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Short Communication

Established GC-FID for simultaneous determination of diterpenes and phytosterols in Plaunoi (*Croton stellatopilosus* Ohba)

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Abstract

A gas chromatography-flame ionization detector (GC-FID) was established and validated for simultaneous determination of the diterpenes (phytol, GGOH, plaunotol) and phytosterols (campesterol, stigmasterol and β-sitosterol) in plant leaves, callus and suspension cultures of Plaunoi (*Croton stellatopilosus*). Under the developed GC-FID conditions, the diterpenes and phytosterols were separated in a single run. The limit of quantification (LOQ) and the limit of detection (LOD) of each standard sample were determined. Interday and intraday precisions were less than 5%. The average recoveries of the method were 102.4-109.9% and all compounds showed linearity with r^2 values all ≥ 0.9994. According to this newly established GC-FID method, the metabolites that accumulated in the leaves, green callus and suspension cultures of Plaunoi were determined. Phytol, GGOH and plaunotol were present at relatively high concentrations in plant leaves, and in considerably reduced amounts in green callus cells. Phytol and plaunotol were not detected in suspension cell cultures.

Keywords: *Croton stellatopilosus*, diterpenes, GC-FID, phytosterols, Plaunoi

1. Introduction

Plaunoi [Thai] or *Croton stellatopilosus* Ohba, formerly known as *C. sublyratus* Kurz. (Euphorbiaceae) (Esser and Chayamarit, 2001) is a Thai medicinal plant that contains a variety of diterpenes such as phytol, geranylgeraniol (GGOH), plaunotol and phytosterols (Figure 1) (Kongduang, 2007). Among them, plaunotol, an acyclic diterpene alcohol, has played an important role in the treatment of chronic ulcers (Ogiso *et al.*, 1985). The leaves of *C. stellatopilosus* have been used as the raw material for extracting plaunotol. It is

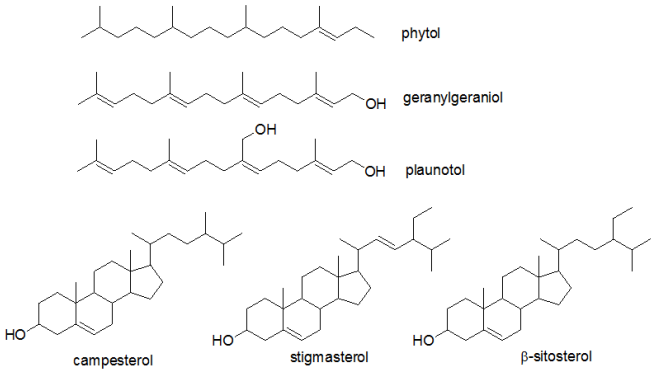

Figure 1. Structures of diterpenes and phytosterols.

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widely used in the treatment of peptic ulcers including *Helicobacter pylori*-associated and non-steroidal anti-inflammatory drug induced ulcers (Koga *et al.*, 1996; Makino *et al.*, 1998). The mechanisms underlying the anti-ulcer effects of plaunotol include an increase of prostaglandin production by the gastric mucosa (Ushiyama *et al.*, 1987). Plaunotol has been registered with the World Health Organization (WHO) under the code CS-684. Its tradename is Kelnac® which has been manufactured by Sankyo Co., Ltd. (Ogiso *et al.,* 1985). Besides plaunotol, geranylgeraniol (GGOH) has potential as an apoptotic agent (Yoshikawa *et al.*, 2009). Phytol, a degradation product of chlorophyll, is a precursor for the synthesis of vitamin A and K1 (Peisker *et al.*, 1989; Daines *et al.*, 2003; Netscher, 2007). Phytosterols have a role in strengthening membranes in plants and also have some pharmacological values e.g. a cholesterol-lowering effect (Hovenkamp *et al.*, 2008). Wungsintaweekul *et al.* (2007) have reported that the diterpenes and phytosterols present in suspension cultures of *C. stellatopilosus* can be extracted with hexane to afford the lipophilic fraction. Simultaneous determination of these compounds not only serves as a quality control method but also provides a profile of an array of metabolites during the development of plant and cell cultures. Previously, TLC-densitometric and GC methods have been developed but only to determine the plaunotol content (Vongcharoensathit and De-Eknamkul, 1998). In the present study, we aimed to establish and validate a GC-FID method that could simultaneously determine the diterpenes and phytosterols.

2. Materials and Methods

2.1 Plant materials and chemicals

The young leaves of Plaunoi used were collected from the botanical garden of the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai Campus, Songkhla, Thailand. Phytol and geranylgeraniol were from Sigma-Aldrich, Singapore. Pure plaunotol was isolated from Kelnac® using a silica gel column (Wungsintaweekul and De-Eknamkul, 2005). Pure phytosterols including campesterol, stigmasterol and β-sitosterol were isolated from suspension cultures of *C. stellatopilosus* using a silica gel column and their structures were confirmed by ^{1}H-NMR and mass spectroscopy (MS) (Kongduang *et al.*, 2008). Organic solvents used in this study were analytical grade.

2.2 Green callus and suspension cultures

Callus induction was induced from young fully expanded leaves and maintained in Murashige and Skoog (MS) medium supplemented with 1.0 mg/L 2,4-D, 1.0 mg/L BA , 3% (w/v) sucrose and 0.8% (w/v) plant agar (Duchefa, The Netherlands) as described by Kongduang *et al.* (2008). For induction of green callus culture, three week-old proliferated calli were placed on MS medium supplemented with 2 mg/L BA, 2 mg/L NAA, 1% (w/v) sucrose and 0.2% (w/v) gellan gum (Phytotech, USA). The green callus culture was initiated after subculture at 25±2°C under light for 16 h/day.

A suspension culture was initiated from the green callus cultures. Calli were transferred into a 250-mL Erlenmeyer flask containing 50 mL of MS liquid medium supplemented with 2 mg/L BA, 2 mg/L NAA, 1% (w/v) sucrose. The suspension culture was agitated on a rotary shaker at 120 rpm and a temperature of 25±2°C, under light for 16 h/day. Suspension cultures were subcultured every 2 weeks. The inoculum size was 20 mL/50 mL of medium .

2.3 Sample preparation for quantification of diterpene and phytosterols

Young leaves (1 g), green callus cells (19-day old) (0.2 g) and suspension cultures (4-day old) (0.2 g) were harvested and dried at 50°C for 14 h, and separately extracted with 10 mL of 95% (v/v) ethanol by heating at 70°C for 1 h then filtered through Whatman no.1. The filtrate was evaporated and re-dissolved in 2 mL of 50% (v/v) ethanol. A solution of 10% (w/v) NaOH was added to a final concentration of 1% (v/v) and the mixture was heated at 70°C for 30 min. The lipophilic components in the solution were partitioned with *n*-hexane (3 ml each, x3). The *n*-hexane fraction, after being concentrated, was then subjected to GC analysis. Samples were analyzed in triplicate.

2.4 Establishment of the GC-FID

GC analyses were carried out with a Hewlett Packard HP6850 series instrument (Wilmington, USA) equipped with a flame-ionization detector (FID) and a chemically bonded fused silica capillary column of methylsiloxane (HP1, 30 m x 0.32 mm i.d., 0.25 μm film thickness). The GC separation of diterpenes and phytosterols was optimized and achieved with the temperature program being set from 220°C (hold 3 min) to 280°C (rate 10°C /min and hold 14 min), the injector temperature was 280°C, the oven temperature was 300°C, and the detector temperature was 280°C, sample size 1.0 μL, flow 1.0 mL/min helium and split (10:1) injection. Total run time was 23 min.

2.5 Method validation

The quantification method was validated according to the APVMA guidelines (Australian Pesticides and Veterinary Medicines Authority, 2004) and AOAC Guidelines (Association of Official Analytical Chemists, 2002). The validation included tests on specificity, linearity, precision and accuracy.

2.5.1 Linearity

The calibration curve was constructed using authentic phytol, GGOH, plaunotol, campesterol, stigmasterol and β-sitosterol. The stock solutions of the authentic compounds were prepared in volumetric flasks to obtain 1152 μg/mL, 768

μg/mL, 1280 μg/mL and 1920 μg/mL stock solutions of phytol, GGOH, plaunotol and phytosterols, respectively. The stock solutions were diluted by the half-dilution technique over the concentration ranges of 4.5-576 μg/mL, 3.0-384 μg/mL and 5.0-620 μg/mL to construct the calibration curves for phytol, GGOH and plaunotol respectively. Analysis of the phytosterols by GC determined that they were a mixture of campesterol, stigmasterol and β-sitosterol in a ratio of 2:3:10. By analysis of the peak areas of the phytosterols, the concentration ranges for campesterol, stigmasterol and β-sitosterol were 1-128 μg/mL, 1.5-192 μg/mL, and 5-640 μg/mL, respectively.

2.5.2 Precision

The precision of the GC-FID system was established by injecting an aliquot of the same sample six times on the same day. The repeatability and the interday intermediate precision were determined by analyzing three samples of different concentrations covering the range of the calibration curve three times a day on three different days. The data were used to calculate the percentage relative standard deviation (%R.S.D.) of intraday and interday precision.

2.5.3 Accuracy

The accuracy of the procedure was demonstrated by the recovery studies, which were carried out by fortifying the sample extracts with standard solutions. The amount of each analyte was determined in triplicate and the percentage recoveries were then calculated. The percentage recoveries of the analyte need not be 100%, but the extent of the recovery of an analyte should be consistent and precise.

2.6 Gas chromatographic-mass spectrometric (GC-MS) analysis

The hexane extract was analyzed by GC-MS in order to confirm the molecular weights of its constituents. Electron impact (70 eV) using a Hewlett Packard HP6890 GC-HP5972 MSD gas chromatograph-mass spectrometer (Hewlett Packard, USA). Mass and fragmentation patterns were compared with either the authentic or Wiley275 database (Scientific equipment center, PSU).

3. Results and Discussion

Although a specific method for the analysis of phytosterols, has been recently reported (Fernandez-Cuesta *et al.*, 2012), their GC method was to determine phytosterol levels of sunflowers produced in a plant breeding program. Since impurities in extracts from different sources may affect the separations differently, we have developed a tailor-made method for analysis of phytosterols from Plaunoi. Our interest was to follow these metabolites in plant leaves and in unorganized cells such as callus and suspension cultures. Methods using TLC-densitometric and GC-FID for determination of the plaunotol content from Plaunoi have been reported (Vongcharoensathit and De-Eknamkul, 1998). In addition, metabolite profiling in these plant models can provide information on the biosynthesis of terpenoids. Thus a simultaneous determination for phytol, GGOH, plaunotol, campesterol, stigmasterol and b-sitosterol contents could provide insights on terpenoid biosynthetic pathway during plant development.

In this present study, a GC-FID method was established and optimized based on an initial previous report (Wungsintaweekul *et al.*, 2007). Separation and detection of phytol, GGOH, plaunotol and phytosterols by GC-FID in the crude hexane extracts of *C. stellatopilosus* were confirmed. Various GC parameters such as the injection temperature, temperature gradient profile and detector temperature were optimized. Eventually, we did optimize the GC conditions to detect all the diterpenes and phytosterols in a single run with a GC injection temperature of 220°C, a temperature program: 220°C for 3 min that was then elevated to 280°C at 10°C/min, then held at 280°C for 14 min (total run time 23 min) and a detector temperature of 300°C. The authentic compounds used included phytol, GGOH, plaunotol, campesterol, stigmasterol and β-sitosterol that were eluted at 5.7, 6.3, 8.0, 17.8, 18.5 and 19.9 min, respectively.

The limit of detection (LOD) and limit of quantification (LOQ) were determined by an empirical method that consisted of analyzing a series of standard solutions containing decreasing amounts of the authentic compounds. The LOD values for detection of standards were approximately 3.0, 2.0, 3.5, 0.8, 1.2 and 4.0 μg/mL of phytol, GGOH, plaunotol, campesterol, stigmasterol and β-sitosterol, respectively. The LOQ is defined as the lowest concentration of a measurable value of the standard solutions and they were 4.5, 3.0, 5.0, 1.0, 1.5 and 5.0 μg/mL of phytol, GGOH, plaunotol, campesterol, stigmasterol and β-sitosterol, respectively. For validation, of linearity the standard solutions were prepared in *n*-hexane. Calibration curves were constructed using authentic phytol, GGOH, plaunotol, campesterol, stigmasterol and β-sitosterol with six concentrations each. All compounds showed linearity with r^2 values ≥ 0.9994. The linear regression and correlation coefficients are presented in Table 1. The assay precision was assessed for its repeatability (intraday) and intermediate precision (interday). Intraday was evaluated by assaying samples, at the same concentration on the same day. The interday precision was studied by comparing the assay of the same concentrations of the samples on different days (3 days). The intraday relative standard deviation (RSD) was $< 1.2\%$ (n=6) and the interday relative standard deviation (RSD) was $< 1.3\%$ (n=9). The results are summarized in Table 2. The accuracy of the optimized GC-FID was evaluated by checking three different concentrations of standard solutions. Three quality control solutions were prepared and spiked into a crude hexane extract from leaves, callus and suspension cultures of Plaunoi. The analytical recoveries of all authentic compounds were 102.4-109.9% (Table 2). From these results, the established GC-FID was approved according to the

Table 1. Linear range and correlation coefficients of calibration curves.

compound	y = ax+b linear model*	r^2	concentrations (μg/mL)	LOQ (μg/mL)	LOD (μg/mL)
phytol	y = 1.4519x + 2.044	0.9998	4.5-576.0	4.5	3.0
GGOH	y = 2.2022x - 0.539	0.9996	3.0-384.0	3.0	2.0
plaunotol	y = 1.9684x - 0.7287	0.9999	5.0-620.0	5.0	3.5
campesterol	y = 2.5628x - 0.6533	0.9994	1.0-128.0	1.0	0.8
stigmasterol	y = 2.4043x - 0.5114	0.9996	1.5-192.0	1.5	1.2
β-sitosterol	y = 2.4281x + 4.8606	0.9998	5.0-640.0	5.0	4.0

*x and y represent concentration and the peak area, respectively.

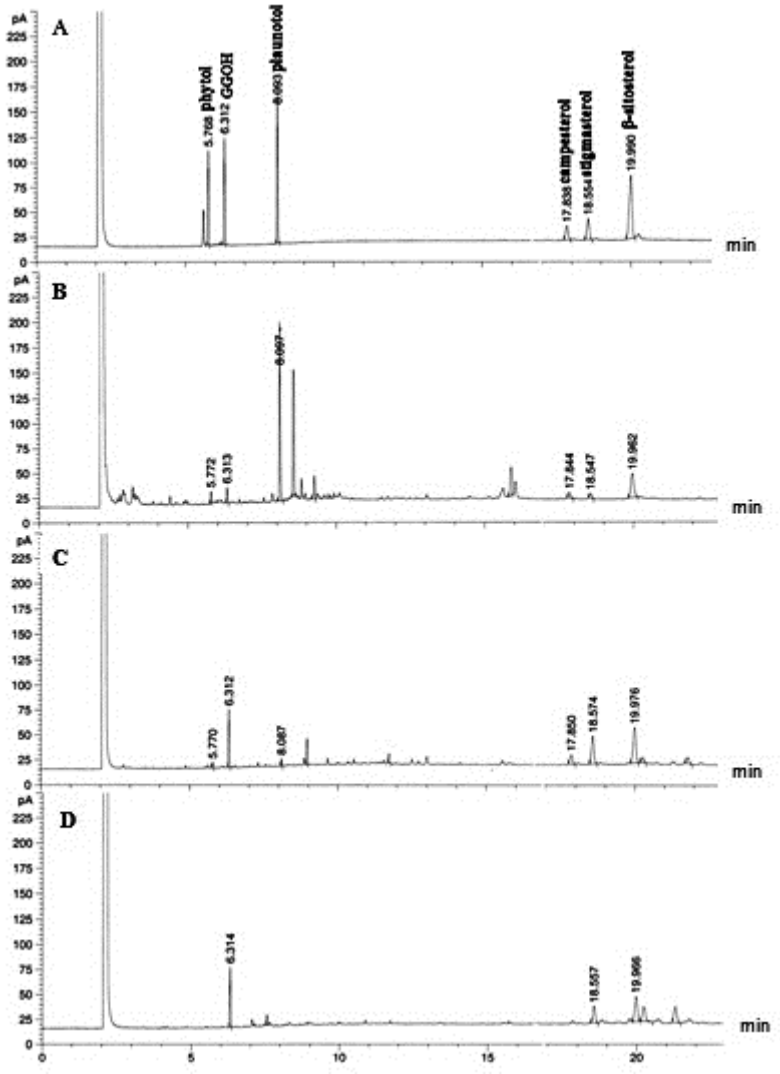

Figure 2. Final GC-FID optimization results showing the GC chromatogram of a crude hexane extracts of *C. stellatopilosus*. A: authentic compounds; B: leaf extract; C: green callus extract ; D: suspension extract.

guidelines and can be used to determine the amounts of phytol, GGOH, plaunotol and phytosterols in the organized and unorganized cells of Plaunoi.

Using the HP1 methylsiloxane capillary column for the GC separation of diterpenes and phytosterols, in this study, provided a good resolution and sensitivity (Figure 2b). As shown in Table 1, the linearity range for plaunotol was 5.0-620.0 μg/mL and had the correlation coefficients of 0.9999. In contrast, plaunotol separated on 2% silicone OV17 (2 m x 2 mm) GC column had the linearity range of 0.05-0.8 mg/mL

Table 2. Repeatability, reproducibility and recoveries of phytol, GGOH, plaunotol, campesterol, stigmasterol and β-sitosterol from *C. stellatopilosus* leaves, callus and suspension extracts.

Compound	R.S.D. (%)						% Recovery (Mean ± S.D.)		
	intraday (n=6)			interday (n=9)					
	leaves	callus	suspension	leaves	callus	suspension	leaves	callus	suspension
phytol	1.03	0.75	n.d.	1.17	0.91	n.d.	105.4±0.92	108.5±0.49	n.d.
GGOH	0.82	0.98	0.94	0.99	1.04	1.08	102.4±0.89	109.9±0.14	103.6±0.57
plaunotol	1.17	1.04	n.d.	1.22	1.29	n.d.	104.4±1.18	105.8±0.23	n.d.
campesterol	0.89	0.75	0.59	0.96	1.06	0.97	107.8±0.99	106.8±0.46	105.1±0.82
stigmasterol	0.78	0.85	0.59	1.00	0.99	0.72	107.3±0.93	108.3±0.24	109.8±0.34
β-sitosterol	1.00	0.91	1.02	1.16	1.26	1.11	102.5±1.06	109.9±0.06	108.9±0.25

*n.d.: not detected

Table 3. Contents of diterpenes and phytosterols.

Samples	contents (μg/g DW ± S.D.)[a]					
	diterpenes			phytosterols		
	phytol	GGOH	plaunotol	campesterol	stigmasterol	β-sitosterol
Young leaves	1726.92 ± 78.61	655.68 ± 30.61	1740.11 ± 63.96	228.98 ± 6.78	255.09 ± 12.42	1026.72 ± 58.66
19-day old green callus	72.81 ± 0.94	168.64 ± 0.27	314.04 ± 0.56	73.54 ± 0.75	301.14 ± 2.13	399.79 ± 3.22
4-day old suspension	n.d.[b]	81.00 ± 4.35	n.d.[b]	253.67 ± 29.28	260.00 ± 24.87	931.33 ± 91.48

[a]Samples were analyzed in triplicate; [b]n.d.: not detected

and the correlation coefficient of 0.998 (Vongcharoensathit and De-Eknamkul, 1998).

The optimized GC condition could be applied to separate all components in the crude hexane extract of plant leaves, callus and suspension cultures of Plaunoi (Figure 2). The mass and fragmentation patterns of compounds were further confirmed by GC-MS. The determined molecular weights of phytol, GGOH, plaunotol, campesterol, stigmasterol and b-sitosterol were 296, 290, 306, 400, 412 and 414, respectively. Furthermore, their mass fragmentations were matched with the patterns of the authentic compounds such as phytol, GGOH and plaunotol. For the phytosterols: campesterol, stigmasterol and b-sitosterol, the mass fragmentation patterns were also matched with the patterns of the WILEY275 database library (data not shown).

Table 3 shows the amounts of diterpenes and phytosterols in the crude hexane extracts of plant leaves, callus and suspension cultures. Based on the information from Kongduang (2007), callus and suspension cultures have different growth characteristics. We showed that callus produced diterpenes and phytosterols during the early stationary phase, whereas suspension cultures produced GGOH and phytosterols in the late lag phase (Kongduang, 2007). Therefore, 19-day old callus and 4-day old suspension cells were selected for the simultaneous determination of diterpenes and phytosterols. Plant leaves accumulated the diterpenes (phytol, GGOH, plaunotol) rather than the phytosterols, whereas similar amounts of diterpenes and phytosterols were accumulated by the green callus culture. However, the accumulation of all diterpenes and phytosterols except for stigmasterol were all considerably lower than in the fresh plant leaves. For the suspension culture, only GGOH and phytosterols were detected in the cells, and there was no detectable production of phytol and plaunotol.

In conclusion, the GC-FID method developed from this study can be used as an analytical method for the measurement of terpenoids (phytol, GGOH, plaunotol) and phytosterols (campesterol, stigmasterol, β-sitosterol) contents simultaneously. We demonstrate that the developed GC-FID method made the quantification of these compounds easy. This result will be useful for plant scientists, who are interested in the analysis of the terpenoid components in organized and unorganized plant cell cultures.

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