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ORIGINAL PAPER

Dynamic Analysis of Phorbol Esters in the Manufacturing Process of Fatty Acid Methyl Esters from *Jatropha curcas* Seed Oil

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Abstract *Jatropha curcas* seed oil, which is unsuitable as an edible oil but has received attention as a novel vegetable fat and oil resource, contains tumor-promoting phorbol esters. Currently, six types of derivatives of 12-deoxy-16-hydroxyphorbol (DHPEs) in *J. curcas* oil have been identified, and their toxicological safety for humans is being discussed. However, it is reported that most DHPEs disappear during the transesterification process. We investigated the dynamics of phorbol esters in the manufacturing process of fatty acid methyl esters from *J. curcas* seed oil. With the assumption that the precursor ion was the fragment ion ($m/z = 311$) from the frame unit of phorbol esters and their derivatives, we developed an LC–MS method for detecting the product ion ($m/z = 165$), which was obtained by cleavage of the fragment ion. The derivatives generated from the structural changes of the phorbol esters existed in fractions of glycerine–water in the manufacturing process; however, phorbol esters and their derivatives were not detected in the fatty acid methyl esters that were produced via a high-vacuum distillation process. Investigation into the dynamics of phorbol esters confirmed that the contents of phorbol esters, including DHPEs, in the fatty acid methyl esters were under detection limits.

Keywords Phorbol esters · Jatropha · Dynamics · Refining · Transesterification · High-vacuum distillation · Liquid chromatography–mass spectrometry

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Introduction

At present, approximately 85% of commercially produced vegetable fats and oils are used in foods, while the rest are used as industrial raw materials (5–6%) and bio-diesel fuels (6–7%). As it is predicted that the demand for edible fats and oils will increase along with the growing world population, non-competing inedible fats and oils should be used for meeting industrial and bio-fuel demands—thus, inedible fats and oils from sources such as micro-algae, *Jatropha curcas*, and *Camelina* have become potential candidates and are being investigated. Among these new fat and oil resources, *J. curcas* which grows in tropical areas has received worldwide attention owing to its strong drought-resistant and salinity-resistant properties [1–3].

Jatropha curcas L. belongs to the family of Euphorbiaceae. *Jatropha* is considered unsuitable for ingestion in certain cases because the oral intake of its leaves and seeds can cause hypercatharsis or diarrhea; moreover, it contains the toxin curcin (a protein with a molecular weight of 28.2 kDa and 251 amino acids) as well as phorbol esters,

which are known to be tumor-promoters [4, 5]. Curcin becomes nontoxic on denaturation by heating [6]; however, phorbol esters persist in oil fractions. Therefore, an effective method for removing phorbol esters from *J. curcas* seed oil is essential for its safe use.

Phorbol esters are diterpene esters of the tigliane type. 12-*O*-tetradecanoylphorbol 13-acetate [TPA, also known as phorbol 12-myristate 13-acetate (PMA)] is well known as a typical diester of phorbol (Fig. 1). TPA has strong tumor-promoting activity, and it has been confirmed to cause papilloma and carcinoma in a two-stage carcinogenesis experiment on mouse skin [7]. While there are no reports that TPA is present in the seed oil of *J. curcas*, six diester derivatives of 12-deoxy-16-hydroxyphorbol (DHPEs) (Fig. 2) have been found in the oil [8]. Hirota et al. [9] demonstrated weak tumor-promoting activity of DHPEs in a two-stage carcinogenesis experiment on mouse skin. The concentration of DHPEs in the seed oil of *Jatropha* is commonly estimated by high-performance liquid chromatography (HPLC) using TPA as a standard reagent [10]. DHPEs content in the *Jatropha* seed oil varies according to the growing district, ranging from 870 to 3,320 μg/g of kernels except for non-toxic variety (Mexico, Papantla) [11].

With this background, various methods for the detoxification of *J. curcas* seed oil have been investigated for industrial use [12]. Gross et al. [13] suggested the detoxification of the seed oil by the extraction of DHPEs using ethanol. Devappa et al. [14] optimized the condition for extraction of DHPEs. Haas et al. [15] demonstrated that DHPEs content decreases following deacidification and bleaching. Furthermore, Makker et al. [16] demonstrated the majority of DHPEs are removed during the transesterification process. However, these studies have not clarified what happens to these decreased DHPEs. Therefore, it is important to investigate whether these DHPEs are destroyed or converted to derivatives with altered structures but persisting toxicity during the refining or transesterification process, in order to ensure the safety of products and manufacturing workers.

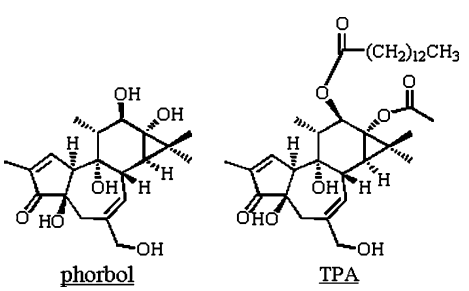

Fig. 1 Structures of phorbol (M_w: 364) and 12-*O*-tetradecanoylphorbol 13-acetate (TPA, M_w: 616)

Vogg et al. [17] suggested a liquid chromatography–mass spectrometry (LC–MS/MS) method for detecting the esters of phorbol and linear fatty acids such as TPA. However, no selective detection methods for the esters of 12-deoxy-16-hydroxyphorbol or alicyclic compounds such as DHPEs have been established.

In this paper, we describe the development of an LC–MS/MS method for detecting the fragment ions from the frame units of TPA, DHPEs, and their derivatives. By applying the LC–MS/MS method, we evaluated whether TPA existed in *J. curcas* seed oil. In the same way, we investigated the dynamics of DHPEs in the manufacturing process of fatty acid methyl esters by analyzing several samples from various stages in the manufacturing process.

Materials and Methods

Reagent

We purchased 75% phosphoric acid aqueous solution, methanol (extra pure grade), and sodium hydroxide from Kanto Chemical Co., Inc. for use during seed oil refinement, transesterification, and distillation processes. Reagents for sample preparation were purchased from Kanto Chemical Co., Inc. and Sigma–Aldrich Co. The former group included methanol [guaranteed (GR) grade], isooctane (GR grade), diethyl ether (GR grade), chloroform (GR grade), and sodium hydroxide, while the latter group included TPA. Reagents for analysis included formic acid (HPLC grade), ammonium formate (GR grade), and acetonitrile (HPLC grade), all of which were purchased from Kanto Chemical Co., Inc.

Preparation of Fatty Acid Methyl Esters

Jatropha curcas Seed Oil

Two different *J. curcas* oils were used for this study. These oils were purchased from Nippon Biodiesel Fuel Co., Ltd. in March 2008. The seeds were obtained from Kalimantan and southern Sumatra (Lampung), both in Indonesia.

Refining Process for the Seed Oil

The two oils were obtained by using an expeller press from seeds at room temperature. Approximately 20 wt% of water was added to the oils, and stirred for 30 min at 80 °C. After decantation, the upper portion was filtered through nonwoven cloth (mean pore size 0.3 mm) at 60 °C. Approximately 0.975 g of 75% phosphoric acid aqueous solution (0.075 wt%) was added to 1,300 g of the oils heated to 65 °C in a 2-L flask, and the mixture was

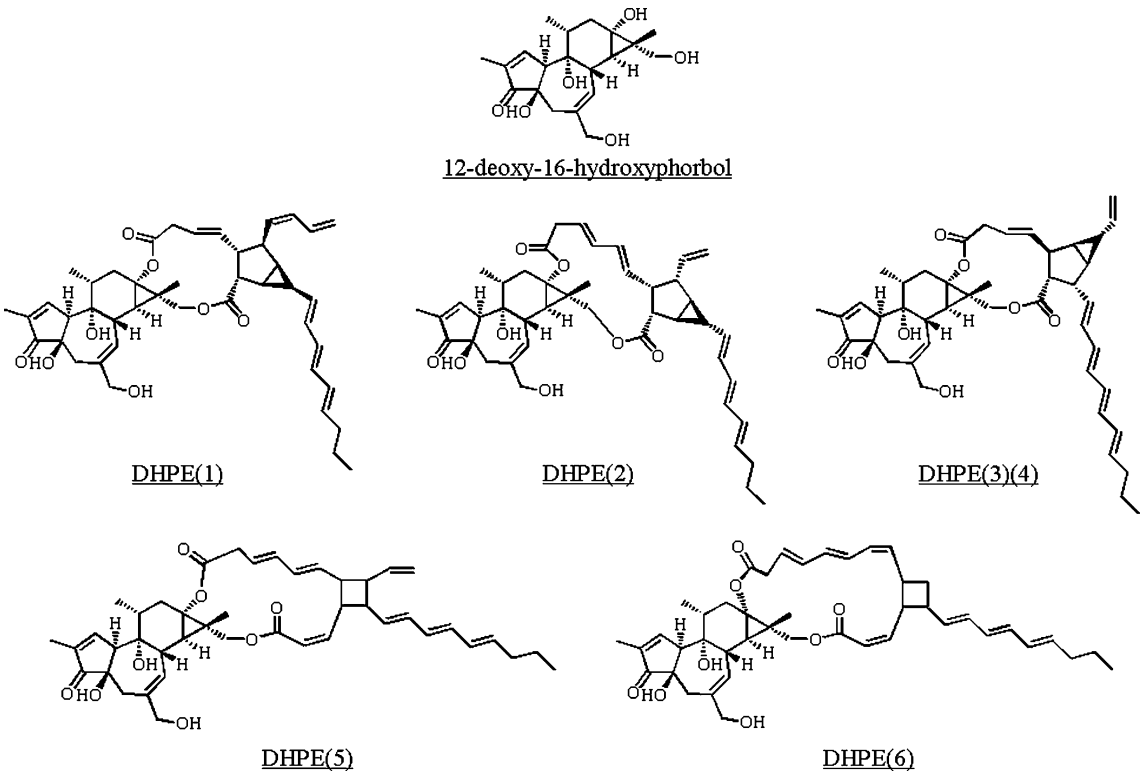

Fig. 2 Structures of 12-deoxy-16-hydroxyphorbol (M_w: 364) and phorbol esters in *J. curcas* (DHPEs, M_w: 710). Haas et al. [8] reported the maximum wavelength of six DHPEs in the UV–Vis absorption spectra (λ_{max} : DHPE(1) 284 nm, DHPE(2) 280 nm, DHPE(3)(4) 290 nm 303 nm 317 nm, DHPE(5) 272 nm, DHPE(6) 276 nm)

stirred for 10 min at 65 °C. Approximately 0.52 g (0.04 wt%) filter aid (Perlite #38), supplied by Toko Perlite Kogyo, was added, and the mixture was stirred for 5 min at 65 °C. Degumming oil was obtained by pressured filtration (Advantec filter). The experiment was performed by the fixed-bed process, wherein 800 mL cation-exchange resin (SK-104H from Mitsubishi Chemicals) was filled in a 1-L water-jacketed column (60 °C). The mixture prepared with 1,000 g of degumming oil and 200 g methanol (20 wt%), and stirred at 60 °C—the temperature maintained by a pumped water bath—under a reaction time of 120 min, and deoxidized oil was finally obtained.

Transesterification Process

A mixture prepared with ~6 g sodium hydroxide (ca. 0.3 wt%) and 780 g methanol (39 wt%, ninefold mol vs. oils and fats) was added to 2,000 g of pre-treated oil, and stirred at 70 °C for 60 min in a 5-L four-necked flask. The reaction mixture was cooled down to room temperature, and the crude methyl ester and glycerine layers were each separated. The crude methyl ester layer was heated and washed with 280 g (14 wt%) water and then cooled to separate the crude methyl ester layer from the water layer.

High-Temperature Vacuum Distillation Process

Methanol and water were removed from 1,500 g of crude methyl ester in a 3-L four-necked flask under 100 mmHg at 80 °C. The crude methyl ester was placed in a 3-L topping flask, to which a Widmer distillation column was subsequently attached. Distillation was performed at 5 mmHg. At ~250 °C, the distillation was considered complete if no distillates were observed. The distillation was taken by 7 h. Fatty acid methyl esters and bottom deposits were obtained at the end of this process.

It should be noted that the processes detailed in Refining Process for the Seed Oil to High-temperature Vacuum Distillation Process were originally developed by the Lion Corporation for producing fatty acid methyl esters from palm oil on an industrial scale [18].

Sample Preparation for HPLC and Mass Spectrometry

Seed Oil, Methyl Esters, and Products from the Manufacturing Process

Sample preparation for measurement was done according to the methods proposed by Wink et al. [10] and Haas et al. [15]. In a centrifuge tube, 2 g of seed oil, deoxidized oil,

and bottom deposits was mixed with 2 mL of methanol. The mixture was stirred sufficiently and centrifuged ($560 \times g$, 10 min). The upper methanol layer was transferred to a measuring flask (10 mL). Following the three-time extraction, the extract was dissolved in methanol to produce a 10-mL sample for assay. The crude methyl esters, glycerin layer, and distilled esters were directly dissolved in methanol to produce corresponding samples. Using a measuring flask, methanol was added to 1 g of the sample until the mixture reached a volume of 5 mL, which was used for the assay.

Concentrated DHPEs Solution

In order to measure the MS spectra of the DHPEs accurately, a concentrated solution of DHPEs was prepared by extraction from *J. curcas* seed oil. In a separating funnel, 50 g of the seed oil and 300 mL methanol were mixed and shaken vigorously. The mixture was left to stand until it separated into two layers. Subsequently, 500 mL of the methanol layer was extracted and transferred to a recovery flask. The extract was distilled by using an evaporator. This extraction and concentration process was repeated four times to obtain yellow residue (7.2 g). The residue was dissolved in isooctane/diethyl ether (95/5), and analyzed by silica gel-column chromatography (silica gel for adsorption 20 g; silica gel for separation 10 g). The column was sequentially exposed to four eluents: 200 mL isooctane/diethyl ether (95/5), 400 mL isooctane/diethyl ether (50/50), 200 mL diethyl ether, and 200 mL chloroform/methanol (50/50). The diethyl ether fraction was evaporated, and then dried under reduced pressure at 60 °C for 3 h to obtain a concentrate containing 8% DHPEs, which chiefly consisted of monoglycerides. In a measuring flask, methanol was added to the DHPEs concentrate until the mixture reached a volume of 5 mL to obtain a concentrated DHPEs solution (1,000 μg/mL).

Next, transesterification was performed using the concentrated DHPEs solution to estimate the structural changes in the DHPEs. Then, 0.25 mL of concentrated DHPEs solution and 0.25 mL of methanol containing 0.2% sodium hydroxide were added to a pressure-tight vial. The vial was placed on a block heater set at 70 °C for a period of 5 min to 20 h, followed by the transesterification reaction. The reaction mixture was diluted by five times to produce an assay sample.

Instruments

NANO SPACE SI-2 (Shiseido), Quattro Micro API (Waters), and SYNAPT MS (Waters) were used for HPLC, tandem mass spectrometry (MS/MS), and time-of-flight mass spectrometry (TOFMS), respectively. Mass spectrometry was analyzed by electrospray ionization in the positive ion mode. UV6000LP (Thermo Finnigan) was used as the photodiode array (PDA).

Analytical Conditions

HPLC Analysis

We hypothesized that DHPEs eventually altered to 12-deoxy-16-hydroxyphorbol through certain intermediates in the manufacturing process of fatty acid methyl esters. HPLC conditions were set to be able to simultaneously detect the most hydrophilic 12-deoxy-16-hydroxyphorbol and the most hydrophobic DHPEs in the expected compounds. The structural changes in DHPEs were then examined. Chromatographic separation was performed on a C18 reversed-phase column (Cadenza CD-C18; 150 × 2 mm; particle size, 3 μm; Imtakt Corp.). The mobile phase consisted of 0.15% formic acid aqueous solution (solvent A) and 0.15% formic acid aqueous solution/acetonitrile (1/9) (solvent B) under the gradient condition: solvent A 90%/solvent B 10%, followed by solvent A 35%/solvent B 65% (20 min, linear gradient), solvent A 0%/solvent B 100% (50 min, linear gradient), solvent A 90%/solvent B 10% (5 min, linear gradient), and solvent A 90%/solvent B 10% (15 min, linear gradient). The column temperature was 30 °C; the flow rate, 0.2 mL/min; and the injection volume, 5 μL.

MS Analysis

To achieve sensitive and selective detection of TPA, DHPEs, and other derivatives by LC–MS/MS, it is necessary to identify the basic fragment ion shared by TPA, DHPEs, and other derivatives and also to optimize the MS/MS conditions accordingly. TPA was used for such identification and optimization due to the unavailability of other substances, i.e., it is almost impossible to obtain DHPEs and other derivatives as pure substances, whereas TPA is available as a standard reagent. Measurement was performed by the flow injection method. The capillary voltage was 3.5 kV; ion source block temperature, 120 °C; and scan range, 50–1,000 *m*/*z*. Where the mobile phase was 0.15% formic acid aqueous solution/acetonitrile (2/8), the desolvation temperature used was 350 °C; the cone voltage, 10–60 V; and the collision energy, 20–60 V. Where the mobile phase was 10 mM ammonium formate aqueous solution/acetonitrile (2/8), the desolvation temperature used was 200 °C; the cone voltage, 10 V; and the collision energy, 15 V.

Next, in order to confirm whether the specified fragment ion was detected for DHPEs, the MS spectra of DHPEs were analyzed using the concentrated DHPEs solution. The

capillary voltage was 3.5 kV; the desolvation temperature, 350 °C; the ion source block temperature, 120 °C; the cone voltage, 40 V; and the scan range, 50–1,000 *m/z*.

Finally, multiple reactions monitoring (MRM) was used for the sensitive and selective detection of TPA, DHPEs, and other derivatives. The precursor ion ($m/z = 311$) originating in the frame unit and the product ion ($m/z = 165$; maximum ion intensity) were used for MRM. The detected peaks were qualitatively analyzed by comparing the MS/MS spectra of the fragments and identified by product ion scanning with TPA and DHPEs. The capillary voltage was 3.5 kV; the desolvation temperature, 350 °C; the ion source block temperature, 120 °C; the cone voltage, 40 V; and the collision energy, 50 V. The detection limit of TPA was 0.002 μg/mL in the MRM mode. In order to ensure the qualitative analysis of the detected peaks in the distilled fatty acid methyl ester, the exact mass and elemental composition of the fragment (precursor) ion of $m/z = 311$ were calculated by TOFMS, where the capillary voltage was 3.5 kV; the desolvation temperature, 350 °C; the ion source block temperature, 120 °C; the cone voltage, 40 V; and the scan range, 50–1,000 *m/z*.

PDA Analysis

The concentration of DHPEs was estimated according to previously described methods [11, 15]. Accordingly, the detector wavelength was set at 280 nm, and quantitative analysis was performed using TPA as the standard reagent.

Results and Discussion

Development of the Selective LC–MS/MS Method

MS spectra of TPA were analyzed with formic acid used for the buffer solution of the mobile phase at a cone voltage between 10 and 60 V (Fig. 3). $[M+H]^+$ ($m/z = 617$) of the molecular-related ion was weakly detected, and a majority of fragment ions were detected in the lower mass range. Similar fragmentation took place at lower cone voltages. This indicated that the fragment ions were produced by in-source decay, in which fragmentation occurred in the ion source during or immediately after ionization. When ammonium formate was used for the buffer solution of the mobile phase, similar fragment ions were also observed in the MS/MS spectrum for the fragmentation of $[M + NH_4]^+$ ($m/z = 634$) of the molecular-related ion. This indicated that the fragment ions originated in TPA. Fragmentation analysis was conducted to specify the fragment ion originating in the frame unit of TPA. The fragment ion of $m/z = 389$ was estimated to be the result of cleavage of $[M + NH_4]^+$ ($m/z = 634$) following the loss of a myristyl group with a proton. The fragment ion of $m/z = 329$ was estimated to be the result of further cleavage following the loss of an acetyl group with a proton. The fragment ion of $m/z = 311$ was estimated to be the result of further cleavage following the loss of a hydroxyl group with a proton (dehydration). The fragment ion of $m/z = 293$ was estimated to be the result of further dehydration. The fragment ions of $m/z = 329$, 311, and 293, originating in the frame unit of TPA, were also detected in the MS spectra of DHPEs, which were composed of a similar frame unit. Hence, it was expected that the fragment ions would also be detected in the derivatives generated by the structural change of DHPEs. In this study, the fragment ion of $m/z = 311$ was used as the primary ion because of its high ionic strength in both TPA and DHPEs, and the cone voltage was set at 40 V.

Many components besides phorbol esters were present in the seed oil, fatty acid methyl ester, and other intermediate products. In the case of detecting phorbol ester by single MS with primary ions, it was unlikely to detect the peaks of inclusions. Therefore, we established an additional selective method—the LC–MS/MS method—for detecting secondary ions generated by the fragmentation of primary ions. MS/MS spectra detected by the fragmentation of $m/z = 311$ are shown in Fig. 4. Analysis with the collision energy ranging from 20 to 60 V found fragment ions with the highest ionic strength at $m/z = 165$. Hence, the fragment ion of $m/z = 165$ was used as the secondary ion, and collision energy was set at 50 V.

Dynamic Analysis of DHPEs in the Manufacturing of Fatty Acid Methyl Ester

Detection of DHPEs in J. curcus Seed Oil

The peaks of DHPEs were detected between 48 and 58 min in the PDA chromatogram of the seed oil. Haas et al. [8] reported the maximum wavelength of six DHPEs in the UV–Vis absorption spectra (Fig. 2). Comparing the previously reported wavelength with the maximum wavelength of DHPEs detected in the PDA chromatogram of the seed oil, the existence of five DHPEs was confirmed in our study (λ_{max} : DHPE(1) 283 nm, DHPE(2) 281 nm, DHPE(3)(4) 291 nm 304 nm 318 nm, DHPE(6) 276 nm). The concentration of DHPEs determined using the molar absorbance coefficient of TPA as the standard was approximately 2,800 ppm and corresponded well with previously reported concentrations (870–3,320 ppm) [11]. Only DHPEs were detected in the MRM chromatogram of the seed oil (Fig. 5), whereas several peaks of inclusions were detected in the PDA chromatogram. The MRM chromatogram indicates that derivatives composed of the frame unit of phorbol esters could be detected selectively. In this study, it was found that phorbol esters modified by complex hydrophobic groups,

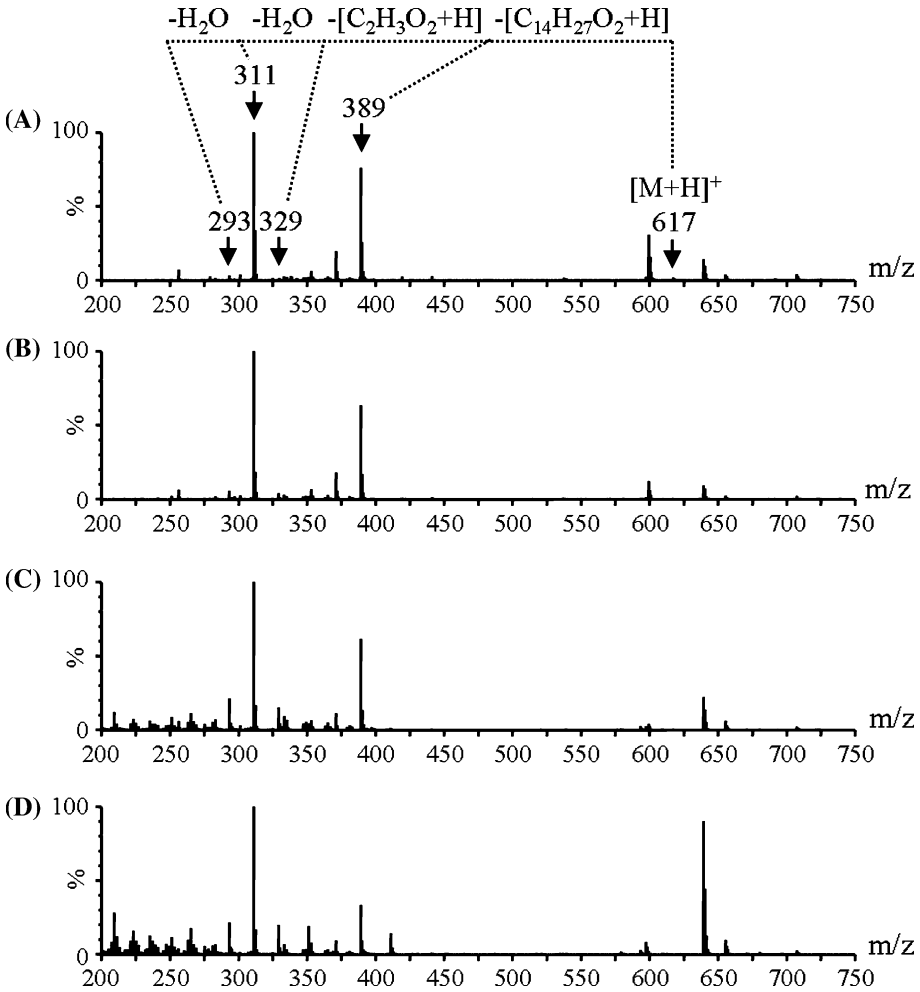

Fig. 3 MS spectra of TPA (cone voltage: **a** 10 V, **b** 20 V, **c** 40 V, **d** 60 V, mobile phase: formic acid aqueous solution)

such as DHPEs, could be detected by the LC–MS/MS method, while Vogg et al. [17] reported a selective detection method for phorbol esters modified by simple hydrophobic groups. It was confirmed that TPA was detected between 62 and 64 min under the analysis conditions of this study. Analysis with the standard reagent confirmed that the lower detection limit of TPA was 0.002 μg/mL, suggesting the possibility of sensitive detection. TPA was not detected by the analysis of the seed oil. This suggests that there was no TPA in *J. curcus* seed oil from Indonesia. MS/MS spectra of five DHPEs are shown in Fig. 5 and resemble the TPA spectrum. It is thus interpreted that comparing the MS/MS spectra of the detected peaks with those of DHPEs better qualifies whether the peaks originated in the frame unit of DHPEs.

Detection of DHPEs in Deoxidized Oil

Figure 6 shows an MRM chromatogram of deoxidized oil. The concentration of refined DHPEs taking the molar absorbance coefficient of TPA as the standard was approximately 1,900 ppm. The concentration was 32% lower than before refinement. Haas et al. [15] reported that the concentration of DHPEs decreased up to 30% with alkaline deoxidation treatment, which removed free fatty acid using NaOH or KOH aqueous solution. The results of our study suggest that the concentration of DHPEs can be decreased with deoxidation treatment using a cation-exchange resin. Peak (1) was newly detected at a hydrophilic retention time greater than that of DHPEs. MS/MS spectra of peak (1) were in good agreement with those of DHPEs (Fig. 6). It seems possible that the compound of peak (1) was the derivative that was generated from the structural change of DHPEs.

Detection of DHPEs in Crude Fatty Acid Methyl Esters

Figure 7 shows the MRM chromatogram of the crude fatty acid methyl ester layer after esterification. The concentration of crude ester DHPEs taking the molar

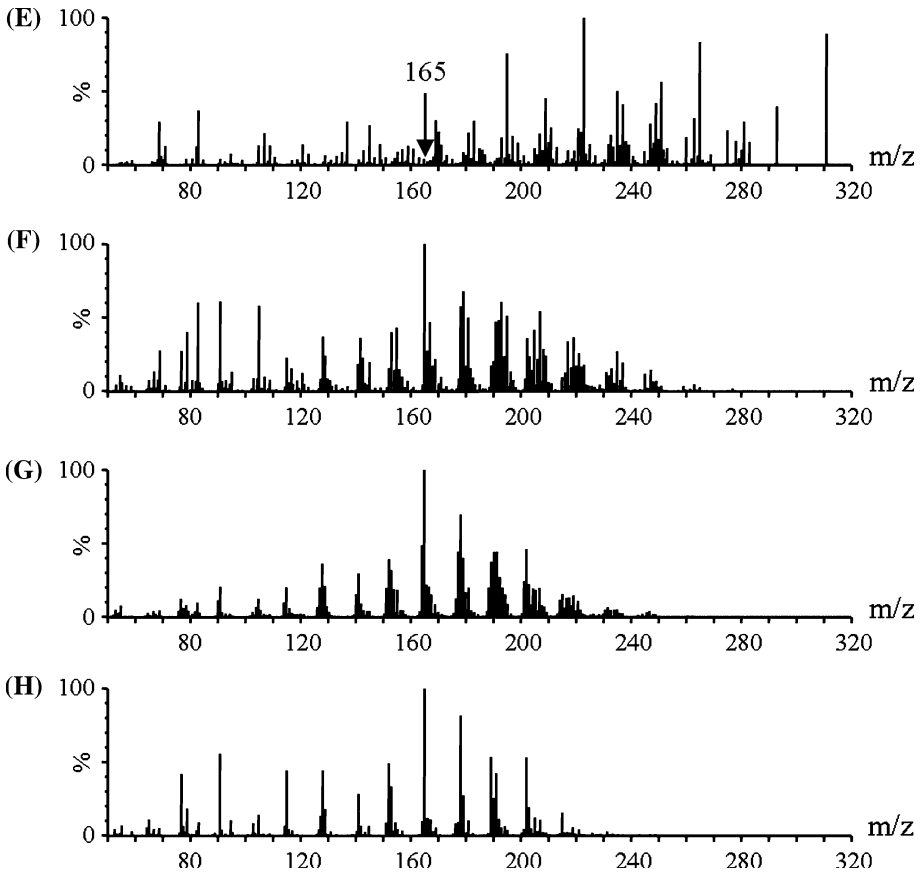

Fig. 4 MS/MS spectra of TPA (cone voltage: 40 V, collision energy: **e** 20 V **f** 40 V **g** 50 V **h** 60 V, mobile phase: formic acid aqueous solution)

absorbance coefficient of TPA as the standard was approximately 30 ppm. This indicates that the concentrations of most of DHPEs decreased by transesterification. DHPEs were not detected in the glycerine layer in the transesterification process, while Makker et al. [16] reported that DHPEs were barely detected. Peak (1) was also detected in the crude methyl ester layer, similar to the case for deoxidized oil. Peak (2) was newly detected at a hydrophobic retention time greater than that for DHPEs. Peaks (3) and (4) were newly detected at a hydrophilic retention time greater than that for peak (1) in the glycerine layer. MS/MS spectra of peaks (3) and (4) were in agreement with those of DHPEs, whereas the MS/MS spectrum of peak (2) did not correspond to those of DHPEs (Fig. 7).

Furthermore, the results of dynamic analysis of the concentrated DHPEs solution in the transesterification process showed that DHPEs changed to 12-deoxy-16-hydroxyphorbol through monoester derivatives produced after transesterification of an ester bond. The monoesters and 12-deoxy-16-hydroxyphorbol were detected at retention times of 42 and 8 min, respectively, although these compounds were not detected in the MRM chromatogram of the crude methyl ester and glycerine layers.

It is possible that two derivatives composed of the frame unit of DHPEs were newly generated in the transesterification process.

Detection of DHPEs in Fatty Acid Methyl Esters After Distillation

Figure 8 shows the MRM chromatogram of fatty acid methyl esters after distillation. DHPEs were not detected in the distilled esters and bottom deposits. Peaks (5) and (6) were newly detected at hydrophilic retention times greater than those for DHPEs in the distilled esters. MS/MS spectra of peaks (5) and (6) did not correspond to those of DHPEs at all, suggesting that the compounds of peaks (5) and (6) were not derivatives composed of the frame unit of DHPEs (Fig. 8). To clarify the qualitative properties of peaks (5) and (6) better, an exact mass measurement was conducted. The results confirmed that the compounds corresponding to peaks (5) and (6) were not DHPE derivatives. The exact mass of the fragment ion ($m/z = 311$) that originated in the frame unit of DHPEs was 311.1647, and the elemental composition of the fragment ion was $C_{20}H_{23}O_3$. The exact mass of the fragment ion ($m/z = 311$) corresponding to peaks (5) and (6) was 311.2379, and the

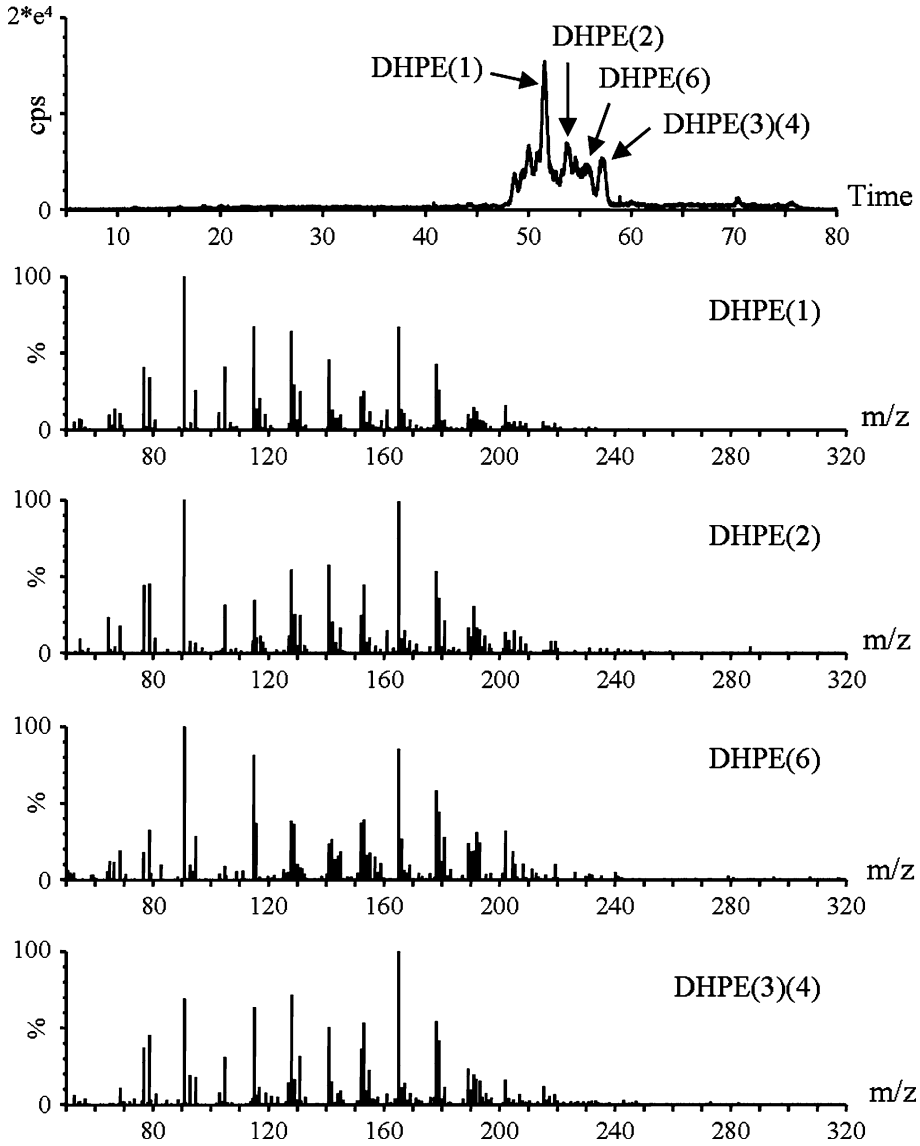

Fig. 5 MRM chromatogram of *J. curcus* seed oil ($m/z = 311 \rightarrow m/z = 165$) and MS/MS spectra of DHPEs detected in the MRM chromatogram

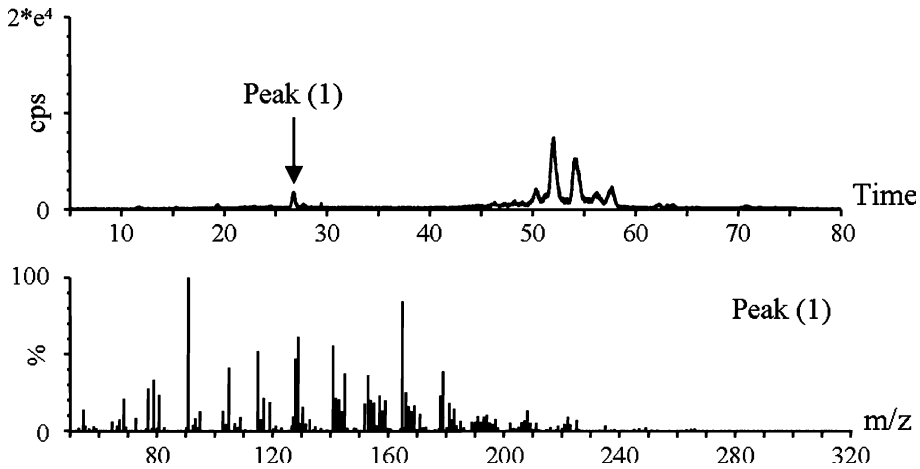

Fig. 6 MRM chromatogram of deoxidized oil ($m/z = 311 \rightarrow m/z = 165$) and MS/MS spectra of peak (*1*) detected in the MRM chromatogram. Peak (*1*) corresponds to the derivative that was generated from the structural change of DHPEs

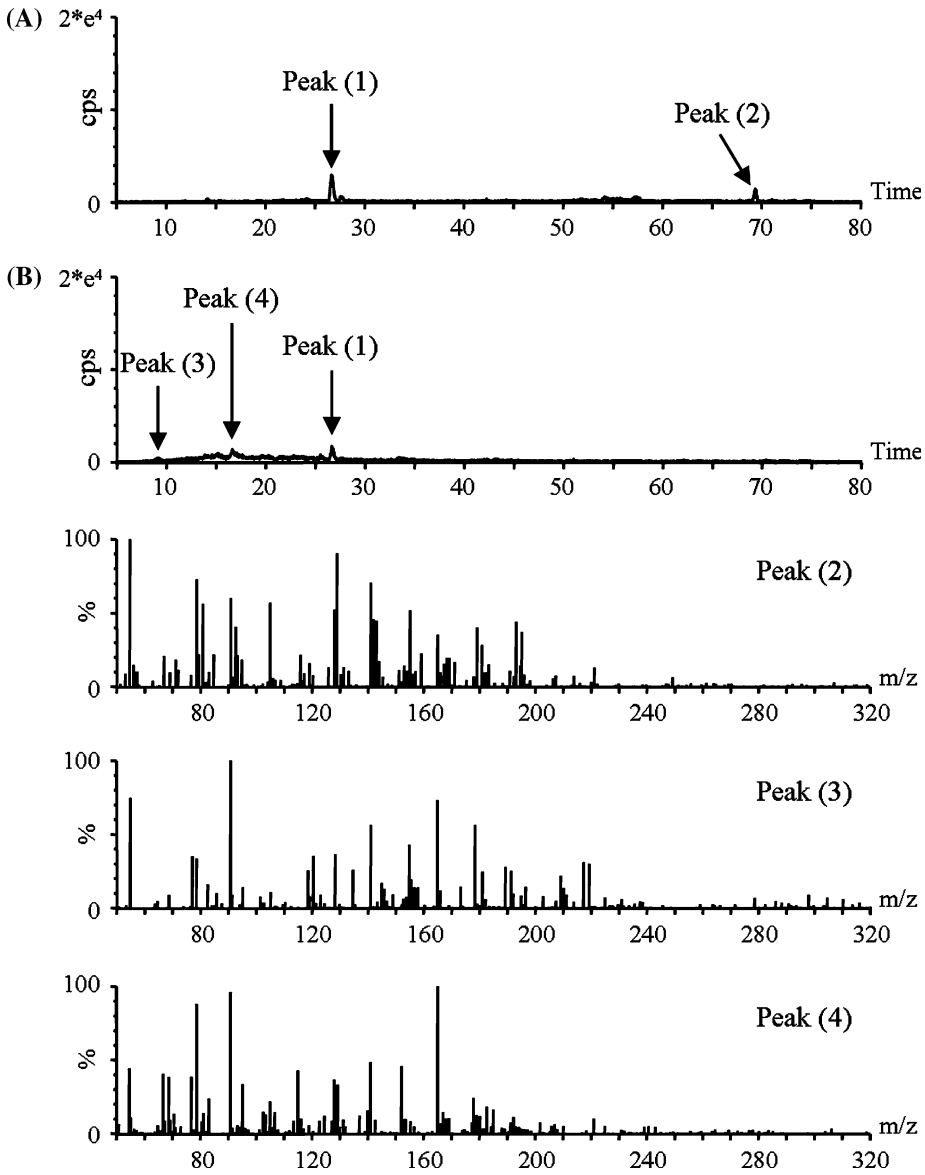

Fig. 7 MRM chromatograms of the crude methyl ester layer (**a**) and glycerine layer (**b**) after transesterification ($m/z = 311 \rightarrow m/z = 165$) and MS/MS spectra of peaks (*2*), (*3*), (*4*) detected in the MRM chromatograms. Peak (*2*) doesn't correspond to the derivative composed of the frame unit of DHPEs. Peaks (*3*) and (*4*) correspond to the derivatives that were generated from the structural change of DHPEs

elemental composition of the fragment ion was $C_{22}H_{31}O$, which both differ from the values for the DHPEs. The results suggest a significant finding that no DHPEs or their derivatives were present in the distilled esters.

The compound of peak (1), detected in crude esters, was strongly present in the MRM chromatogram of the bottom. This reveals that the DHPE derivatives observed in crude esters were finally concentrated in the fraction of the bottom, which was discharged during distillation.

Conclusions

Dynamic analysis of DHPEs during the manufacturing of fatty acid methyl esters was achieved by the LC–MS/MS method, which was able to detect TPA, DHPEs, and their derivatives.

The concentration of DHPEs was approximately 2,800 ppm in *J. curcus* seed oil, whereas TPA was not detected. The concentration of DHPEs decreased to 1,900 ppm during the refining process, and one compound was found. It seems most likely that it was a DHPE derivative. After the transesterification process, nearly all DHPEs disappeared and two compounds were found in the glycerine layer, and again, it seems likely that they were DHPE derivatives. The compounds detected during the manufacturing process were concentrated in the fraction of the bottom discharged during high-temperature distillation under reduced pressure. The structure and toxicity of DHPE derivatives were not clarified. However, it was

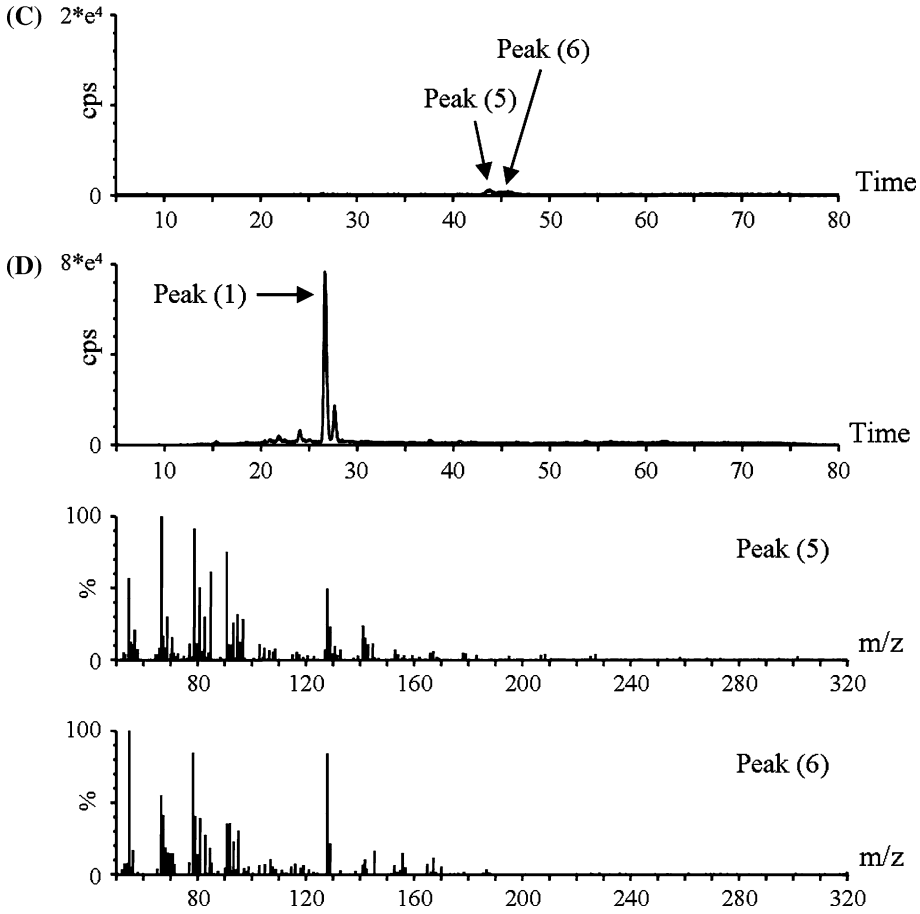

Fig. 8 MRM chromatograms of fatty acid methyl ester (**c**) and the bottom deposits (**d**) after distillation ($m/z = 311 \rightarrow m/z = 165$) and MS/MS spectra of peaks (*5*) and (*6*) detected in the MRM chromatograms. Peaks (*5*) and (*6*) don't correspond to the derivatives composed of the frame unit of DHPEs

revealed that there were no DHPEs, TPA or their derivatives in the methyl esters.

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