0616
Palmitic acid (C_{16})	16.275	14.6228	16.277	14.5714	16.281	14.6349
Steric acid (C_{18})	18.092	6.4521	18.098	6.5983	18.101	6.5252
Total		21.1378		21.2308		21.2217
Unsat. Fatty acids:						
Palmitoleic acid ($C_{16:1}$)	16.464	0.8333	16.464	0.8719	16.465	0.8908
Oleic acid ($C_{18:1}$)	18.274	42.9208	18.280	42.8154	18.283	42.7941
Linoleic acid ($C_{18:2}$)	18.654	34.6462	18.661	34.6924	18.663	34.7159
Linolenic acid ($C_{18:3}$)	19.096	0.1851	19.097	0.1903	19.098	0.1903
Total FA		78.5854		78.5700		78.5911
Others		0.2768		0.1992		0.1872

RT = retention time

Figure 37 and Table 9 showed the chromatogram of *Jatropha* seed oil fatty acids before and after adsorption. The fatty acids found in all samples included both saturated fatty acids (myristic acid; C_{14}, palmitic acid, C_{16} and steric acid; C_{18}) and unsaturated fatty acids (palmitoleic acid; $C_{16:1}$, oleic acid; $C_{18:1}$, linoleic acid; $C_{18:2}$ and linolenic acid, $C_{18:3}$). The major fatty acids of the three samples were oleic acid (C18:1) and linoleic acid (C18:2), whereas less amount fatty acid were myristic acid (C14), palmitioleic acid (C16:1) and linolenic acid (C18:3). The results demonstrated no effect of adsorption by bentonite 200 on fatty acids composition in *Jatropha* seed oil. The yield of saturated and unsaturated fatty acids in the oil after adsorption still remained as about 21% and 78%, respectively, as those before adsorption.

9. Removal of Phorbol Esters from *Jatropha* Press Cake

The content of phorbol esters in *Jatropha* press cake was about 1-2 mg/g. Phorbol esters were heat stable and therefore could not be destroyed by heat as high as 160°C for 30 min. However, it was possible to reduce the concentration of phorbol esters by chemical treatments (Makkar and Becker, 1997). This experiment compared the capacibility of NaOH and KOH for the phorbol esters removal of *Jatropha* press cake.

The two-step washing was applied in this experiment. The first step, the press cake was washed with alkaline chemical and the optimum condition of washing was determined. The second step, the press cake from the first step was immersed in 95% ethanol solution for removing phorbol esters to the safety level. The comparison of NaOH and KOH solutions for phorbol esters removal in *Jatropha* press cake was shown in Figure 38.

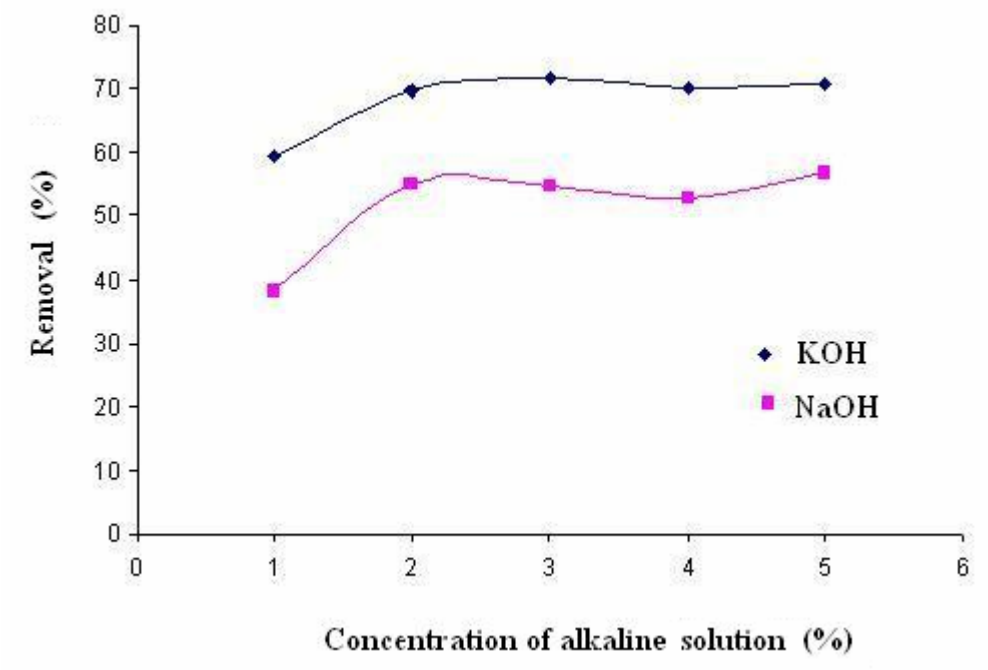


Figure 38 Removal capabilities of phorbol esters from *Jatropha* press cake by base solution treatment; KOH and NaOH solutions.

Figure 38 showed that the phorbol esters removal by potassium hydroxide solution was better than sodium hydroxide solution. When the KOH concentration increased from 1% to 5 %, the phorbol esters removal was also improved. However, at KOH concentration more than 3% (w/w), the removal capacity had flattened out at about 70%.

The highest percentage of phorbol esters removal from *Jatropha* press cake was obtained when 3% KOH was used (71.68% or 0.4173 mg/g) whereas 5% NaOH had to be used to obtain the highest phorbol esters removal (56.80% or 0.6365 mg/g). Thus, KOH was selected for the next experiment when the removal condition was optimized and the optimization of KOH concentration effect was 3% by weight (Figure 39 (a)).

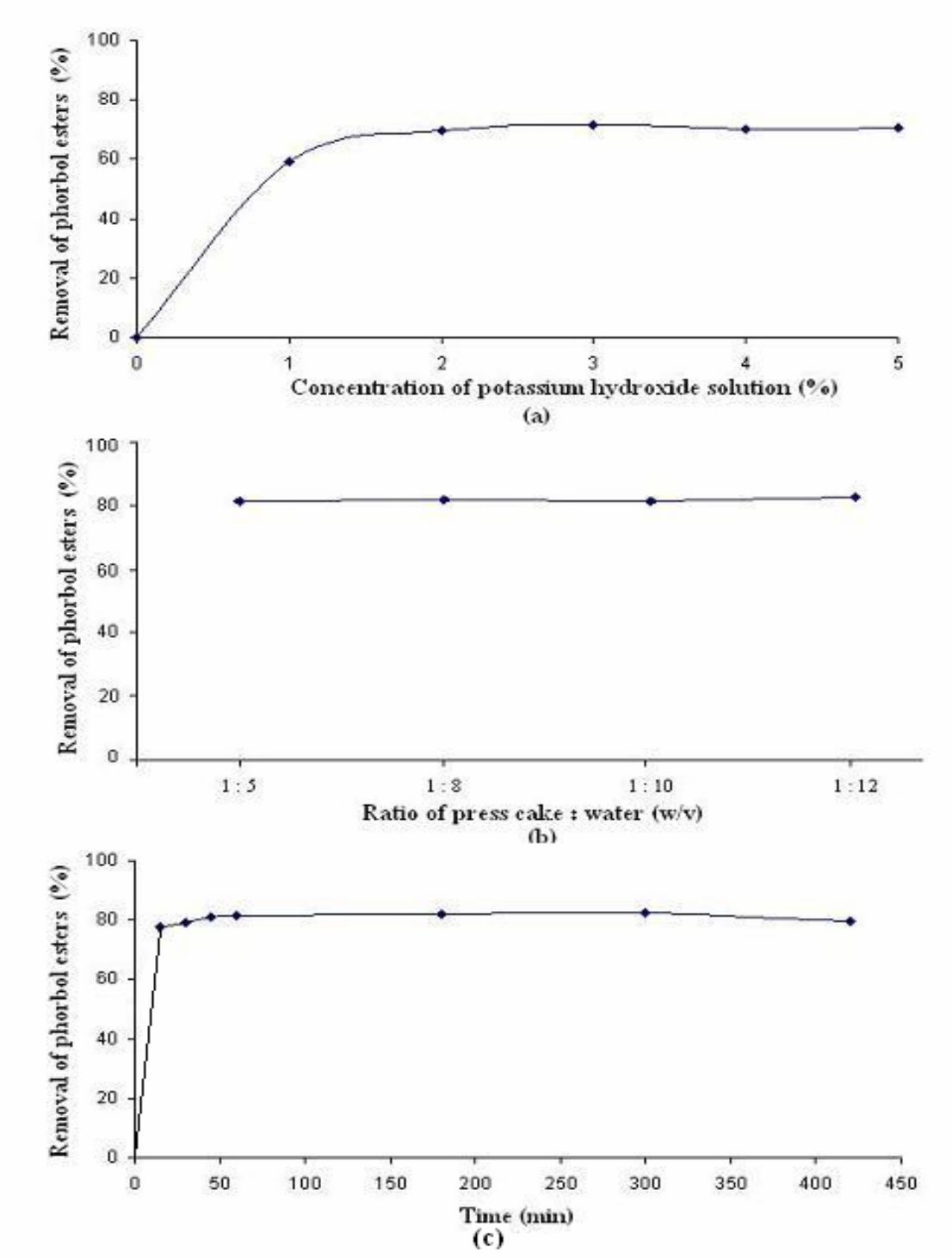


Figure 39 The effect of KOH concentration (a), press cake to water ratio (b), stirring time (c), stirring rate (d) and temperature (e) on removal percentage of phorbol esters in press cake.

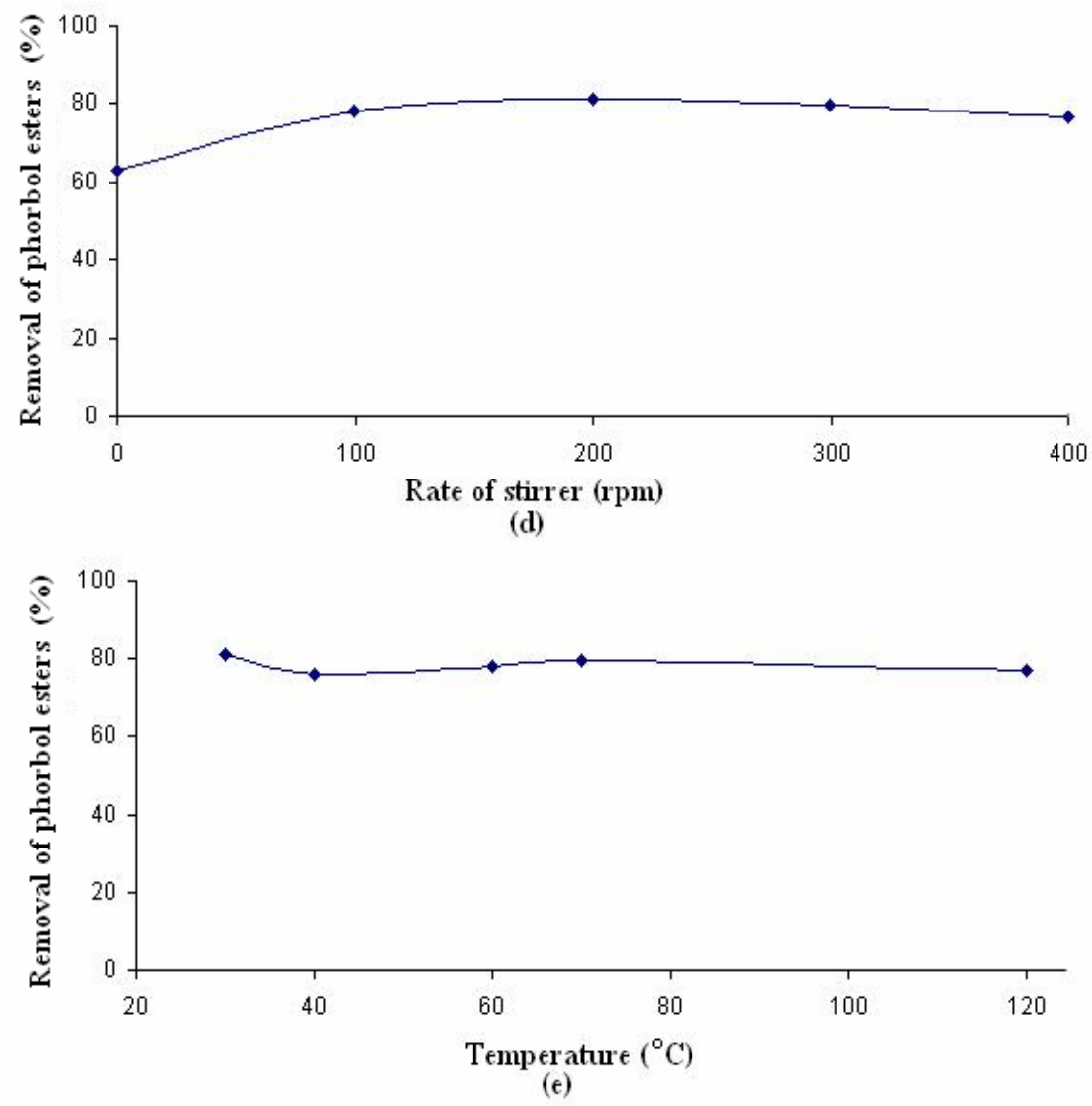


Figure 39 (Continuted)

Figure 39 (b) showed that phorbol esters removal from press cake was not affected by the ratio of press cake to water. All experiments of ratio could remove phorbol esters about 81.00% or 0.27 mg/g of phorbol esters remained in press cake. As a result, the optimum ratio of press cake to water ratio was 1:5 by weight.

Figure 39 (c) showed the effect of stirring time on phorbol esters removal. Increasing of stirring time from 15 min to 420 min resulted in an increased removal of phorbol esters. However, the removal capability had flattened out with maximum

phorbol esters removal about 80.00% or 0.2 mg/g of phorbol esters content remained in press cake after 420 min. As a result, the optimum stirring time for phorbol esters removal was 45 min.

Figure 39 (d) showed the effect of stirring rate on phorbol esters removal. The results demonstrated an increase in removal efficiency with an increasing stirring rate. However, at stirring rate faster than 200 rpm, the removal capability was reduced from 81.29% to 79.78%. As a result, 200 rpm of stirring rate was selected for the next experiment.

Figure 39 (e) showed the effect of temperature on phorbol esters removal. The results illustrated no difference in removal capability for all tested temperatures with maximum phorbol esters removal at 81.29% or 0.2756 mg/g phorbol esters remained in press cake. Therefore, the temperature at 32°C was chosen as the most optimum temperature for the next experiment.

Figure 39 summarized the removal of phorbol esters from press cake by washing with potassium hydroxide solution. It was shown that the most optimum condition was 3% (w/w) KOH, press cake: water ratio 1:5, 45 min washing time, 32 °C and 200 rpm stirring rate with maximum 70-80% phorbol esters removal or 0.2-0.3 mg/g of phorbol esters remained in press cake. However, the concentration of phorbol esters remained in press cake was about 0.2-0.3 mg/g which was still higher than that in the non-toxic *Jatropha curcas* L.. Thus, the further step of removal was needed. The press cake from the 1st step removal was subjected to the 2nd step removal by 95% ethanol. The result was shown in Table 10.

Table 10 The effect of stirring rate on phorbol esters removal by 95% ethanol.

Stirring rate	Phorbol esters content in press cake (mg/g)		
	Before ethanol washing	After ethanol washing	Removal of phorbol esters (%)
Without stirring rate	0.3201	0.1097	65.7607
100 rpm of stirring rate	0.2976	0.0839	71.8078

The results showed 65.7067% removal or 0.1097 mg/g phorbol esters remained in press cake when no stirring was applied in the reaction. An increase to 71.8079% phorbol esters removal was observed when the reaction was stirred at 100 rpm. This indicated that the safe phorbol esters concentration for animal feed could be obtained from the removal experiments either with or without stirring. This was consistent with Aregheore (2003) who worked on washing press cake with 4% NaOH followed by washing with 95% ethanol. The advantage of this experiment was that KOH waste could be used as plant fertilizer.

The yield of press cake after phorbol esters removal was also studied and the result was shown in Table 11. It indicated about 25% loss of press cake after the experiment.

Table 11 Weight of *Jatropha* press cake before and after KOH washing.

No.	sample	Weight of press cake (g)		Yield (%)
		Before washing	After washing	
1	Press cake no.1	20.0329	15.0449	75.1009
2	Press cake no.2	20.0941	14.9880	74.5891
3	Press cake no.3	20.0836	14.9056	74.2178
4	Press cake no.4	20.0214	14.9842	74.8409
5	Press cake no.5	20.0734	15.2135	75.7894
			Average	74.9076

The oil content related to phorbol esters in press cake was also evaluated and the result was demonstrated in Table 12.

Table 12 The content of phorbol esters and oil in *Jatropha* press cake and seed oil.

No.	Type of sample	Oil content (%)	Concentration of phorbol esters	
			ppm	mg/g
1	Press cake from screw press (wet)	10.0-15.0	295.0121	1.4733
2	Press cake from screw press (dry)	0.8-1.0	124.5414	0.5170
3	Oil extracted from press cake by hexane	0.0	62.6378	0.2982
4	Seed oil	100.0	1781.4550	4.5084

After screw press extraction, 25-30% of oil and 70-75% of press cake were obtained. However, there was still 10-15% of oil or about 1.4733 mg/g phorbol esters remained in the press cake. After drying, the press cake contained 0.8-1.0% of oil or 0.5170 mg/g phorbol esters remained in dry press cake. This press cake was further oil extracted using hexane until there was no oil left in press cake or only 0.2982 mg/g remaining phorbol ester. This result indicated the decrease in phorbol esters content when the oil was removed.

10. Protein in *Jatropha* Press Cake

A total 19-27% crude protein obtained from *Jatropha* press cake (Makkar et al., 1997) could be an ideal source for essential amino acids which were even higher (except lysine) than the Food and Agriculture Organization (FAO) reference protein (Makkar andBecker, 1997). Protein and its essential amino acids from *Jatropha* press cake were nutrients for animal feed if the phorbol esters were eliminated or reduced to the safe level. Thus, the contents of protein and its amino acids after chemical treatments must be studied and its result was shown in Table 13.

Table 13 Protein content in *Jatropha* press cake.

Press cake	Protein content (%)	Phorbol esters content (mg/g)
Press cake	26.07	1.4733
Press cake after 3% KOH washing	24.66	0.3201
Press cake after 95% ethanol immersion	16.03	0.1097

The *Jatropha* press cake contained 26.07% protein. After the 1st and 2nd detoxification by 3% KOH and 95% ethanol, the press cake contained 24.66% and 16.03% protein, respectively. This indicated the more effect of ethanol than KOH on *Jatropha* protein as resulting in a decrease in protein content after treatment. This could be easily observed by the precipitation or coagulation of protein in the reaction.

11. Amino Acids Content in *Jatropha* Press Cake

Essential amino acids including isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophane and valine are necessary for the animal feed. The amino acids in press cake, 3% KOH-washed press cake and 95% ethanol-immersed press cake were shown in Table 14.

Table 14 Amino acids in raw material and detoxified *Jatropha* press cake.

Amino acids	Total Amino acids (mg/100mg)		
	Raw material of press cake	Washing with 3% KOH solution	Immersion in 95% ethanol
Aspartic acid	1.96	1.71	0.95
Serine	1.03	0.89	0.48
Glutamic acid	3.30	2.73	1.46
Glycine	0.92	0.80	0.46
Histidine	0.41	0.34	0.17
Arginine	2.37	2.02	1.02
Threonine	0.92	0.81	0.48
Alanine	1.08	0.96	0.57
Proline	1.01	0.90	0.51
Tyrosine	0.41	0.34	0.15
Valine	1.00	0.86	0.51
Lysine	0.76	0.61	0.27
Isoleucine	0.82	0.69	0.41
Leucine	1.51	1.33	0.75
Phenylalanine	0.95	0.84	0.47

The results also suggested the negative effect of ethanol on *Jatropha* press cake amino acids. It indicated the decrease in amino acids after the 1st and 2nd chemical treatments. Amino acids after the 2nd washing were reduced due to the denaturation of protein by ethanol.

CONCLUSIONS

The seed oil and press cake of *J. curcas* L. contained phorbol esters at 3.0-6.0 mg/g and 1.0-2.0 mg/g, respectively. HPLC separation was greatly enhanced by mass spectrometric detection, which allowed to identify the compounds in *Jatropha* seed oil. The methanol extracts were analyzed by combined HPLC/PDA/MS spectrometry. This method confirmed that phorbol esters in seed hull, kernel, seed oil and press cake of *Jatropha curcas* L. were DHPB whereas TPA phorbol esters were found in fruit bark, bark and wood.

Bentonite 200 was the most suitable adsorbent for phorbol esters adsorption from seed oil when compared among the activated carbon, bentonite 150, chitin and chitosan. The optimum condition was 15 min adsorption time, 3.2% (w/v) bentonite 200, 32°C temperature and 100 rpm stirring rate with maximum removal up to 98.00% or 0.09 mg/g phorbol esters remained in seed oil. The 1st adsorption fitted well with the Freundlich and Langmuir isotherms equations. The 2nd adsorption showed the optimum condition at 0.8% (w/v) bentonite 200, 15 min stirring time at 32°C temperature and 100 rpm stirring rate with maximum removal up to 99.50% or 0.02 mg/g phorbol esters remained in seed oil.

Elimination of phorbol esters from *Jatropha* press cake could be achieved by washing press cake with 3% (w/w) KOH at 1:5 ratio of press cake to water, 32°C temperature, 45 min stirring time and 200 rpm stirring rate followed by immersed in 95% ethanol for one night. This could remove phorbol esters down to 0.08-0.11 mg/g which was safe for animal feed. However, 95% ethanol destroyed the protein in press cake resulting in its quality change.

In addition, The *Jatropa* press cake could be dried or oil extracted with hexane before washing with KOH solution because the protein content in *Jatropha* press cake was not destroyed. The high protein content of *Jatropha* press cake used for the animal feed. The phorbol esters adsorption with bentonite 200 in *Jatropha* seed oil

had much utilization such as elimination of phorbol esters in the seed oil before biodiesel production. The phorbol esters may be remained in glycerol which it was by product of biodiesel industrial for cosmetic or pharmaceutical. Elimination of the phorbol esters in the *Jatropha* press cake used in fertilizer industrial which protected phorbol esters substances to remain in soil, water or plants.

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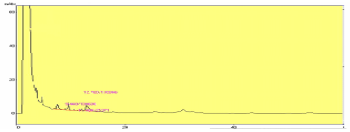
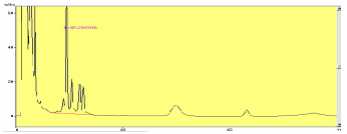
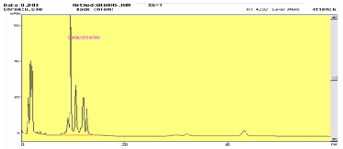
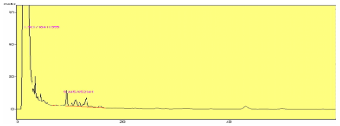
APPENDICES

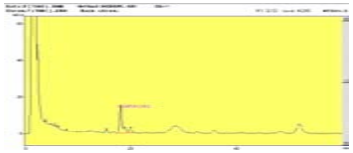
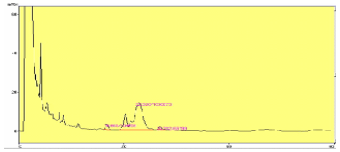
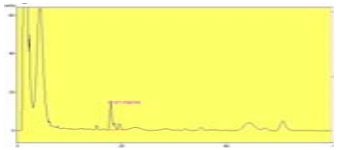
Appendix A

Adsorption results

Appendix Table A1 Phorbol esters in different parts of *Jatropha curcas* L. at two retention times of chromatogram.

Parts of *Jatropha curcas* L.	Weight (g)	Concentration of Phorbol esters						Total concentration of phorbol esters		
		RT of peak 8-12 min			RT of peak 18-21 min					
		mg/25ml	mg/g	%	mg/25ml	mg/g	%; (w/w)	mg/25ml	mg/g	%;
Seed hull	12.5022	4.4670	0.3573	0.0357	-	-	-	4.4670	0.3573	0.0357
										
Kernel	11.7793	43.0225	3.6524	0.3652	-	-	-	43.0225	3.6524	0.3652
										
Oil	9.8789	44.5365	4.5084	0.4508	-	-	-	44.5365	4.5084	0.4508
										
Press cake	12.5026	12.9096	1.0326	0.1033	-	-	-	12.9096	1.0326	0.1033
										

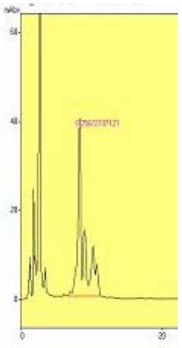
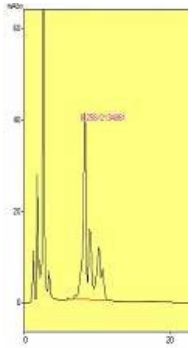
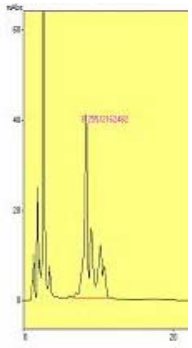
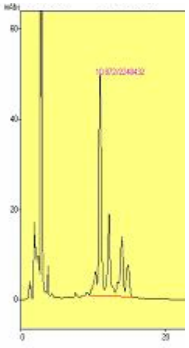
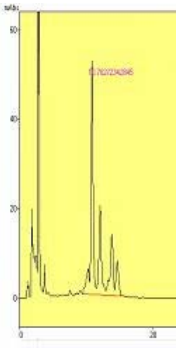
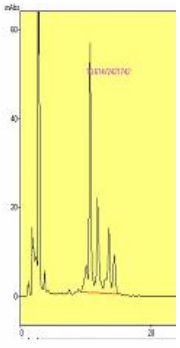
Appendix Table A1 (Continued)

Parts of *Jatropha curcas* L.	Weight (g)	Concentration of Phorbol esters						Total concentration of phorbol esters		
		RT of peak 8-12 min			RT of peak 18-21 min					
		mg/25ml	mg/g	%; (w/w)	mg/25ml	mg/g	%	mg/25ml	mg/g	%
Fruit bark	12.0754	-	-	-	9.2833	0.7688	0.0769	9.2833	0.7688	0.0769
										
Bark	3.5374	-	-	-	8.6485	2.4449	0.2445	8.6485	2.4449	0.2445
										
Wood	3.9475	-	-	-	9.0685	2.9730	0.2973	9.0685	2.973	0.2973
										

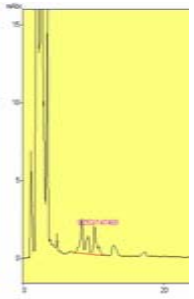
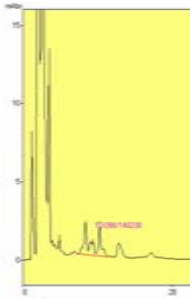
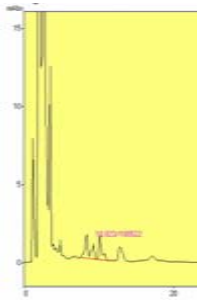
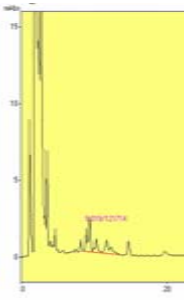
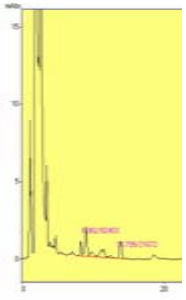
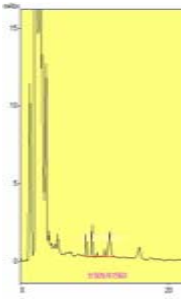
RT = retention time.

% = weight by weight.

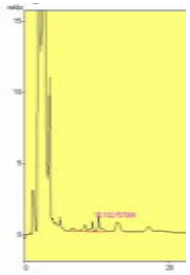
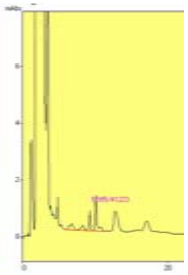
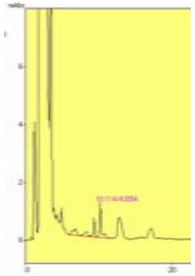
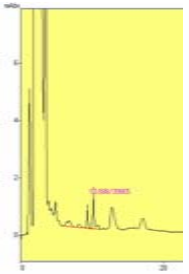
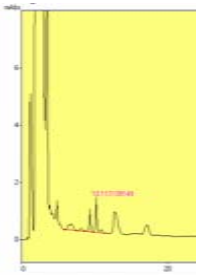
Appendix Table A2 Phorbol esters in *Jatropha* seed oil adsorbed by different adsorbents.

Activated carbon		Stirring time; (min)					
		15	30	45	60	120	180
Weight	Adsorbent	0.8033	0.8870	0.8163	0.8049	0.8034	0.8039
(g)	Seed oil	10.1003	10.0585	10.0718	10.0259	10.0763	10.0846
HPLC	RT; (min)	8.256	8.256	8.255	10.872	10.782	10.614
	Area	2107121	2134861	2162482	2248432	2342845	2421742
	Chromatogram						
Concentration	ppm	1276.5788	1293.3848	1310.1187	1362.1907	1419.3899	1467.1889
of	mg/25ml	31.9145	32.3346	32.7530	34.0548	35.4847	36.6797
phorbol esters	mg/g	3.1598	3.2147	3.2519	3.3967	3.5216	3.6372
	%; (w/w)	0.3160	0.3215	0.3252	0.3397	0.3522	0.3637
	Adsorption; (%)	18.0125	16.5880	15.6227	11.8656	8.6248	5.6253

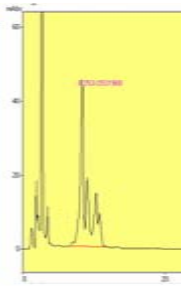
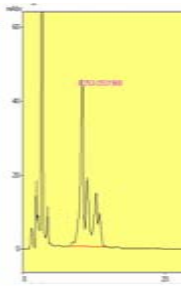
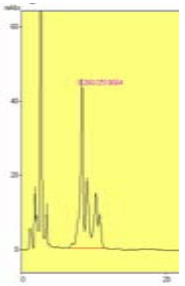
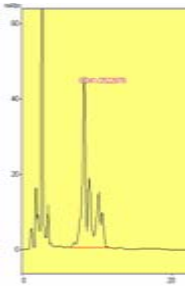
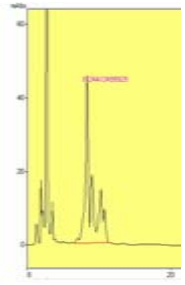
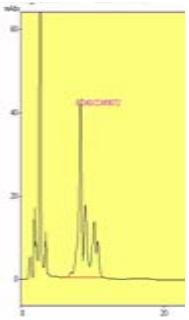
Appendix Table A2 (Continued)

Bentonite 150		Stirring time; (min)					
		15	30	45	60	120	180
Weight (g)	Adsorbent	0.8133	0.8116	0.8053	0.8060	0.8041	0.8036
	Seed oil	10.0150	10.1037	10.0879	10.0671	10.0478	10.0276
HPLC	RT; (min)	8.253	10.098	10.023	9.319	8.982	9.214
	Area	147469	140230	100522	121714	104075	96540
	Chromatogram						
Concentration of phorbol esters	ppm	89.34267	84.9570	60.9003	73.7392	63.0528	58.4878
	mg/25ml	2.2336	2.1239	1.5225	1.8435	1.5763	1.4622
	mg/g	0.2230	0.2102	0.1509	0.1831	0.1569	0.1458
	% (w/w)	0.0223	0.0210	0.0151	0.0183	0.0157	0.0146
	Adsorption; (%)	94.2138	94.5459	96.0846	95.2491	95.9285	96.2112

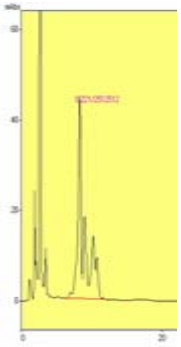
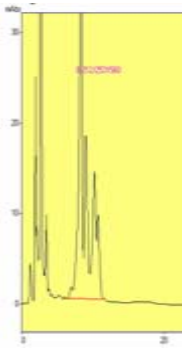
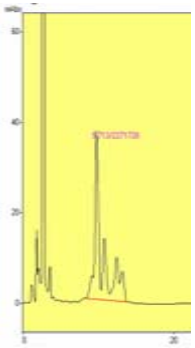
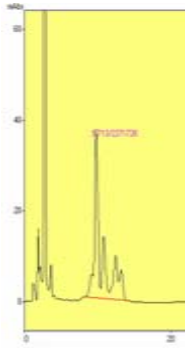
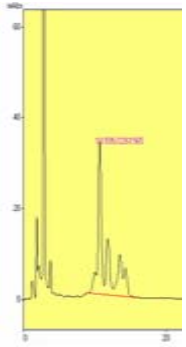
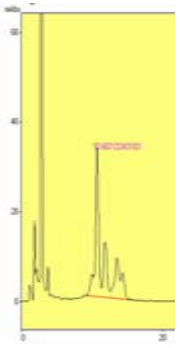
Appendix Table A2 (Continued)

Bentonite 200		Stirring time; (min)					
		15	30	45	60	120	180
Weight	Adsorbent	0.8064	0.8124	0.8010	0.8071	0.8078	0.8054
(g)	Seed oil	10.0439	10.0660	10.0285	10.0938	10.0236	10.0766
HPLC	RT; (min)	9.981	10.102	9.995	10.114	10.088	10.117
	Area	83891	57008	41223	43054	39065	39149
	Chromatogram						
Concentration	ppm	50.8245	34.5377	24.9746	26.0838	23.6672	23.7180
of	mg/25ml	1.2706	0.8634	0.6244	0.6521	0.5917	0.5930
phorbol esters	mg/g	0.1265	0.0858	0.0623	0.0646	0.0590	0.0588
	%; (w/w)	0.0127	0.0086	0.0062	0.0065	0.0059	0.0059
	Adsorption; (%)	96.7177	97.7737	98.3835	98.3238	98.4691	98.4743

Appendix Table A2 (Continued)

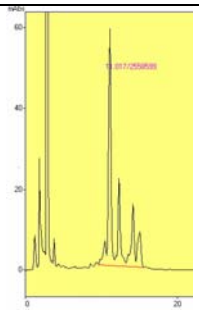
Chitosan		Stirring time; (min)					
		15	30	45	60	120	180
Weight	Adsorbent	0.8078	0.8015	0.8026	0.8070	0.8042	0.8074
(g)	Seed oil	10.1199	10.0132	10.0549	10.0007	10.0041	10.0512
HPLC	RT; (min)	8.253	8.261	8.260	8.241	8.244	8.240
	Area	2531968	2499326	2518664	2522078	2499929	2349872
	Chromatogram						
Concentration	ppm	1533.9683	1514.1924	1525.9082	1527.9765	1514.5577	1423.6472
of	mg/25ml	38.3492	37.8548	38.1477	38.1994	37.8639	35.5912
phorbol esters	mg/g	3.7895	3.7805	3.7939	3.8197	3.7848	3.5410
	%; (w/w)	0.3789	0.3780	0.3794	0.3820	0.3785	0.3541
	Adsorption; (%)	1.6736	1.9071	1.5594	0.8900	1.7955	8.1214

Appendix Table A2 (Continued)

Chitin		Stirring time; (min)					
		15	30	45	60	120	180
Weight	Adsorbent	0.7973	0.7985	0.8013	0.8074	0.8002	0.8087
(g)	Seed oil	10.0596	10.0397	10.0182	10.0431	10.0319	10.0501
HPLC	RT; (min)	8.221	8.212	8.228	9.713	10.595	10.607
	Area	2512512	2501560	2319380	2271728	2261565	2243183
	Chromatogram						
Concentration	ppm	1522.1810	1515.5459	1405.1739	1376.3044	1370.1472	1359.0107
of	mg/25ml	38.0545	37.8886	35.1293	34.4076	34.2537	33.9753
phorbol esters	mg/g	3.7829	3.7739	3.5066	3.4260	3.4145	3.3806
	%; (w/w)	0.3783	0.3774	0.3507	0.3426	0.3414	0.3381
	Adsorption; (%)	1.8448	2.0784	9.0140	11.1053	11.3934	12.2833

Appendix Table A2 (Continued)

Note**:** Phorbol esters in *Jatropha* seed oil before experiment (3.8540 mg/g). Experiment condition (0.8 g adsorbent, 200 rpm stirring rate at room temperature (32˚C)**)**.

Weight of seed oil (g)	HPLC			Phorbol esters content			
	Chromatogram	RT (min)	Area	ppm	mg/25ml	mg/g	% (w/w)
10.0552		11.017	2558599	1550.1024	38.7526	3.8540	0.3854

Appendix Table A3 Effect of parameters on one-time adsorption of phorbol esters from seed oil.

Effect of bentonite 200 amount on phorbol esters adsorption under condition (0.8 g adsorbent, 15 min stirring time, 200 rpm stirring rate at room temperature (32°C)).

		Amount of bentonite 200 (g)						
		0.4	0.6	0.8	1.0	1.2	2.0	3.0
Weight; (g)	Seed oil	10.0333	10.0020	10.0352	10.0931	10.1424	10.0482	10.0615
Chromatogram	RT; (min)	9.080	9.064	9.055	9.044	9.050	9.046	9.042
	Area	410132	173083	61266	34227	25524	18187	14692
Concentration of phorbol esters	ppm	248.4745	104.8607	37.1174	20.7361	15.4635	11.0184	8.9010
	mg/25ml	6.2119	2.6215	0.9279	0.5184	0.3866	0.2755	0.2225
	mg/g	0.6191	0.2621	0.0925	0.0514	0.0381	0.0274	0.0221
	%; (w/w)	0.0619	0.0262	0.0093	0.0051	0.0038	0.0027	0.0022
	Adsorption; (%)	89.6246	95.6075	98.4498	99.1386	99.3615	99.5408	99.6296

Note: Phorbol esters in *Jatropha* seed oil before experiment (5.9670 mg/g).

Weight of seed oil (g)	Chromatogram		Phorbol esters			
	RT (min)	Area	ppm	mg/25ml	mg/g	% (w/w)
10.4139	9.089	4102686	2485.5725	62.1393	5.9670	0.5967

Appendix Table A3 (Continued)

Effect of temperature on phorbol esters adsorption under condition (0.8 g adsorbent, 15 min stirring time and 200 rpm stirring rate).

		Temperature (°C)				
		32	45	65	85	120
Weight; (g)	Seed oil	10.0642	10.0590	10.0683	10.0028	10.0357
Chromatogram	RT; min	9.033	9.092	9.086	11.768	11.142
	Area	69359	20692	25789	29941	37393
Concentration of phorbol esters	ppm	42.0205	12.5360	15.6240	18.1395	22.6542
	mg/25ml	1.0505	0.3134	0.3906	0.4535	0.5664
	mg/g	0.1044	0.0312	0.0388	0.0453	0.0564
	%; (w/w)	0.0104	0.0031	0.0039	0.0045	0.0056
	Adsorption; (%)	98.2504	99.4771	99.3498	99.2408	99.0548

Note: Phorbol esters in *Jatropha* seed oil before experiment (5.9670 mg/g).

Weight of seed oil (g)	Chromatogram		Phorbol esters			
	RT (min)	Area	ppm	mg/25ml	mg/g	% (w/w)
10.4139	9.089	4102686	2485.5725	62.1393	5.9670	0.5967

Appendix Table A3 (Continued)

Effect of stirring rate on phorbol esters adsorption under condition (0.8 g adsorbent at room temperature (32°C) and 15 min stirring).

		Rate of stirrer (rpm)					
		0	100	150	200	250	300
Weight; (g)	Seed oil	10.0224	10.0969	10.1268	10.0352	10.0244	10.0588
Chromatogram	RT; min	9.037	9.038	9.038	9.055	9.042	9.034
	Area	1657869	64500	70523	61266	55543	47485
Concentration of phorbol esters	ppm	1004.4038	39.0767	42.7257	37.1174	33.6502	28.7683
	mg/25ml	25.1101	0.9769	1.0681	0.9279	0.8413	0.7192
	mg/g	2.5054	0.0968	0.1055	0.0925	0.0839	0.0715
	%; (w/w)	0.2505	0.0097	0.0105	0.0092	0.0084	0.0072
	Adsorption; (%)	58.0124	98.3777	98.2319	98.4498	98.5939	98.8017

Note: Phorbol esters in *Jatropha* seed oil before experiment (5.9670 mg/g).

Weight of seed oil (g)	Chromatogram		Phorbol esters content			
	RT (min)	Area	ppm	mg/25ml	mg/g	%; (w/w)
10.4139	9.089	4102686	2485.5725	62.1393	5.9670	0.5967

Appendix Table A4 Effect of parameters on two-time adsorption of phorbolesters from seed oil.

Effect of bentonite 200 on phorbol esters adsorption under condition (15 min stirring time, 200 rpm stirring rate at room temperature (32°C)).

		Amount of bentonite 200; (g)				
		0.2	0.4	0.6	0.8	1.0
Weight; (g)	Seed oil	10.0253	10.0752	10.0751	10.0057	10.0329
Chromatogram	RT; (min)	11.509	11.488	11.504	11.514	11.054
	Area	14075	12356	13541	14200	14012
Concentration of phorbol esters	ppm	8.5272	7.4858	8.2037	8.6029	8.4890
	mg/25ml	0.2132	0.1871	0.2051	0.2151	0.2122
	mg/g	0.0213	0.0187	0.0204	0.0215	0.0212
	%; (w/w)	0.0021	0.0019	0.0020	0.0021	0.0021
Adsorption (%)	One time	99.6430	99.6883	99.6581	99.6397	99.6447
	Two times	77.0391	79.8405	78.0088	76.8236	77.1469

Adsorption1 is calculated based on phorbol esters concentration from raw seed oil before experiment; 5.9670 mg/g.

Adsorption2 is calculated based on phorbol esters concentration from the seed oil after one-time adsorption; 0.0928 mg/g.

Note: Phorbol esters in *Jatropha* seed oil before and after one-time adsorption (5.9670 and 0.0928 mg/g, respectively).

	Weight of seed oil (g)	Chromatogram		Phorbol esters			
		RT (min)	Area	ppm	mg/ 25ml	mg/g	% (w/w)
Before adsorption	10.4139	9.089	4102686	2485.5725	62.1393	5.9670	0.5967
After one-time adsorption	10.0222	9.020	61381	37.1871	0.9297	0.0928	0.0093

Appendix Table A4 (Continued)

Effect of washing time on phorbol esters adsorption under condition (0.8 g bentonite 200, room temperature and 200 rpm of stirring rate).

		Time; (min)		
		15	30	45
Weight; (g)	Seed oil	10.0638	10.0711	10.7520
Chromatogram	RT; min	11.489	11.489	11.488
	Area	14376	14161	12356
Concentration of phorbol esters	ppm	7.4858	8.5793	7.4858
	mg/25ml	0.1871	0.2177	0.1871
	mg/g	0.0216	0.0213	0.0174
	%; (w/w)	0.0022	0.0021	0.0017
Adsorption (%)	One-time	99.6380	99.6430	99.7084
	Two-time	76.7241	77.0474	81.2500

Adsorption1 is calculated based on phorbol esters concentration from raw seed oil before experiment; 5.9670 mg/g.

Adsorption2 is calculated based on phorbol esters concentration from the seed oil after one - time adsorption; 0.0928 mg/g.

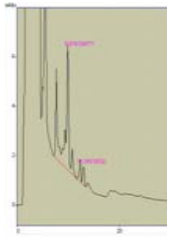
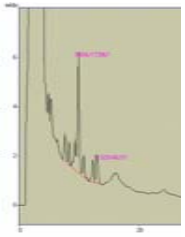
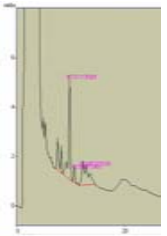
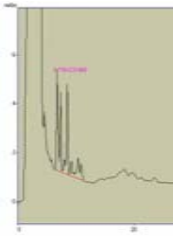
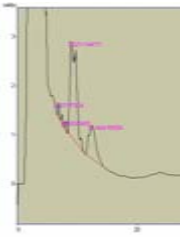
Note: Condition of one-time adsorption was 0.8 g bentonite 200, 15 min stirring time, 100 rpm stirring rate at room temperature (32˚C).

Condition of two-time adsorption was 0.2 g bentonite 200, 15 min stirring time, 100 rpm stirring rate at room temperature (32˚C).

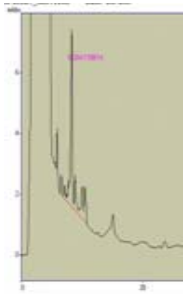
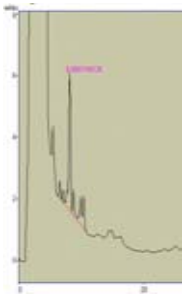
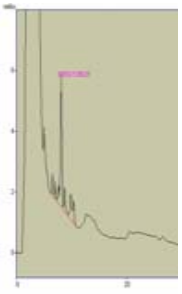
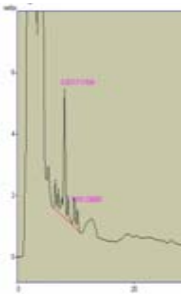
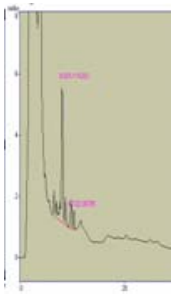
	Weight of seed oil (g)	Chromatogram		Phorbol esters			
		RT (min)	Area	ppm	mg/ 25ml	mg/g	% (w/w)
Before adsorption	10.4139	9.089	4102686	2485.5725	62.1393	5.9670	0.5967
After one-time adsorption	10.0222	9.020	61381	37.1871	0.9297	0.0928	0.0093

Appendix Table A5 Effect of parameters on phorbol esters removal from press cake.

The effect of chemicals concentration on phorbol esters removal from press cake.

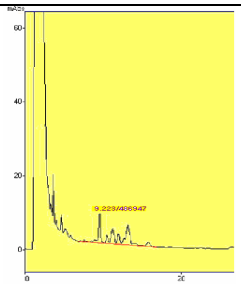
Chemical			Concentration; (w/v)				
			1	2	3	4	5
NaOH	Weight of press cake; (g)	Before washing	20.1103	20.1277	20.0345	20.0996	20.2044
		After of washing	15.4321	15.4323	15.4231	14.9872	14.9877
		Extraction	5.0069	5.0438	5.0153	5.0284	5.0373
	HPLC	RT; (min)	9.874	9.806	9.737	6.716	6.871
		Area	301303	220178	220868	231488	211684
		Chromatogram					
	Concentration of phorbol esters	ppm	182.5415	133.3927	133.8107	140.2448	128.2467
		mg/25ml	4.5635	3.3348	3.3453	3.5061	3.2062
		mg/g	0.9114	0.6612	0.6670	0.6973	0.6365
		%; (w/w)	0.0911	0.0661	0.0667	0.0697	0.0637
		Removing; (%)	38.1389	55.1212	54.7275	52.6709	56.7977

Appendix Table A5 (Continued)

Chemical			Concentration; (w/v)				
			1	2	3	4	5
KOH	Weight of press cake (g)	Before of washing	20.0329	20.0941	20.0836	20.0214	20.0734
		After of washing	15.0449	14.9880	14.9056	14.9842	15.2135
		Extraction	5.0364	5.0284	5.0461	5.0136	5.0386
	HPLC	RT; (min)	8.204	8.093	8.053	8.037	8.021
		Area	199614	148126	139029	146099	142987
		Chromatogram					
	Concentration of phorbol esters	ppm	120.9342	89.7407	84.2294	88.5127	86.6273
		mg/25ml	3.0234	2.2435	2.1057	2.2128	2.1657
		mg/g	0.6003	0.4462	0.4173	0.4414	0.4298
		%; (w/w)	0.0600	0.0446	0.0417	0.0441	0.0423
		Removing; (%)	59.2547	69.7142	71.6758	70.0400	70.8274

Appendix Table A5 (Continued)

Note: Phorbol esters in *Jatropha* press cake before experiment was 1.4733 mg/g. Experiment condition was 0.8 g adsorbent, 200 rpm stirring rate at room temperature (32˚C).

Weight of press cake (g)	HPLC			Phorbol esters			
	Chromatogram	RT (min)	Area	ppm	mg/ 25ml	mg/g	% (w/w)
5.0060		9.223	486947	295.0121	7.3753	1.4733	0.1473

Appendix Table A5 (Continued)

Effect of press cake : water ratio on phorbol esters removal (3% KOH at room temperature (32°C) and 60 min stirring time).

		press cake : water			
		1:5	1:8	1:10	1:12
Weight of press cake (g)	Before washing	20.0229	20.1785	20.0789	20.0618
	After washing	11.7558	10.5507	13.0750	10.8934
	Extraction	6.0192	6.0234	6.0197	6.0167
Chromatogram	RT; min	9.428	9.492	9.490	9.499
	Area	108768	107858	110616	100572
Concentration of phorbol esters	ppm	65.8960	65.3447	67.0156	60.9306
	mg/25ml	1.6474	1.6336	1.6754	1.5233
	mg/g	0.2737	0.2712	0.2783	0.2532
	%; (w/w)	0.0274	0.0271	0.0278	0.0253
	Removing; (%)	1.6474	1.6336	1.6754	1.5233

Note: Phorbol esters in *Jatropha* seed oil before experiment (1.4733 mg/g).

Weight of seed oil (g)	Chromatogram		Phorbol esters			
	RT (min)	Area	ppm	mg/25ml	mg/g	%; (w/w)
5.0060	9.223	486947	295.0121	7.3753	1.4733	0.1473

Appendix Table A5 (Continued)

Effect of stirring time on phorbol esters removal (3% of KOH, 1:10 press cake to water ratio at room temperature (32°C) and 200 rpm stirring rate).

		Stirring time; (min)						
		15	30	45	60	180	300	420
Weight of press cake (g)	Before washing	20.0199	20.0690	20.1332	20.2182	20.1110	20.1014	20.1260
	After washing	12.5017	12.5165	12.5360	12.8185	12.7160	12.9090	14.9332
	Extraction	6.0235	6.0158	6.0128	6.0341	6.0035	6.0239	6.0134
Chromatogram	RT; min	8.930	7.993	8.674	10.276	8.526	8.466	8.771
	Area	130795	123413	112114	109801	105956	102114	119663
Concentration of phorbol esters	ppm	79.2397	74.7686	67.9232	66.5219	64.1924	61.8648	72.4967
	mg/25ml	1.9810	1.8692	1.6981	1.6630	1.6048	1.5466	1.8124
	mg/g	0.3289	0.3107	0.2824	0.2756	0.2673	0.2567	0.3014
	%; (w/w)	0.0329	0.0311	0.0282	0.0276	0.0267	0.0257	0.0301
	Removing; (%)	77.6760	78.9113	80.8321	81.2937	81.8571	82.5765	79.5425

Note: Phorbol esters in *Jatropha* seed oil before experiment (1.4733 mg/g).

Weight of seed oil (g)	Chromatogram		Phorbol esters			
	RT (min)	Area	ppm	mg/25ml	mg/g	% (w/w)
5.0060	9.223	486947	295.0121	7.3753	1.4733	0.1473

Appendix Table A5 (Continued)

Effect of stirring rate on phorbol esters removal (3% of KOH, 1:10 ratio press cake to water at room temperature (32°C) and 60 min stirring time).

		Stirring rate, (rpm)				
		0	100	200	300	400
Weight	Before washing	20.2198	20.2388	20.2182	20.1482	20.1813
Of press cake	After washing	13.6216	13.1589	12.8185	13.1055	12.8868
(g)	Extraction	6.0185	6.0472	6.0341	6.0162	6.0100
Chromatogram	RT; min	9.840	9.844	10.276	9.850	9.846
	Area	217306	128205	109801	118315	135140
Concentration	ppm	131.6527	77.6718	66.5219	71.6800	81.8733
of	mg/25ml	3.2913	1.9418	1.6630	1.7920	2.0468
phorbol esters	mg/g	0.5469	0.3211	0.2756	0.2979	0.3406
	%; (w/w)	0.0547	0.0321	0.0276	0.0298	0.0341
	Removing; (%)	62.8793	78.2054	81.2937	79.7801	76.8818

Note: Phorbol esters in *Jatropha* seed oil before experiment (1.4733 mg/g).

Weight	Chromatogram		Phorbol esters			
of	RT	Area	ppm	mg/25ml	mg/g	%
seed oil	(min)					(w/w)
(g)						
5.0060	9.223	486947	295.0121	7.3753	1.4733	0.1473

Appendix Table A5 (Continued)

Effect of temperature on phorbol esters removal (3% KOH, 1:10 ratio of press cake to water at 200 rpm and 60 min stirring time).

		Temperature, (°C)				
		32	40	60	70	120
Weight of press cake (g)	Before washing	20.2182	20.0462	20.2686	20.1553	20.0451
	After washing	12.8185	12.8864	11.2554	11.5293	12.7654
	Extraction	6.0341	6.0358	6.0087	6.0302	6.0509
Chromatogram	RT; min	10.276	7.411	6.693	6.362	10.624
	Area	109801	139595	126988	118490	134551
Concentration of phorbol esters	ppm	66.5219	84,5723	76.9344	71.7860	81.5164
	mg/25ml	1.6630	2.1146	126988	118490	134551
	mg/g	0.2756	0.3505	0.3201	0.2976	0.3368
	%; (w/w)	0.0276	0.0351	0.0320	0.0298	0.0337
	Removing; (%)	81.2937	76.2234	78.2733	79.8004	77.1398

Note: Phorbol esters in *Jatropha* seed oil before experiment (1.4733 mg/g).

Weight of seed oil (g)	Chromatogram		Phorbol esters			
	RT (min)	Area	ppm	mg/25ml	mg/g	% (w/w)
5.0060	9.223	486947	295.0121	7.3753	1.4733	0.1473

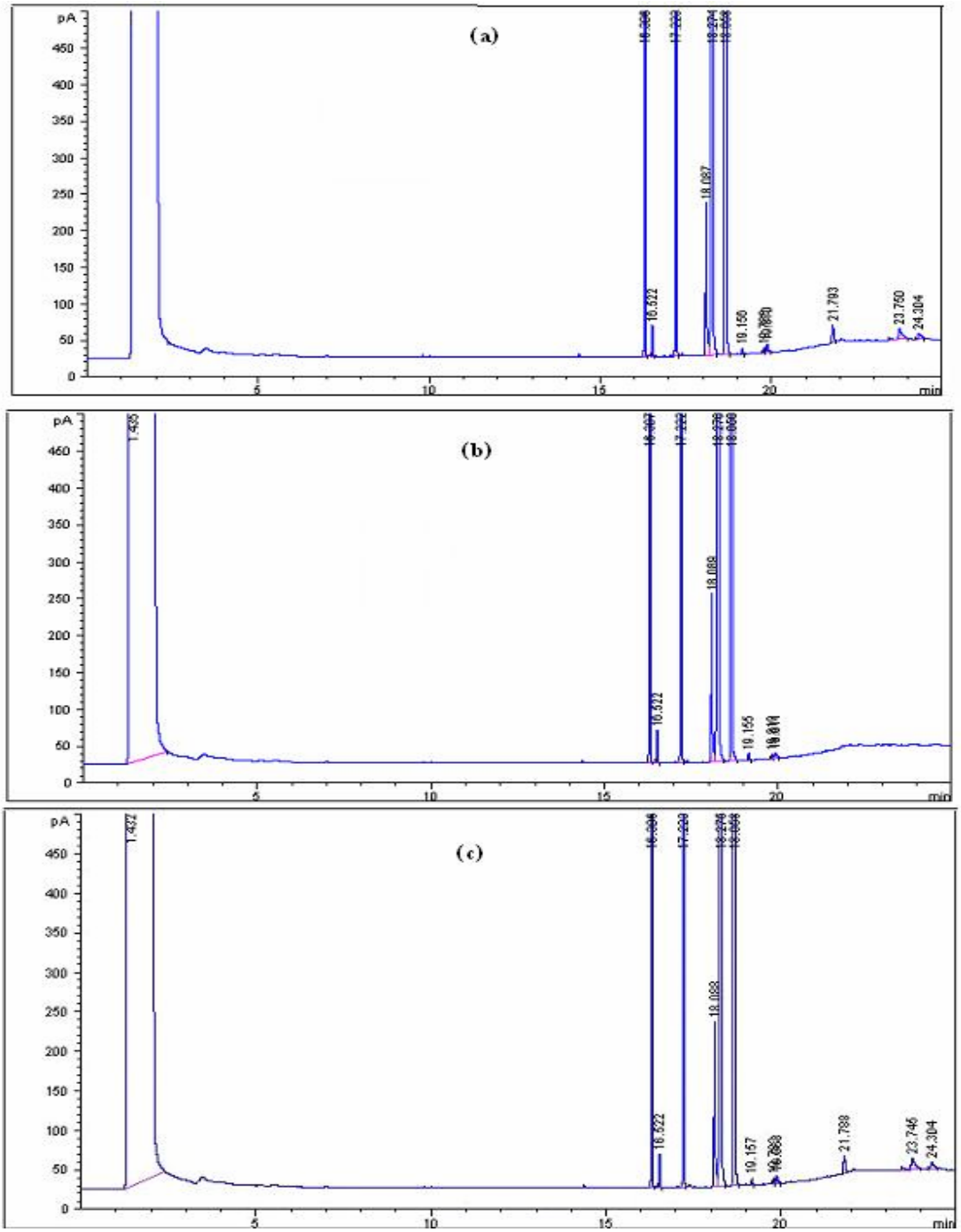
Appendix Table A6 Effect of stirring rate on phorbol esters removal from press cake using 95% ethanol.

No.	Stirring rate (rpm)	PEs in press cake after washing KOH (mg/g)	Weight of press cake (g)	Concentration of phorbol esters							
				Solution				Press cake			
				Chromatogram		ppm	mg/g	Chromatogram		ppm	mg/g
				RT (min)	Area			RT (min)	Area		
1	0	0.3201	5.0169	6.338	69681	420.7790	0.2104	8.248	36383	22.0423	0.1098
2	100	0.2976	4.9091	5.215	62860	379.5894	0.1939	10.570	27185	16.4698	0.0839

APPENDIX B

Chemical and physical properties

Appendix Figure B1 Fatty acids composition of *Jatropha* seed oil detected by GC; raw seed oil (a), seed oil after one-time adsorption (b) and seed oil after two-time adsorption (c).



Appendix Table B1 Area peak of fatty acids from *Jatropha* seed oil (see appendix Figure B1).

Fatty Acids	Raw *Jatropha* seed oil				One-time adsorption			
	Sample 1		Sample 2		Sample 1		Sample 2	
	RT (min)	Area (%)	RT (min)	Area (%)	RT (min)	Area (%)	RT (min)	Area (%)
Myristic (C_{14})	14.305	0.05965	14.307	0.06615	14.305	0.06134	14.307	0.06092
Palmitic (C_{16})	16.275	14.51486	16.253	14.73073	16.277	14.5284	16.256	14.61432
Palmitoleic ($C_{16:1}$)	16.464	0.84519	16.457	0.82143	16.464	0.84988	16.458	0.89384
Steric (C_{18})	18.092	6.70430	18.042	6.19991	18.098	6.67706	18.050	6.51960
Oleic ($C_{18:1}$)	18.274	42.62792	18.233	43.21371	18.280	42.65733	18.240	42.97339
Linoleic ($C_{18:2}$)	18.654	34.81258	18.614	34.47984	18.661	34.81762	18.621	34.56708
Linolenic ($C_{18:3}$)	19.096	0.19896	19.089	0.17126	19.097	0.19549	19.090	0.18508

Appendix Table B1 (Continued)

Fatty Acids	Raw *Jatropha* seed oil			
	Sample 1		Sample 2	
	RT (min)	Area (%)	RT (min)	Area (%)
Myristic (C_{14})	14.304	0.06498	14.306	0.05824
Palmitic (C_{16})	16.281	14.74025	16.254	14.52950
Palmitoleic ($C_{16:1}$)	16.465	0.93661	16.570	0.84492
Steric (C_{18})	18.101	6.55530	18.049	6.49503
Oleic ($C_{18:1}$)	18.283	42.62867	18.237	42.95955
Linoleic ($C_{18:2}$)	18.663	34.69085	18.619	34.74104
Linolenic ($C_{18:3}$)	19.098	0.19550	19.088	0.18503

RT = retention time

Appendix Table B2 Amino acids from *Jatropha* press cake before and after phorbol esters removal.

Amino acid	Amino acid (mg/100mg)		
	press cake	3% KOH washing press cake	95% ethanol immersing press cake
Aspartic acid	1.96	1.71	0.95
Serine	1.03	0.89	0.48
Glutamic acid	3.30	2.73	1.46
Glycine	0.92	0.80	0.46
Histidine	0.41	0.34	0.17
Arginine	2.37	2.02	1.02
Threonine	0.92	0.81	0.48
Alanine	1.08	0.96	0.57
Proline	1.01	0.90	0.51
Tyrosine	0.41	0.34	0.15
Valine	1.00	0.86	0.51
Lysine	0.76	0.61	0.27
Isoleucine	0.82	0.69	0.41
Leucine	1.51	1.33	0.75
Phenylalanine	0.95	0.84	0.47

Appendix Table B2 (Continued)

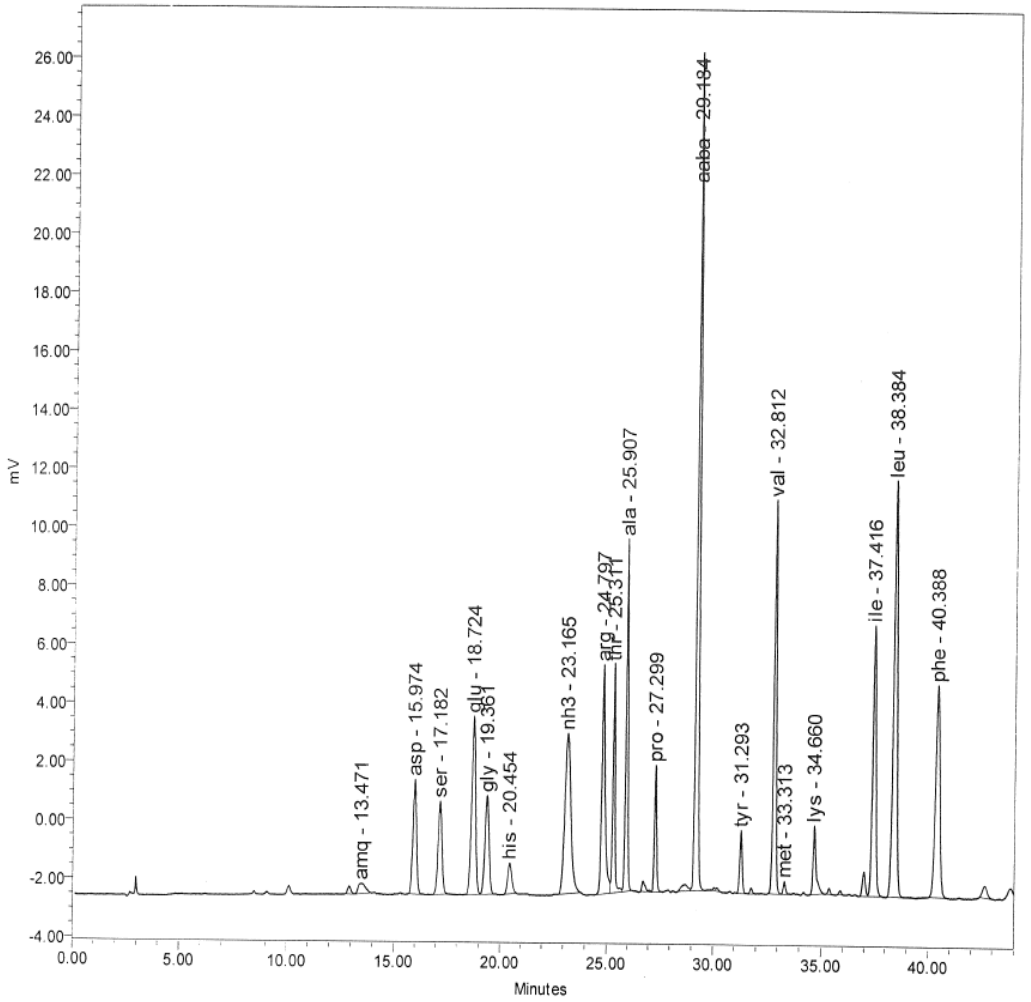
Individual Sample Report

Reported by User: System

Project Name: cif002_2551

SAMPLE INFORMATION

Sample Name:	TB 003-1	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	23/11/2550 1:19:10
Vial:	31	Acq. Method Set:	HypersilGold_54_35C
Injection #:	1	Date Processed:	23/11/2550 10:21:36
Injection Volume:	5.00 ul	Processing Method:	231107
Run Time:	54.0 Minutes	Channel Name:	SATIN
Sample Set Name:		Proc. Chnl. Descr.:	



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Appendix Table B2 (Continued)

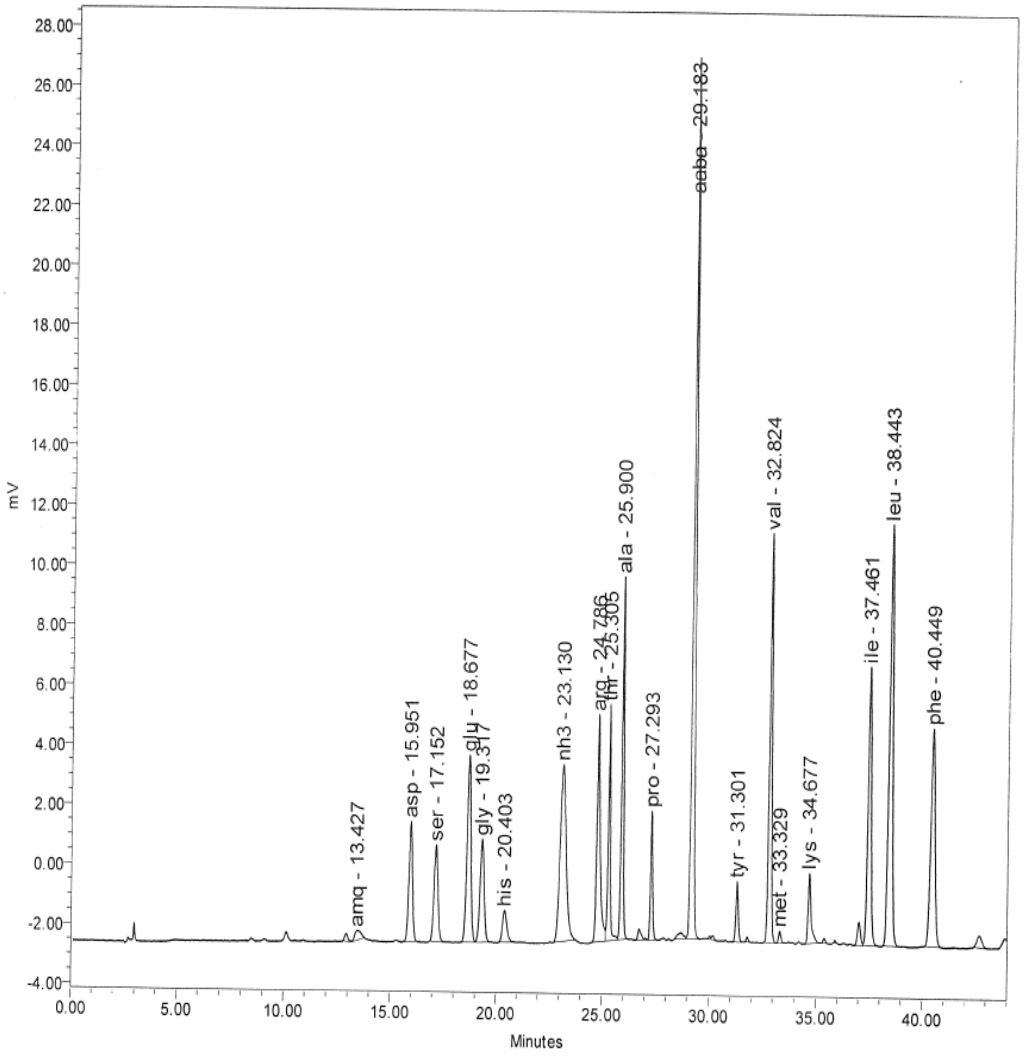
Individual Sample Report

Reported by User: System

Project Name: cif002_2551

SAMPLE INFORMATION

Sample Name:	TB 003-2	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	23/11/2550 2:14:04
Vial:	32	Acq. Method Set:	HypersilGold_54_35C
Injection #:	1	Date Processed:	23/11/2550 10:21:36
Injection Volume:	5.00 ul	Processing Method:	231107
Run Time:	54.0 Minutes	Channel Name:	SATIN
Sample Set Name:		Proc. Chnl. Descr.:	



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Appendix Table B2 (Continued)

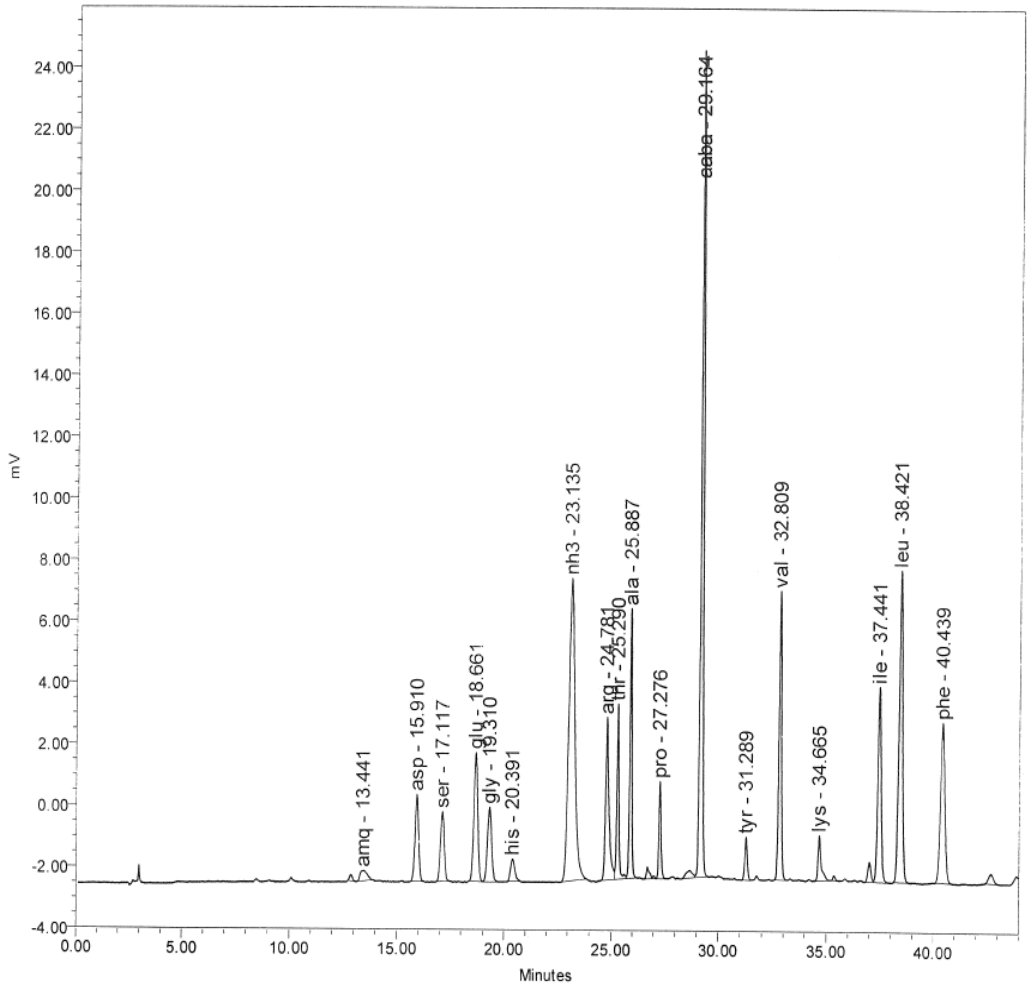
Individual Sample Report

Reported by User: System

Project Name: cif002_2551

SAMPLE INFORMATION

Sample Name:	TB 004-1	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	23/11/2550 3:09:00
Vial:	34	Acq. Method Set:	HypersilGold_54_35C
Injection #:	1	Date Processed:	17/12/2550 18:29:51
Injection Volume:	5.00 ul	Processing Method:	231107
Run Time:	54.0 Minutes	Channel Name:	SATIN
Sample Set Name:		Proc. Chnl. Descr.:	



Report Method: Default Individual Report Printed 18:30:40 17/12/2550 Page: 1 of 1

Appendix Table B2 (Continued)

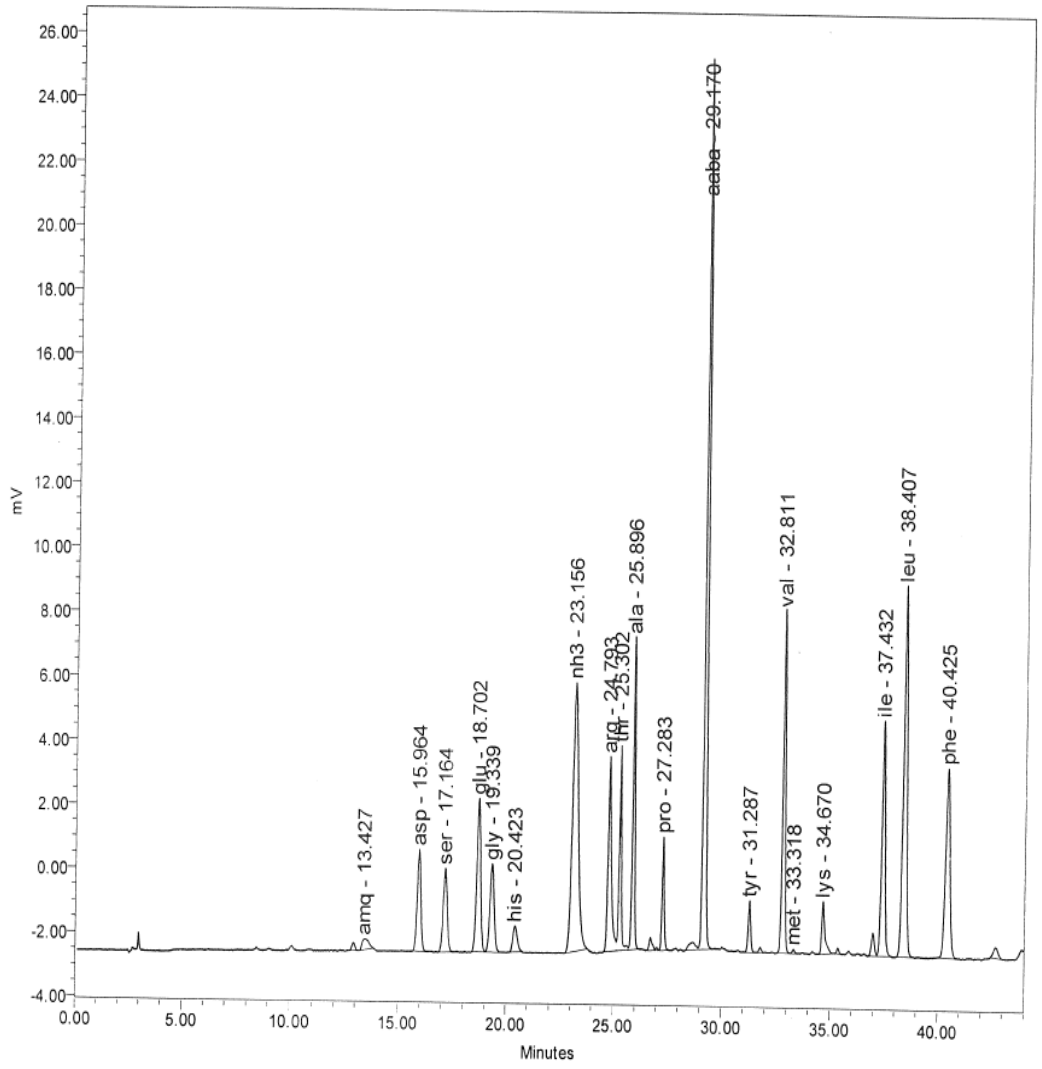
Individual Sample Report

Reported by User: System

Project Name: cif002_2551

SAMPLE INFORMATION

Sample Name:	TB 004-2	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	23/11/2550 4:03:57
Vial:	35	Acq. Method Set:	HypersilGold_54_35C
Injection #:	1	Date Processed:	23/11/2550 10:21:37
Injection Volume:	5.00 ul	Processing Method:	231107
Run Time:	54.0 Minutes	Channel Name:	SATIN
Sample Set Name:		Proc. Chnl. Descr.:	



Report Method: Default Individual Report Printed 18:31:50 17/12/2550 Page: 1 of 1

Appendix Table B2 (Continued)

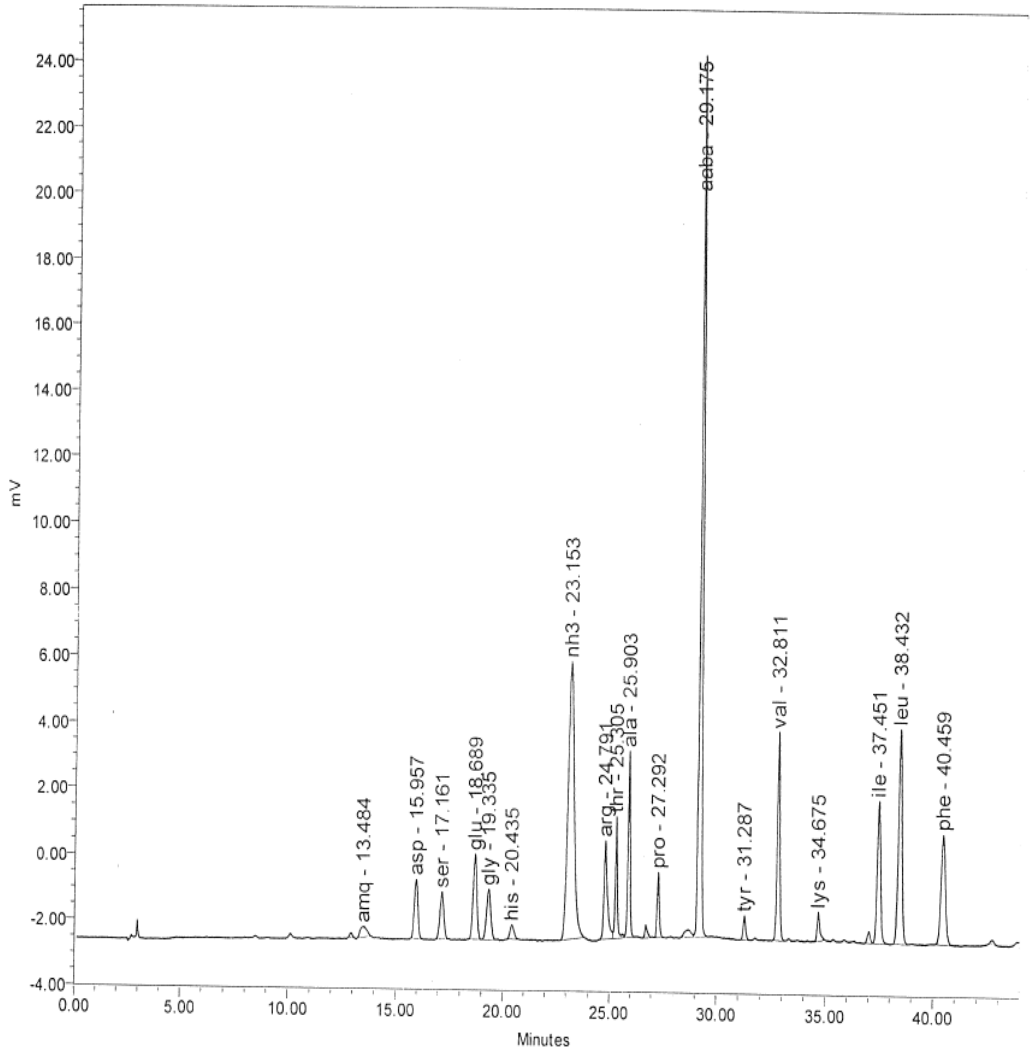
Individual Sample Report

Reported by User: System

Project Name: cif002_2551

SAMPLE INFORMATION

Sample Name:	TB 005-1	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	23/11/2550 4:58:55
Vial:	37	Acq. Method Set:	HypersilGold_54_35C
Injection #:	1	Date Processed:	23/11/2550 10:21:37
Injection Volume:	5.00 ul	Processing Method:	231107
Run Time:	54.0 Minutes	Channel Name:	SATIN
Sample Set Name:		Proc. Chnl. Descr.:	



Report Method: Default Individual Report Printed 18:27:51 17/12/2550 Page: 1 of 1

Appendix Table B2 (Continued)

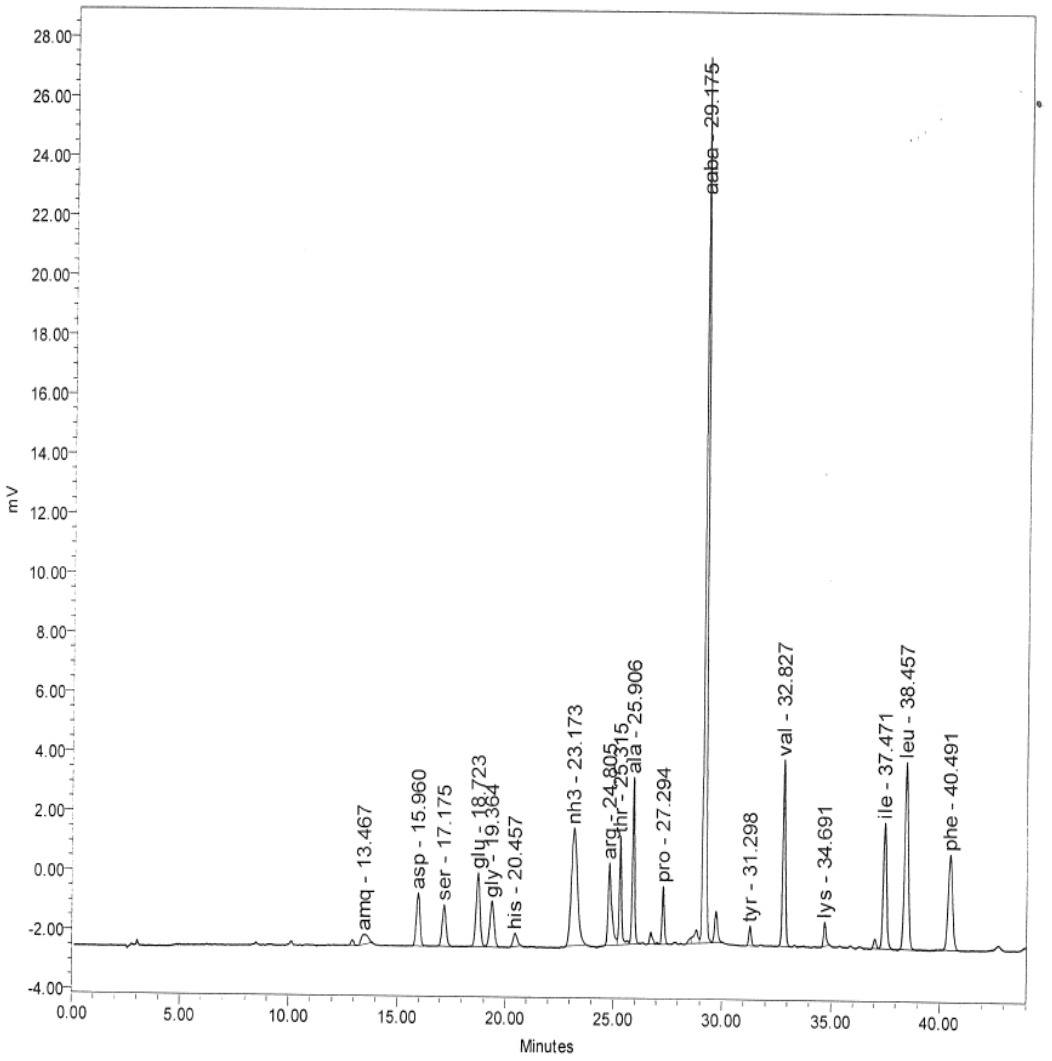
Individual Sample Report

Reported by User: System

Project Name: cif002_2551

SAMPLE INFORMATION

Sample Name:	TB 005-2	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	23/11/2550 5:53:53
Vial:	38	Acq. Method Set:	HypersilGold_54_35C
Injection #:	1	Date Processed:	23/11/2550 10:21:37
Injection Volume:	5.00 ul	Processing Method:	231107
Run Time:	54.0 Minutes	Channel Name:	SATIN
Sample Set Name:		Proc. Chnl. Descr.:	



Report Method: Default Individual Report Printed 18:27:51 17/12/2550 Page: 1 of 1

Appendix Table B2 (Continued)

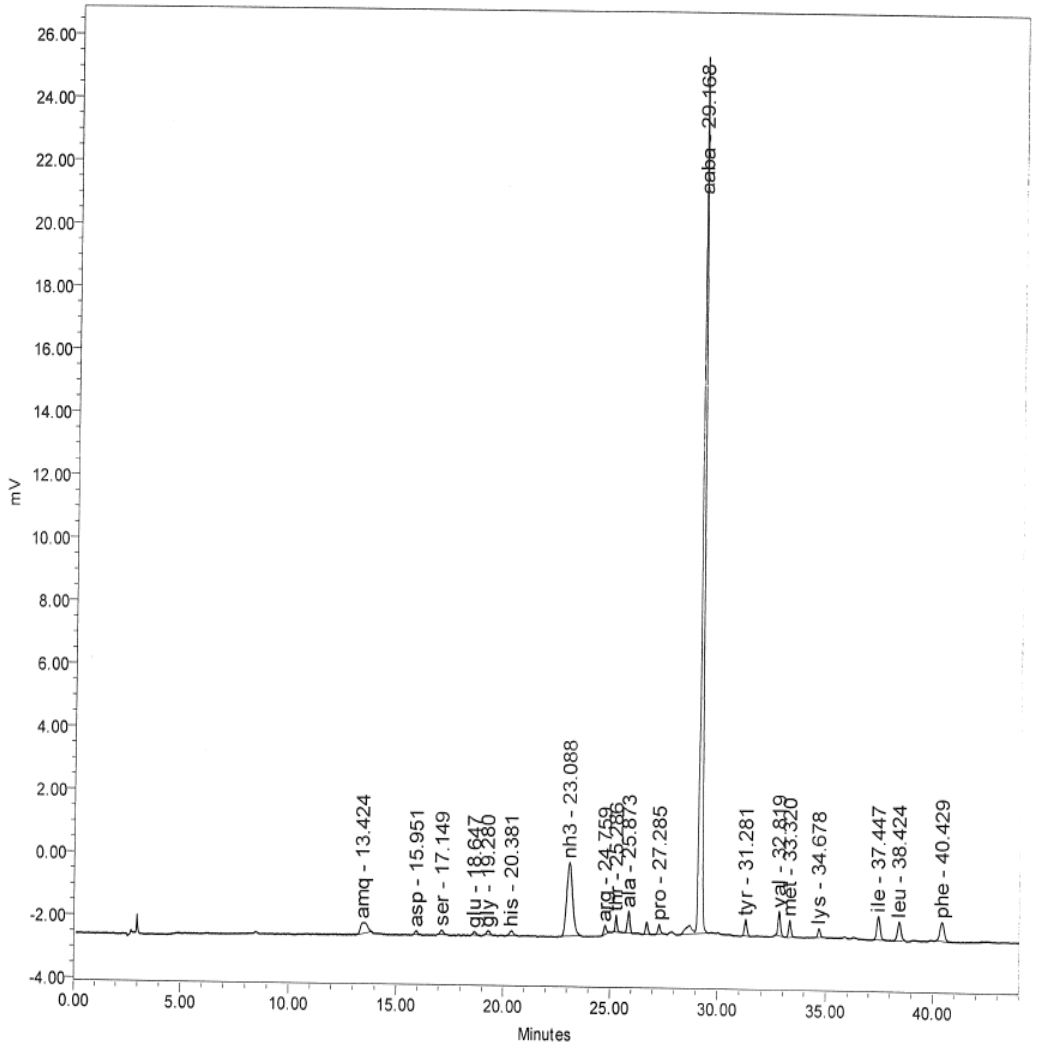
Individual Sample Report

Reported by User: System

Project Name: cif002_2551

SAMPLE INFORMATION

Sample Name:	H-STD 1 pmol	Acquired By:	System
Sample Type:	Standard	Date Acquired:	22/11/2550 17:59:11
Vial:	2	Acq. Method Set:	HypersilGold_54_35C
Injection #:	1	Date Processed:	23/11/2550 10:20:02
Injection Volume:	5.00 ul	Processing Method:	231107
Run Time:	54.0 Minutes	Channel Name:	SATIN
Sample Set Name:		Proc. Chnl. Descr.:	



Report Method: Default Individual Report Printed 14:58:36 17/12/2550 Page: 1 of 1

Appendix Table B2 (Continued)

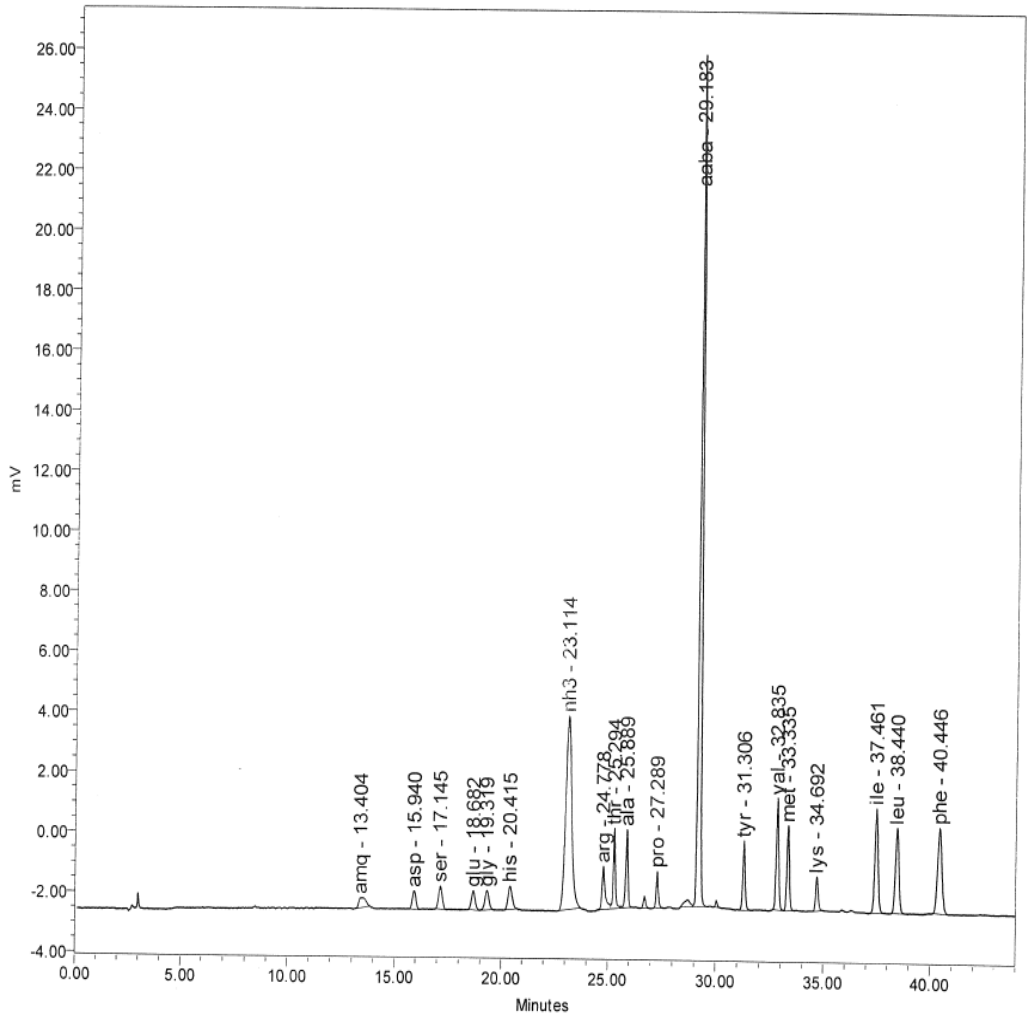
Individual Sample Report

Reported by User: System

Project Name: cif002_2551

SAMPLE INFORMATION

Sample Name:	H-STD 5 pmol	Acquired By:	System
Sample Type:	Standard	Date Acquired:	22/11/2550 18:54:22
Vial:	6	Acq. Method Set:	HypersilGold_54_35C
Injection #:	1	Date Processed:	23/11/2550 10:20:04
Injection Volume:	5.00 ul	Processing Method:	231107
Run Time:	54.0 Minutes	Channel Name:	SATIN
Sample Set Name:		Proc. Chnl. Descr.:	



Report Method: Default Individual Report Printed 14:58:37 17/12/2550 Page: 1 of 1

Appendix Table B2 (Continued)

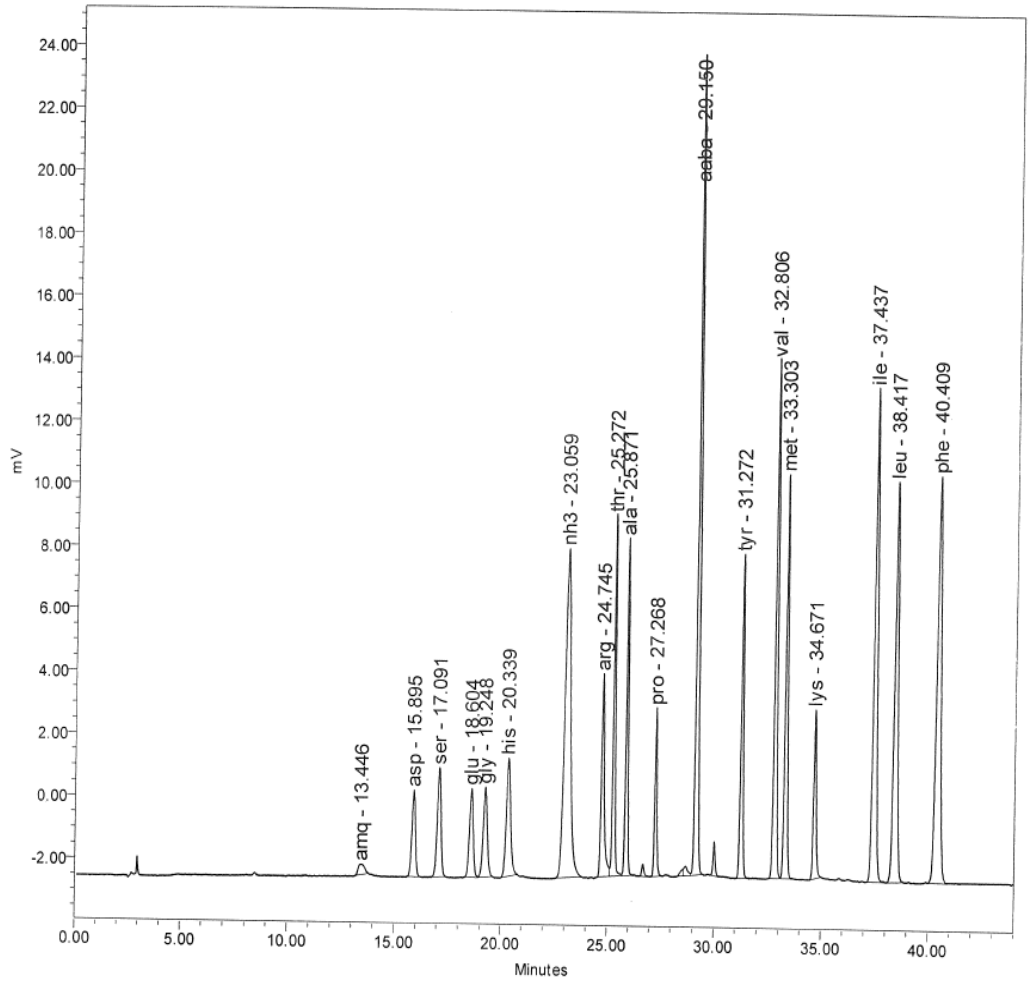
Individual Sample Report

Reported by User: System

Project Name: cif002_2551

SAMPLE INFORMATION

Sample Name:	H-STD 25 pmol	Acquired By:	System
Sample Type:	Standard	Date Acquired:	22/11/2550 15:14:07
Vial:	3	Acq. Method Set:	HypersilGold_54_35C
Injection #:	1	Date Processed:	23/11/2550 10:20:10
Injection Volume:	5.00 ul	Processing Method:	231107
Run Time:	54.0 Minutes	Channel Name:	SATIN
Sample Set Name:		Proc. Chnl. Descr.:	



Report Method: Default Individual Report Printed 14:58:38 17/12/2550 Page: 1 of 1

Appendix Table B2 (Continued)

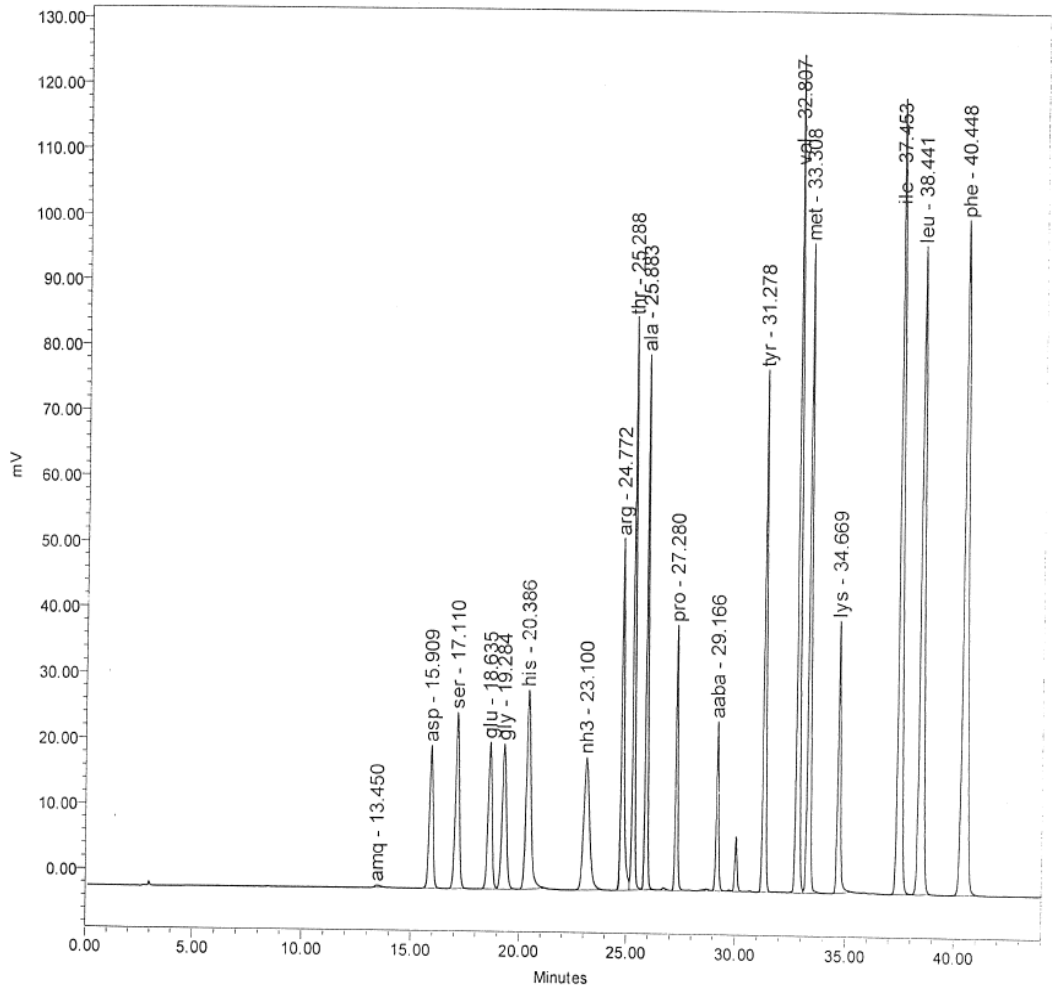
Individual Sample Report

Reported by User: System

Project Name: cif002_2551

SAMPLE INFORMATION

Sample Name:	H-STD 200 pmol	Acquired By:	System
Sample Type:	Standard	Date Acquired:	22/11/2550 17:04:02
Vial:	5	Acq. Method Set:	HypersilGold_54_35C
Injection #:	1	Date Processed:	23/11/2550 10:20:06
Injection Volume:	5.00 ul	Processing Method:	231107
Run Time:	54.0 Minutes	Channel Name:	SATIN
Sample Set Name:		Proc. Chnl. Descr.:	



Report Method: Default Individual Report Printed 14:58:37 17/12/2550 Page: 1 of 1

Appendix Table B2 (Continued)

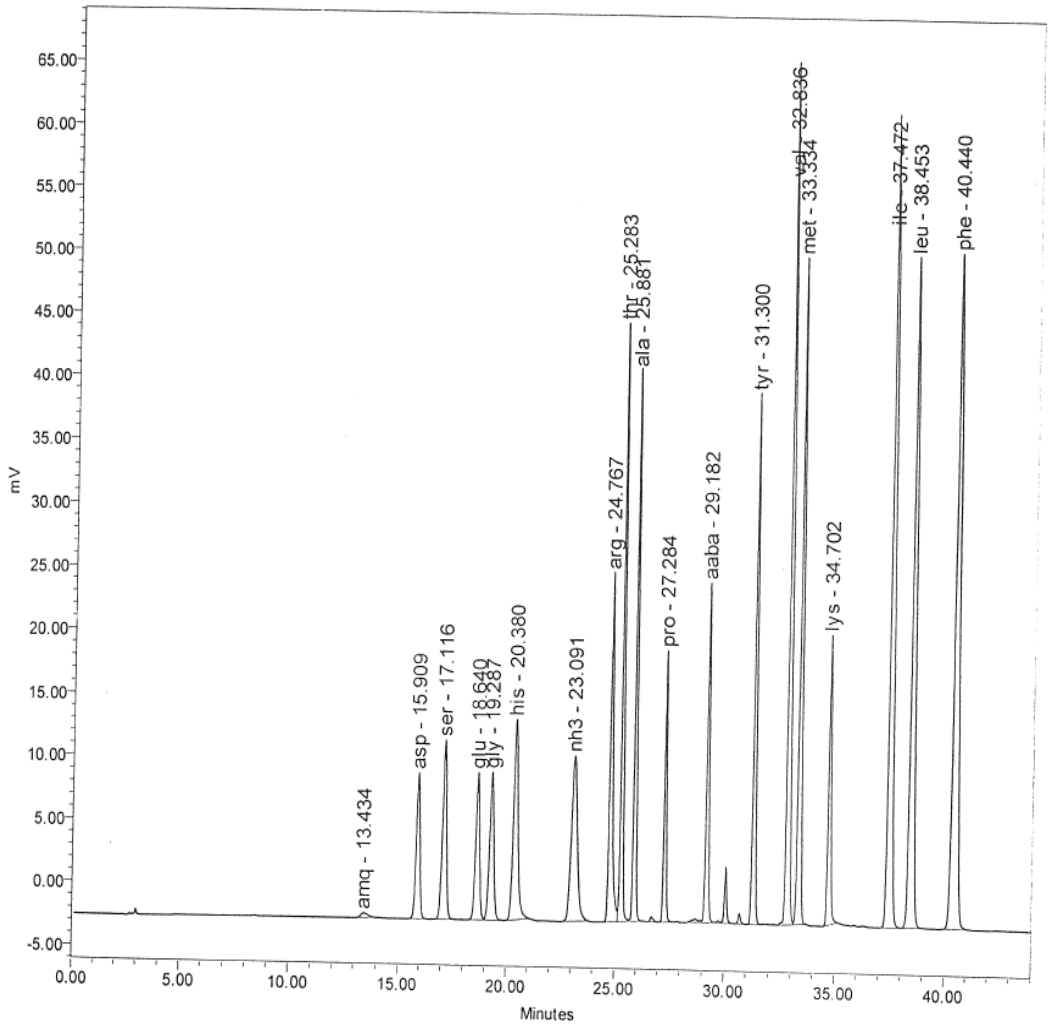
Individual Sample Report

Reported by User: System

Project Name: cif002_2551

SAMPLE INFORMATION

Sample Name:	H-STD 100 pmol	Acquired By:	System
Sample Type:	Standard	Date Acquired:	22/11/2550 16:09:04
Vial:	4	Acq. Method Set:	HypersilGold_54_35C
Injection #:	1	Date Processed:	23/11/2550 10:20:08
Injection Volume:	5.00 ul	Processing Method:	231107
Run Time:	54.0 Minutes	Channel Name:	SATIN
Sample Set Name:		Proc. Chnl. Descr.:	



Report Method: Default Individual Report Printed 14:58:38 17/12/2550 Page: 1 of 1

Appendix B3 Surface area of adsorbents in experiment detected by Autosorb1 by Thailand Institute of Scientific and Technological Research.

sample	Surface area	
	Single point BET (m^2/g)	Multipoint BET (m^2/g)
Activated carbon	932.10	923.80
Bentonite 150	186.20	190.40
Bentonite 200	318.80	327.30
Chitosan	-	-
Chitin	1.13	1.13

Chitosan cannot detect.

Appendix Table B3 (Continued)

Date: 10/11/2006

Quantachrome Corporation
Quantachrome Autosorb Automated Gas Sorption System Report
Autosorb for Windows® Version 1.19

Sample ID	Actvated Carbon				
Description	TISTR				
Comments					
Sample Weight	0.1060 g				
Adsorbate	NITROGEN	Outgas Temp	300.0 °C	Operator	Rungrueng
Cross-Sec Area	16.2 Å²/molecule	Outgas Time	3.0 hrs	Analysis Time	116.9 min
NonIdeality	6.580E-05	P/Po Toler	0	End of Run	09/27/2006 1
Molecular Wt	28.0134 g/mol	Equil Time	3	File Name	AS6B0826.RAW
Station #	1	Bath Temp.	77.40		

AREA-VOLUME-PORE SIZE SUMMARY

SURFACE AREA DATA

Multipoint BET	9.238E+02	m²/g
Single Point BET	9.321E+02	m²/g
Langmuir Surface Area	1.371E+03	m²/g
t-Method External Surface Area	2.680E+02	m²/g
t-Method Micro Pore Surface Area	6.558E+02	m²/g
DR Method Micro Pore Area	1.356E+03	m²/g

PORE VOLUME DATA

t-Method Micro Pore Volume	3.446E-01	cc/g
DR Method Micro Pore Volume	4.818E-01	cc/g
HK Method Cumulative Pore Volume	4.573E-01	cc/g
SF Method Cumulative Pore Volume	4.598E-01	cc/g

PORE SIZE DATA

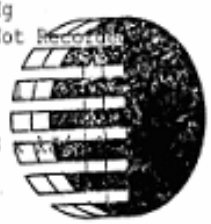
DR Method Micro Pore Width	6.106E+01	Å
DA Method Pore Diameter (Mode)	1.600E+01	Å
HK Method Pore Width (Mode)	1.388E+01	Å
SF Method Pore Diameter (Mode)	2.602E+01	Å

DATA REDUCTION PARAMETERS

Thermal Transpiration : ON
Effective Molecule Diameter (D) 3.5400 Å
Effective Cell Stem Inner Diameter (d) 4.0000 mm
Last Po Acquired 797.59 mm Hg
Additional Initialization Information Not Recorded

BJH/DH Moving Average Size :

Interaction Constant (K) 2.9600 nm^3



Appendix Table B3 (Continued)

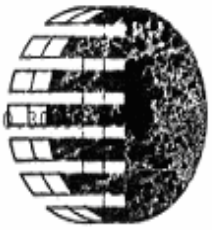
Date: 10/11/2006

Quantachrome Corporation
Quantachrome Autosorb Automated Gas Sorption System Report
Autosorb for Windows® Version 1.19

Sample ID	Actvated Carbon				
Description	TISTR				
Comments					
Sample Weight	0.1060 g				
Adsorbate	NITROGEN	Outgas Temp	300.0 °C	Operator	Rungrueng
Cross-Sec Area	16.2 Å²/molecule	Outgas Time	3.0 hrs	Analysis Time	116.9 min
NonIdeality	6.580E-05	P/Po Toler	0	End of Run	09/27/2006 1
Molecular Wt	28.0134 g/mol	Equil Time	3	File Name	AS6B0826.RAW
Station #	1	Bath Temp.	77.40		

SINGLE POINT BET

Single point area = 932.07 m²/g at P/Po = [illegible]



Appendix Table B3 (Continued)

Date: 10/11/2006

Quantachrome Corporation
Quantachrome Autosorb Automated Gas Sorption System Report
Autosorb for Windows® Version 1.19

Sample ID	Actvated Carbon				
Description	TISTR				
Comments					
Sample Weight	0.1060 g				
Adsorbate	NITROGEN	Outgas Temp	300.0 °C	Operator	Rungrueng
Cross-Sec Area	16.2 Å²/molecule	Outgas Time	3.0 hrs	Analysis Time	116.9 min
NonIdeality	6.580E-05	P/Po Toler	0	End of Run	09/27/2006 1
Molecular Wt	28.0134 g/mol	Equil Time	3	File Name	AS6B0826.RAW
Station #	1	Bath Temp.	77.40		

MULTIPOINT BET

P/Po	Volume [cc/g] STP	1/(W((Po/P)-1))
5.5930e-02	275.2217	1.722E-01
7.5473e-02	281.7811	2.318E-01
1.0190e-01	287.9547	3.153E-01
1.2545e-01	292.0292	3.930E-01
1.4969e-01	295.3330	4.769E-01
1.7538e-01	298.1273	5.708E-01
2.0159e-01	300.4491	6.724E-01
2.2449e-01	302.1708	7.665E-01
2.4979e-01	303.7604	8.770E-01
2.7560e-01	305.1575	9.975E-01
3.0098e-01	306.3462	1.125E+00

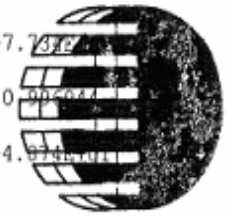
Area = 9.238E+02 m²/g

Slope = 3.847E+00

Y - Intercept = -7.[illegible]

Correlation Coefficient = 0.[illegible]

C = -4.[illegible]



Appendix Table B3 (Continued)

Date: 10/11/2006

Quantachrome Corporation
Quantachrome Autosorb Automated Gas Sorption System Report
Autosorb for Windows® Version 1.19

Sample ID	Bentonite 150				
Description	TISTR				
Comments					
Sample Weight	0.1399 g				
Adsorbate	NITROGEN	Outgas Temp	300.0 °C	Operator	Rungrueng
Cross-Sec Area	16.2 Å²/molecule	Outgas Time	3.0 hrs	Analysis Time	86.2 min
NonIdeality	6.500E-05	P/Po Toler	0	End of Run	09/27/2006 1
Molecular Wt	28.0134 g/mol	Equil Time	3	File Name	AS6BC827.RAW
Station #	1	Bath Temp.	77.40		

AREA-VOLUME-PORE SIZE SUMMARY

SURFACE AREA DATA

Multipoint BET	1.904E-02	m²/g
Single Point BET	1.852E-02	m²/g
Langmuir Surface Area	3.003E-02	m²/g
t-Method External Surface Area	1.815E-02	m²/g
t-Method Micro Pore Surface Area	8.908E-00	m²/g
DR Method Micro Pore Area	2.489E+02	m²/g

PORE VOLUME DATA

t-Method Micro Pore Volume	3.509E-03	cc/g
DR Method Micro Pore Volume	8.845E-02	cc/g
HK Method Cumulative Pore Volume	7.718E-02	cc/g
SF Method Cumulative Pore Volume	7.891E-02	cc/g

PORE SIZE DATA

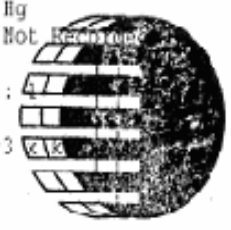
DR Method Micro Pore Width	1.015E+02	Å
DA Method Pore Diameter (Mode)	1.820E+01	Å
HK Method Pore Width (Mode)	1.383E+01	Å
SF Method Pore Diameter (Mode)	2.593E+01	Å

DATA REDUCTION PARAMETERS

Thermal Transpiration : ON
Effective Molecule Diameter (D) 3.5400 Å
Effective Cell Stem Inner Diameter (d) 4.0000 mm
Last Po Acquired 796.22 mm Hg
Additional Initialization Information Not Entered

BJH/DH Moving Average Size : 4

Interaction Constant (K) 2.9600 nm^3



Appendix Table B3 (Continued)

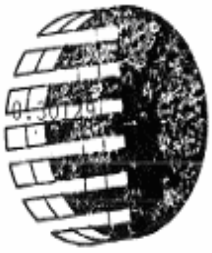
Date: 10/11/2006

Quantachrome Corporation
Quantachrome Autosorb Automated Gas Sorption System Report
Autosorb for Windows® Version 1.19

Sample ID	Bentonite 150				
Description	TISTR				
Comments					
Sample Weight	0.1399 g				
Adsorbate	NITROGEN	Outgas Temp	300.0 °C	Operator	Rungrueng
Cross-Sec Area	16.2 Å²/molecule	Outgas Time	3.0 hrs	Analysis Time	86.2 min
NonIdeality	6.580E-05	P/Po Toler	0	End of Run	09/27/2006 1
Molecular Wt	28.0134 g/mol	Equil Time	3	File Name	AS6B0827.RAW
Station #	1	Bath Temp.	77.40		

SINGLE POINT BET

Single point area = 186.15 m²/g at P/Po = 0.30129



Appendix Table B3 (Continued)

Date: 10/11/2006

Quantachrome Corporation
Quantachrome Autosorb Automated Gas Sorption System Report
Autosorb for Windows® Version 1.19

Sample ID	Bentonite 150				
Description	TISTR				
Comments					
Sample Weight	0.1399 g				
Adsorbate	NITROGEN	Outgas Temp	300.0 °C	Operator	Rungrueng
Cross-Sec Area	16.2 Å²/molecule	Outgas Time	3.0 hrs	Analysis Time	86.2 min
NonIdeality	6.580E-05	P/Po Toler	0	End of Run	09/27/2006 1
Molecular Wt	28.0134 g/mol	Equil Time	3	File Name	AS6B0827.RAW
Station #	1	Bath Temp.	77.40		

MULTIPOINT BET

P/Po	Volume [cc/g] STP	1/(W((Po/P)-1))
5.3550e-02	40.3645	1.122E+00
7.5308e-02	43.1916	1.509E+00
1.0110e-01	45.6779	1.970E+00
1.2646e-01	47.8451	2.421E+00
1.4950e-01	49.6722	2.832E+00
1.7436e-01	51.5742	3.276E+00
1.9936e-01	53.4380	3.728E+00
2.2656e-01	55.4671	4.225E+00
2.4953e-01	57.1793	4.653E+00
2.7645e-01	59.2313	5.161E+00
3.0129e-01	61.2104	5.637E+00

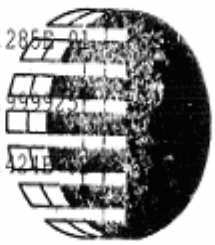
Area = 1.904E+02 m²/g

Slope = 1.816E+01

Y - Intercept = 1.28[illegible]

Correlation Coefficient = 0.[illegible]

C = 1.42[illegible]



Appendix Table B3 (Continued)

Date: 10/11/2006

Quantachrome Corporation
Quantachrome Autosorb Automated Gas Sorption System Report
Autosorb for Windows® Version 1.19

Sample ID	Bentonite 200C				
Description	TISTR				
Comments					
Sample Weight	0.2732 g				
Adsorbate	NITROGEN	Outgas Temp	300.0 °C	Operator	Rungrueng
Cross-Sec Area	16.2 Å²/molecule	Outgas Time	3.0 hrs	Analysis Time	134.6 min
NonIdeality	6.580E-05	P/Po Toler	0	End of Run	10/05/2006 1
Molecular Wt	28.0134 g/mol	Equil Time	3	File Name	AS6B0332.RAW
Station #	1	Bath Temp.	77.40		

AREA-VOLUME-PORE SIZE SUMMARY

SURFACE AREA DATA

Multipoint BET	3.273E+02	m²/g
Single Point BET	3.188E+02	m²/g
Langmuir Surface Area	5.182E+02	m²/g
t-Method External Surface Area	3.240E+02	m²/g
t-Method Micro Pore Surface Area	3.276E+00	m²/g
DR Method Micro Pore Area	4.188E+02	m²/g

PORE VOLUME DATA

t-Method Micro Pore Volume	-8.255E-04	cc/g
DR Method Micro Pore Volume	1.488E-01	cc/g
HK Method Cumulative Pore Volume	1.304E-01	cc/g
SF Method Cumulative Pore Volume	1.335E-01	cc/g

PORE SIZE DATA

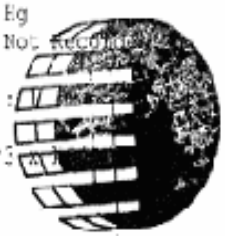
DR Method Micro Pore Width	1.014E+02	Å
DA Method Pore Diameter (Mode)	1.840E+01	Å
HK Method Pore Width (Mode)	1.402E+01	Å
SF Method Pore Diameter (Mode)	2.630E+01	Å

DATA REDUCTION PARAMETERS

Thermal Transpiration : ON
Effective Molecule Diameter (D) 3.5400 Å
Effective Cell Stem Inner Diameter (d) 4.0000 mm
Last Po Acquired 793.01 mm Hg
Additional Initialization Information Not Recorded

BJH/DH Moving Average Size :

Interaction Constant (K) 2.960E nm^3



Appendix Table B3 (Continued)

Date: 10/11/2006

Quantachrome Corporation
Quantachrome Autosorb Automated Gas Sorption System Report
Autosorb for Windows® Version 1.19

Sample ID	Bentonite 200C				
Description	TISTR				
Comments					
Sample Weight	0.2792 g				
Adsorbate	NITROGEN	Outgas Temp	300.0 °C	Operator	Rungrueng
Cross-Sec Area	16.2 Å²/molecule	Outgas Time	3.0 hrs	Analysis Time	134.6 min
NonIdeality	6.580E-05	P/Po Toler	0	End of Run	10/05/2006 1
Molecular Wt	28.0134 g/mol	Equil Time	3	File Name	AS6B0832.RAW
Station #	1	Bath Temp.	77.40		

SINGLE POINT BET

Single point area = 318.83 m²/g at P/Po = 0.301

Appendix Table B3 (Continued)

Date: 10/11/2006

Quantachrome Corporation
Quantachrome Autosorb Automated Gas Sorption System Report
Autosorb for Windows® Version 1.19

Sample ID	Bentonite 200C				
Description	TISTR				
Comments					
Sample Weight	0.2792 g				
Adsorbate	NITROGEN	Outgas Temp	300.0 °C	Operator	Rungrueng
Cross-Sec Area	16.2 Å²/molecule	Outgas Time	3.0 hrs	Analysis Time	134.6 min
NonIdeality	6.580E-05	P/Po Toler	0	End of Run	10/05/2006 1
Molecular Wt	28.0134 g/mol	Equil Time	3	File Name	AS6B0832.RAW
Station #	1	Bath Temp.	77.40		

MULTIPOINT BET

P/Po	Volume [cc/g] STP	1/(W((Po/P)-1))
5.2667e-02	67.9155	6.550E-01
7.5045e-02	72.3782	8.969E-01
1.0070e-01	76.6848	1.168E+00
1.2439e-01	80.2887	1.416E+00
1.4936e-01	83.8750	1.675E+00
1.7454e-01	87.3635	1.936E+00
1.9981e-01	90.8037	2.200E+00
2.2453e-01	94.1906	2.460E+00
2.4923e-01	97.5835	2.722E+00
2.7542e-01	101.2103	3.005E+00
3.0105e-01	104.8009	3.288E+00

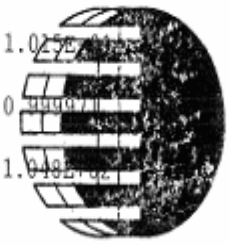
Area = 3.273E+02 m²/g

Slope = 1.054E+01

Y - Intercept = 1.0[illegible]

Correlation Coefficient = 0.[illegible]

C = 1.0[illegible]



Appendix Table B3 (Continued)

Date: 10/11/2006

Quantachrome Corporation
Quantachrome Autosorb Automated Gas Sorption System Report
Autosorb for Windows® Version 1.19

Sample ID	Chitin				
Description	TISTR				
Comments					
Sample Weight	0.0688 g				
Adsorbate	NITROGEN	Outgas Temp	150.0 °C	Operator	Rungrueng
Cross-Sec Area	16.2 Å²/molecule	Outgas Time	3.0 hrs	Analysis Time	60.6 min
NonIdeality	6.5800E-05	P/Po Toler	0	End of Run	09/29/2006
Molecular Wt	28.0134 g/mol	Equil Time	3	File Name	AS6B0628.RAW
Station #	1	Bath Temp.	77.40		

AREA-VOLUME-PORE SIZE SUMMARY

SURFACE AREA DATA

Multipoint BET	1.134E+00	m²/g
Single Point BET	1.131E+00	m²/g
Langmuir Surface Area	1.713E+00	m²/g
t-Method External Surface Area	6.345E-01	m²/g
t-Method Micro Pore Surface Area	4.992E-01	m²/g
DR Method Micro Pore Area	1.645E+00	m²/g

PORE VOLUME DATA

t-Method Micro Pore Volume	2.651E-04	cc/g
DR Method Micro Pore Volume	5.846E-04	cc/g
HK Method Cumulative Pore Volume	5.278E-04	cc/g
SF Method Cumulative Pore Volume	5.319E-04	cc/g

PORE SIZE DATA

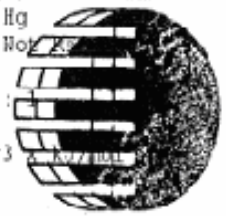
DR Method Micro Pore Width	8.332E-01	Å
DA Method Pore Diameter (Mode)	1.740E-01	Å
HK Method Pore Width (Mode)	1.358E-01	Å
SF Method Pore Diameter (Mode)	2.547E-01	Å

DATA REDUCTION PARAMETERS

Thermal Transpiration : ON
Effective Molecule Diameter (D) 3.5400 Å
Effective Cell Stem Inner Diameter (d) 4.0000 mm
Last Po Acquired 796.10 mm Hg
Additional Initialization Information Not

BJH/DH Moving Average Size

Interaction Constant (K) 2.9600 nm^3



Appendix Table B3 (Continued)

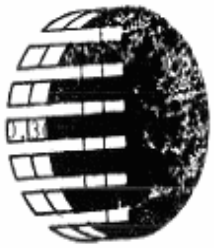
Date: 10/11/2006

Quantachrome Corporation
Quantachrome Autosorb Automated Gas Sorption System Report
Autosorb for Windows® Version 1.19

Sample ID	Chitin				
Description	TISTR				
Comments					
Sample Weight	0.0688 g				
Adsorbate	NITROGEN	Outgas Temp	150.0 °C	Operator	Rungrueng
Cross-Sec Area	16.2 Å²/molecule	Outgas Time	3.0 hrs	Analysis Time	60.6 min
NonIdeality	6.580E-05	P/Po Toler	0	End of Run	09/29/2006 1
Molecular Wt	28.0134 g/mol	Equil Time	3	File Name	AS6B0828.RAW
Station #	1	Bath Temp.	77.40		

SINGLE POINT BET

Single point area = 1.13 m²/g at P/Po = 0.30



Appendix Table B3 (Continued)

Date: 10/11/2006

Quantachrome Corporation
Quantachrome Autosorb Automated Gas Sorption System Report
Autosorb for Windows® Version 1.19

Sample ID	Chitin				
Description	TISTR				
Comments					
Sample Weight	0.0688 g				
Adsorbate	NITROGEN	Outgas Temp	150.0 °C	Operator	Rungrueng
Cross-Sec Area	16.2 Å²/molecule	Outgas Time	3.0 hrs	Analysis Time	60.6 min
NonIdeality	6.580E-05	P/Po Toler	0	End of Run	09/29/2006 1
Molecular Wt	28.0134 g/mol	Equil Time	3	File Name	AS6B0828.RAW
Station #	1	Bath Temp.	77.40		

MULTIPOINT BET

P/Po	Volume [cc/g] STP	1/(W((Po/P)-1))
5.0784e-02	0.2940	1.456E+02
7.7206e-02	0.3147	2.127E+02
1.0248e-01	0.3264	2.799E+02
1.2755e-01	0.3362	3.479E+02
1.5271e-01	0.3413	4.225E+02
1.7765e-01	0.3464	4.990E+02
2.0275e-01	0.3553	5.727E+02
2.2771e-01	0.3604	6.546E+02
2.5277e-01	0.3616	7.485E+02
2.7761e-01	0.3680	8.355E+02
3.0263e-01	0.3725	9.320E+02

Area = 1.134E+00 m²/g

Slope = 3.110E+03

Y - Intercept = -3.81

Correlation Coefficient = 0.

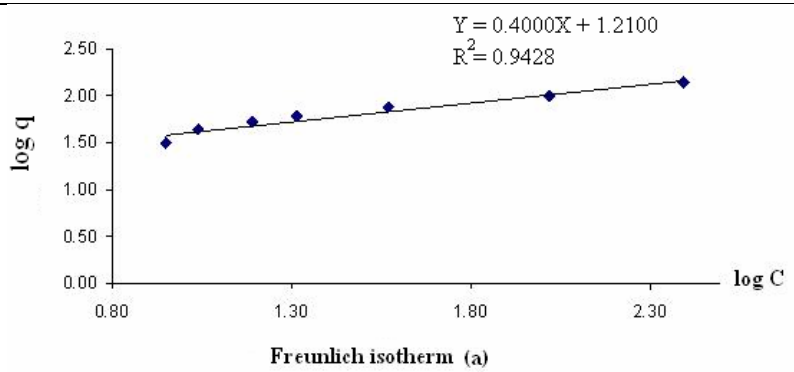
C = -8.05

APPENDIX C

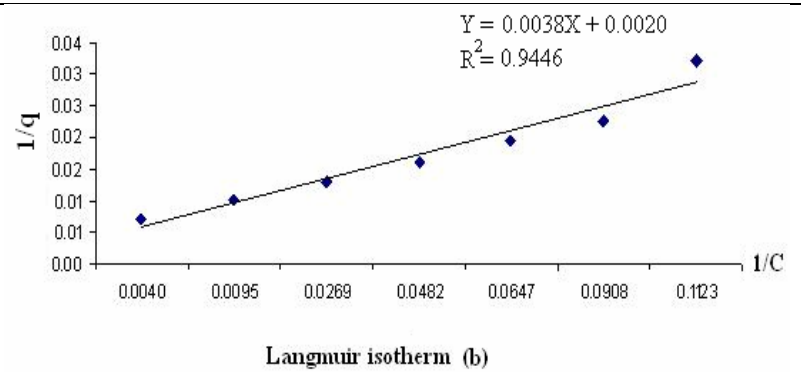
Result of Freundlich and Langmuir isotherms

Appendix Table C1 Freundlich and Langmuir isotherms for phorbol esters after one-time adsorption with bentonite 200.

	Freundlich isotherm $q = KC^{1/n}$ $\log q = \log K + 1/n \log C$						Langmuir isotherm $q = q_m KC / (1+KC)$ $1/q = 1/q_m + 1/ (K q_m C)$	
W (g)	C_0 (mg/L)	C (mg/L)	X (mg)	q (mg/g)	log q (mg/g)	log C (mg/L)	1/q (g/mg)	1/C (L/mg)
0.4000	2485.5725	248.4745	55.9275	139.8186	2.1456	2.3953	0.0072	0.0040
0.6000	2485.5725	104.8607	59.5178	99.1963	1.9965	2.0206	0.0101	0.0095
0.8000	2485.5725	37.1174	61.2114	76.5142	1.8837	1.5696	0.0131	0.0269
1.0000	2485.5725	20.7361	61.6209	61.6209	1.7897	1.3167	0.0162	0.0482
1.2000	2485.5725	15.4635	61.7527	51.4606	1.7115	1.1893	0.0194	0.0647
1.4000	2485.5725	11.0184	61.8639	44.1885	1.6453	1.0421	0.0226	0.0908
2.0000	2485.5725	8.9010	61.9168	30.9584	1.4908	0.9494	0.0323	0.1123



Freunlich isotherm (a)

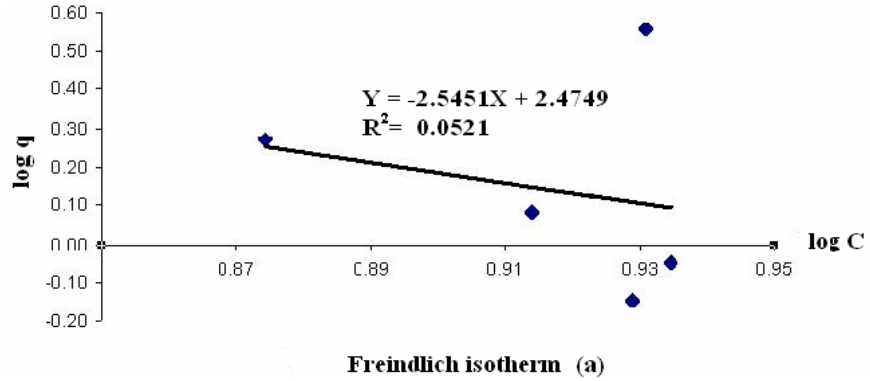


Langmuir isotherm (b)

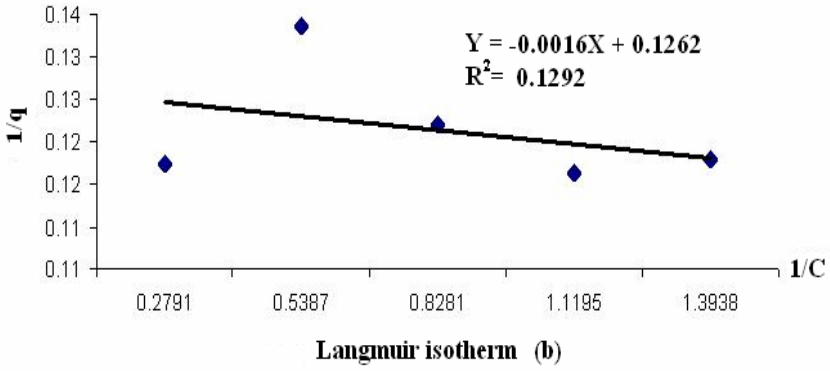
Appendix Table C2 Freundlich and Langmuir isotherms for phorbol esters after two-time adsorption with bentonite200.

		Freundlich isotherm					Langmuir isotherm	
		$q = KC^{1/n}$					$q = q_m KC / (1+KC)$	
		$\log q = \log K + 1/n \log C$					$1/q = 1/q_m + 1/(K q_m C)$	
W	C_0	C	X	q	log q	log C	1/q	1/C
(g)	(mg/L)	(mg/L)	(mg)	(mg/g)	(mg/g)	(mg/L)	(g/mg)	(L/mg)
0.2000	37.1871	8.5272	0.7165	3.5825	0.5542	0.9308	0.2791	0.1173
0.4000	37.1871	7.4858	0.7425	1.8563	0.2687	0.8742	0.5387	0.1336
0.6000	37.1871	8.2037	0.7246	1.2076	0.0819	0.9140	0.8281	0.1219
0.8000	37.1871	8.6029	0.7146	0.8933	-0.0490	0.9346	1.1195	0.1162
1.0000	37.1871	8.4890	0.7175	0.7175	-0.1442	0.9289	1.3938	0.1178

X = adsorbate (mg), q = X/W



Freindlich isotherm (a)



Langmuir isotherm (b)

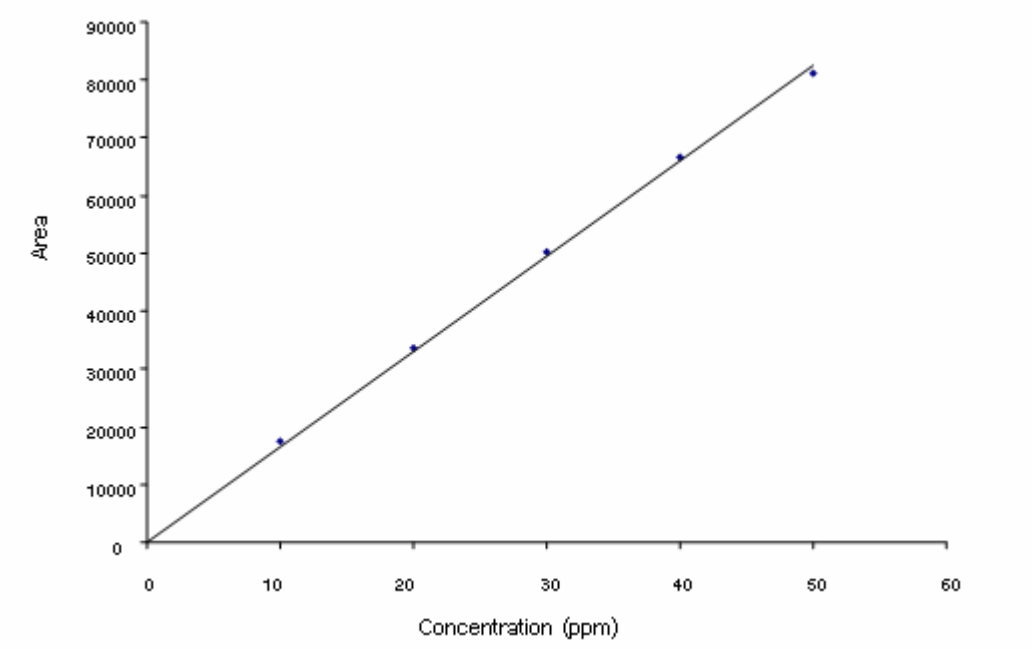
Appendix Table C3 Freundlich and Langmuir parameters of phorbol esters adsorption by bentonite 200.

Adsorption	Freundlich parameters			Langmuir parameters		
	Equation	R^2	1/n	Equation	R^2	q_{max} (mg/g)
One-time	Y=0.4000X + 1.2100	0.9428	0.4000	Y= 0.0038X + 0.0020	0.9446	500.0000
Two-time	Y=-2.5451X + 2.4749	0.0521	-2.5451	Y= -0.0116X + 0.1262	0.1292	7.9239

APPENDIX D

Calibration curve of standardization

Appendix Figure D1 The HPLC calibration curve of standard tetradecanoylphorbolacetate (TPA).



TPA calibration curve

Correlation = 0.9984

Formular : y = mx+b

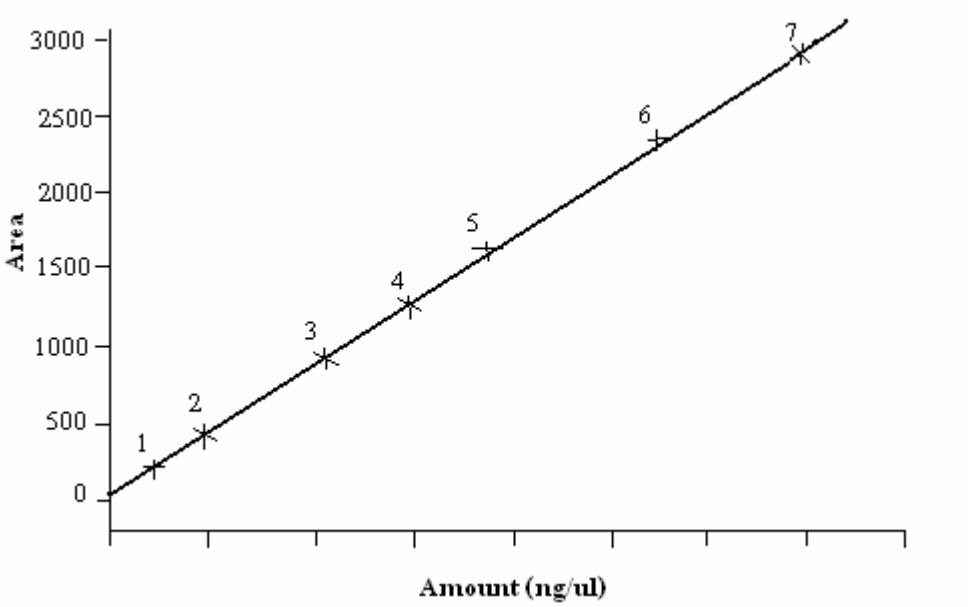
: m = 1650.6000

: b = 0

: x = Concentration of standard TPA, (ppm)

: y = Area

Appendix Figure D2 The GC calibration curve of standard fatty acid methyl esters mixture (C_8-C_{24}).



Fatty acid methyl esters mixture calibration curve

Correlation = 0.9989

Formular : y = mx+b

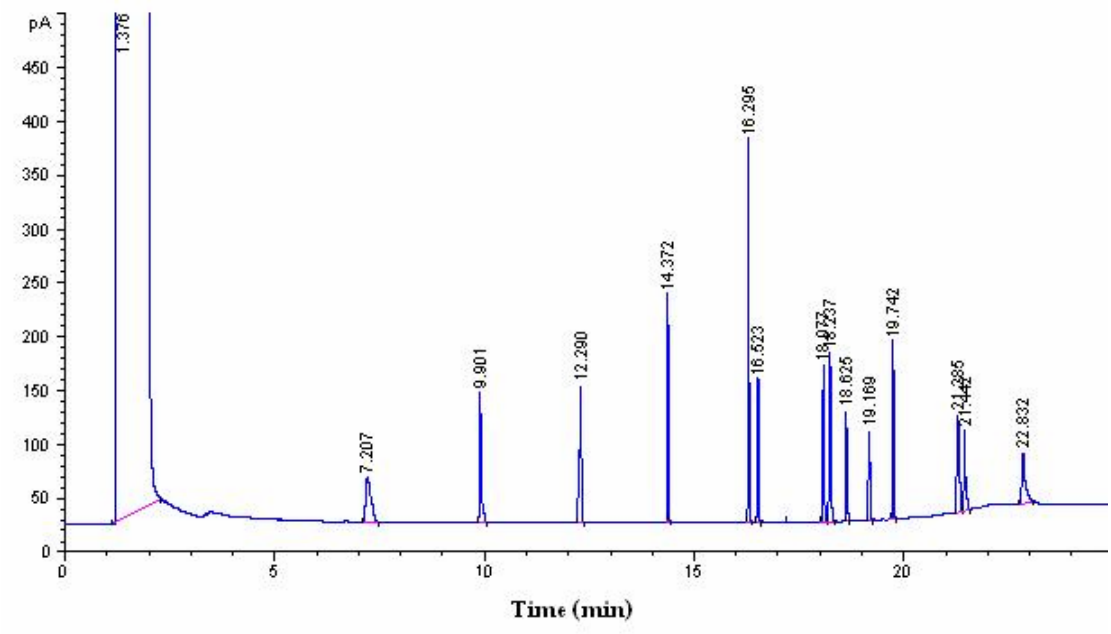
: m = 19.0346

: b = 22.9784

: x = Amount of standard fatty acid methyl esters mixture (C_8-C_{24}),(ng/μl)

: y = Area

Appendix Figure D3 The chromatogram of standard fatty acid methyl esters mixture (C_8-C_{24}).



RT, (min)	Name
7.207	Methyl octanoate
9.901	Methyl decanoate
12.290	Methyl laurate
14.372	Methyl myristate
16.295	Methyl palmitate
16.523	Methyl palmitoleate
18.077	Methyl stearate
18.237	Methyl oleate
18.625	Methyl linoleate
19.169	Methyl linolenate
19.724	Methyl arachidate
21.285	Methyl behenate
21.442	Methyl erucate
22.832	Methyl lignocerate

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ORAL PRESENTRATION AT CONFERENCE	: Eliminated Phorbol Esters in Seed Oil and Press Cake of *Jatropha curcas* L., Proceeding of PACCON 2008, 30th Jan.-1st Feb. 2008, Sofitel Centara Grand Bangkok, Thailand.
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