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Rapid detection of *Ganoderma*-infected oil palms by microwave ergosterol extraction with HPLC and TLC

M.S. Muniroh [a], M. Sariah [a,*], M.A. Zainal Abidin [a], N. Lima [b], R.R.M. Paterson [b]

[a] Department of Plant Protection, Faculty of Agriculture, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia
[b] IBB — Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, Universidade do Minho, 4710-057 Braga, Portugal

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ABSTRACT

Detection of basal stem rot (BSR) by *Ganoderma* of oil palms was based on foliar symptoms and production of basidiomata. Enzyme-Linked Immunosorbent Assays-Polyclonal Antibody (ELISA-PAB) and PCR have been proposed as early detection methods for the disease. These techniques are complex, time consuming and have accuracy limitations. An ergosterol method was developed which correlated well with the degree of infection in oil palms, including samples growing in plantations. However, the method was capable of being optimised. This current study was designed to develop a simpler, more rapid and efficient ergosterol method with utility in the field that involved the use of microwave extraction. The optimised procedure involved extracting a small amount of *Ganoderma*, or *Ganoderma*-infected oil palm suspended in low volumes of solvent followed by irradiation in a conventional microwave oven at 70 °C and medium high power for 30 s, resulting in simultaneous extraction and saponification. Ergosterol was detected by thin layer chromatography (TLC) and quantified using high performance liquid chromatography with diode array detection. The TLC method was novel and provided a simple, inexpensive method with utility in the field. The new method was particularly effective at extracting high yields of ergosterol from infected oil palm and enables rapid analysis of field samples on site, allowing infected oil palms to be treated or culled very rapidly. Some limitations of the method are discussed herein. The procedures lend themselves to controlling the disease more effectively and allowing more effective use of land currently employed to grow oil palms, thereby reducing pressure to develop new plantations.

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1. Introduction

Palm oil is a major commodity used in ca. 30% of foods and cosmetics. Increasingly the oil is used as a biofuel and contributes considerably to the economies of many nations and particularly Malaysia and Indonesia (Paterson et al., 2013). Oil palms suffer from the major disease, basal stem rot (BSR), caused by the white rot fungus *Ganoderma*. According to Idris et al. (2011), 632 out of 1061 estates (59.57%) that responded to a survey, reported incidences of BSR disease. The average incidence of BSR in Malaysia was 3.71% with an affected area of 59,148 ha. The economic losses are between \$68 and \$455 million a year in Malaysia alone (Chong, 2012).

The disease is currently detected based on development of foliar symptoms and production of basidiomata in mature fields. However, visible symptoms indicate that the (a) palms are already at a serious stage of infection and (b) fungus has killed approximately half of the basal tissue. More effective methods to detect the disease early are required urgently so that remedial action (e.g. culling) can be taken quickly to prevent spread to healthy oil palms causing even more disease. Better control of the disease may create ecological benefits from reduced pressure to create new plantations as the yields from existing fields increase.

The Enzyme-Linked Immunosorbent Assay-Polyclonal Antibody (ELISA-PAB) and PCR have been proposed (Mohd Aswad et al., 2011), but these methods are not applicable for large scale field monitoring because they are complex, time consuming and have accuracy limitations. For example, PCR can be subjected to inhibition (Paterson, 2007a) and ELISA-PAB suffers from cross reactivity (Idris and Rafidah, 2008). *Ganoderma boninense*, the causal fungal pathogen of BSR in oil palm, colonises the roots and leads to an incremental increase in fungal biomass during disease development. Also, the fungus appears to colonise from one oil palm to another by producing vast numbers of spores (Sanderson, 2005). One of the common cell membrane components, ergosterol, is sufficiently specific (Mille-Lindblom et al., 2006) that it is used to quantify fungi in soil (Grant and West, 1986; Frostegard and Baath, 1996), roots (Bindler et al., 1988), cereal grains (Seitz et al., 1977), and decaying plant material (Newell et al., 1988). The compound is used to quantify ectomycorrhizae (Salmanowicz and Nylund, 1988;

* Corresponding author. Tel.: +60 3 894 8947; fax: +60 3 89381014.
E-mail address: muniroh_mdsaad@yahoo.com (M. Sariah).

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Wallander et al., 1997). Also, ergosterol in the white rot fungi *Hydnum* and *Polyporus* was correlated with growth of these fungi in oil palm in vitro (Paterson et al., 2000). Paterson (2007b) recommended the use of ergosterol to determine the levels of BSR in oil palm trunk and recent analysis of ergosterol in *Ganoderma* infected oil palm tissue showed a strong correlation between ergosterol concentration and disease severity, indicating the possible application of ergosterol in the quantification of BSR in palms (Mohd Aswad et al., 2011). In this study, apparently diseased and healthy oil palms were felled and assessed for disease, from none to high, by conventional methods. The symptoms correlated well with ergosterol levels, and ergosterol was undetected in the healthy palm samples. In addition, standing oil palms were assessed for disease and drilled for samples: similar results were obtained to the felled oil palms. These procedures are similar to those that would occur during normal plantation management of the disease. The method of analysis of ergosterol was chromatography which is a chemical method involving solid/liquid phase separations and more robust inherently than the biochemical methods, such as PCR and antibodies, which involve potentially more variable biochemicals such as proteins and nucleic acids.

Naturally, there are limitations to the method: It will not differentiate between the target *Ganoderma* and other fungi which may be present. However, no other fungi will contribute significantly to the concentration of ergosterol, as *Ganoderma* is the only fungus involved in this type of disease in oil palm, where a large amount of fungal biomass is produced as infection proceeds (Paterson, 2007b). *Fusarium* wilt causes disease of oil palms by a quite different mechanism involving production of extracellular enzymes rather than large increases in biomass. Fatal yellowing or lethal bud rot of oil palm is caused by *Thielaviopsis paradoxa* (teleomorph *Ceratocystis paradoxa*) which attacks non-lignified tissue. This disease is infrequent and will not be involved in high growth throughout the palm stem or roots as is the case with *Ganoderma*. The most important fungal disease in Colombia is bud rot by *Phytophthora palmivora*. This fungus does not cause disease by extensive growth throughout the roots or stem and by implication is limited in its effect to buds (Paterson et al., 2013). Hence, these fungi will not interfere with the ergosterol analyses. Although not a limitation per se, another issue is the concentration of ergosterol that would merit culling an oil palm, which will require careful investigations to ensure the cost-effective values are obtained.

However, the extraction method in Mohd Aswad et al. (2011) is time consuming, and requires large amounts of costly equipment and high volumes of expensive and potentially-dangerous solvents. In plantations, BSR censuses are carried out regularly and preferably at six month intervals: a robust, rapid and reliable early detection method is highly desirable for large scale BSR monitoring, which could be adapted to field analyses. A particularly desirable method would be one that could be undertaken on-site (e.g. near to an oil palm plantation).

Microwave energy has been shown to greatly accelerate a wide variety of (a) chemical reactions at up to as much as 240 times (Mingos and Baghurst, 1991) and (b) extractions of organic chemicals from various matrices (Ganzler et al., 1986; Onuska and Terry, 1993; Lopez-Avila et al., 1994). Young (1995) developed a microwave-assisted extraction (MAE) technique for analysis of ergosterol in fungi and samples contaminated with fungi. The recovery of ergosterol was more efficient than that of classical and supercritical fluid extraction methods. Thus this present study was conducted to determine the practicability of using the MAE method in monitoring BSR infections in field samples and represents the first such report.

2. Materials and methods

2.1. Mycelial culture of Ganoderma

A pure culture of *G. boninense* (strain UPM 13) was isolated from a basidiomata of an infected oil palm trunk in Gua Musang Felda, Malaysia using *Ganoderma* selective medium (Ariffin and Idris, 1991) and the identification was confirmed by experts based on mycelial and spore morphology. The pure culture was maintained on malt extract agar (MEA) (Merck) and deposited in the culture collection of Institute of Bioscience (IBS), UPM, Malaysia as a stock culture. A mycelial disc (0.5 cm) from a 14-day culture was cut and grown in static 250 mL Erlenmeyer flasks containing 100 mL Malt Extract Broth (MEB) (Merck) at room temperature (25 ± 2 °C). The mycelium was removed from the flask after 2 weeks, rinsed with sterile distilled water, air dried and stored at −80 °C as inocula for subsequent experiments.

2.2. Ergosterol extraction using microwave assisted extraction

Extraction of *G. boninense* was based on the procedure of Young (1995) with modifications. A sample of 0.5 g fresh weight (FW) mycelium from 2 week-old *Ganoderma* mycelium grown in MEB as mentioned above, was macerated in liquid nitrogen using a mortar and pestle into a powder, and transferred to a Pyrex test tube with a Teflon screw cap. Two millilitres of methanol (Merck, Chromatography grade) and 0.5 mL of 2 M sodium hydroxide were added and the tube was tightly sealed. The test tubes were placed in a culture jar at the centre of a conventional microwave (Sharp Jet Convectional Grill, model TTAG A437 with a capacity of 1.5 ft^3) and subjected to various levels of microwaves that formed the basis of experiments. The solutions were left to cool and were neutralized with concentrated hydrochloric acid. Finally, the solutions were extracted three times with 2 mL of pentane (Fisher chemicals, analytical reagent grade). The combine pentane extracts were then evaporated to dryness by using a Buchi rotary evaporator and then dissolved in 500 μL methanol for the detection of ergosterol using thin layer chromatography (TLC) and quantified using high performance liquid chromatography (HPLC) against an ergosterol standard (Sigma, purity ≥ 95.0%).

2.2.1. Optimisation of extraction

To determine the optimum microwave extraction settings, different temperatures were used according to the temperature settings available on the microwave (40 and 70 °C), power (medium, medium high and high) and exposure times (10, 20 and 30 s) and which were tested on 0.5 g FW *Ganoderma* mycelium.

2.2.2. Ergosterol from different weights of Ganoderma

Freshly harvested mycelium from 2 week old *Ganoderma* mycelium grown in MEB as mentioned above (0.025, 0.05, 0.10, 0.20, 0.40, 0.60, 0.80 and 1.00 g) was used for the ergosterol extraction to determine the linearity of response of the extraction.

2.2.3. Ergosterol from different weights of oil palm

The infected tissues were drilled from 15 infected palms obtained from the Gua Musang Felda plantation and exhibiting stage 3 of BSR infection. The level of infection was determined by the disease pictorial key developed by Mohd Aswad et al. (2011), characterised by unopened spear leaves and basidiomata of the fungus appearing at the trunk or roots near soil level; infected drilled tissues appeared dark brown with a strong fermented and rancid odour. Different weights of three sub-samples from each palm (0.25, 0.50, 1.00, 1.50, 2.00, 2.50, 3.00, 3.50 and 4.00 g) were macerated in liquid nitrogen and ergosterol extracted based on the predetermined optimised microwave settings. The identification of ergosterol was achieved by HPLC diode array detection and TLC.

2.2.4. Comparison between different methods of ergosterol extraction

Comparisons were made between the MAE, the non-alkaline extraction (NAE) (Gong et al., 2001), and the ultra-sonication extraction (USE) methods (Yuan et al., 2007) with 0.5 g FW of *Ganoderma* mycelium.

2.3. Detection and quantification of ergosterol

2.3.1. Thin layer chromatography

Six microlitres of the 100 μg mL^{-1} standard ergosterol (Sigma, purity ≥ 95.0%) and the extracted ergosterol was spotted on RP-18 Silica coated TLC plates (Merck, 20 × 20 cm) with a micropipetter (Eppendorf) with a 10 mm space between each spot. The spots were then air dried for 5 min before development. The plates were developed in 25 mL of n-hexane/ethyl acetate (75/25; v/v) for approximately 60 min in a flat bottom developing chamber (CAMAG). The plates were dried for 5 min and viewed under a UV lamp at 365 nm. TLC detection was undertaken in duplicate for all samples. The identification of ergosterol was based on comparison with the R_f value with the ergosterol standard and the concentrations were obtained from the HPLC analyses.

2.3.2. High performance liquid chromatography

An Agilent 1100 series HPLC equipped with a Diode Array Detector (G1315B), a pump (G1311A), and an autosampler (G1313A) was used for quantification of ergosterol using an Ascentis express 2.7 μ C18 reverse-phase column (Supelco, USA). Operating conditions consisted of an isocratic HPLC-grade methanol mobile phase at a flow rate of 1 mL min^{-1}. The mobile phase was degassed for 30 min in an ultrasonicator (Cole-palmer) at full power. The UV detection was at 282 nm and injection volume of 10 μL per sample was set for quantification. The average retention time of ergosterol was 7 min. An ergosterol standard was prepared for constructing the standard curve. The ergosterol peak was determined by comparison of the retention time and UV absorbance at 282 nm against the pure ergosterol standard. The ergosterol concentration for each run was determined by comparison against the ergosterol standard calibration curve. All ergosterol concentrations are reported on a per unit weight basis and each sample was analysed in triplicate.

3. Results

3.1. Ergosterol extraction using microwave assisted extraction

3.1.1. Optimisation of ergosterol extraction

The HPLC responses (peak areas) were checked for linearity within the level 50–120 μg ergosterol standard. The peaks were correlated with ergosterol concentrations, which gave a good correlation coefficient (R^2) of 0.9978. The ergosterol standard peak was well resolved in all runs. The concentrations of ergosterol for the different microwave settings are illustrated in Fig. 1. The setting of medium high at 70 °C for 30 s exposure time was the most efficient, which resulted in the highest concentrations of ergosterol extracted from 0.5 g fresh weight of *Ganoderma* mycelium.

3.1.2. Ergosterol from different weights of Ganoderma

Ergosterol concentration increased proportionally with mycelial biomass (Fig. 2). The lowest and the highest *Ganoderma* biomass of 0.025 g and 1.0 g resulted in the lowest and highest ergosterol concentration of 22.70 μg g^{-1} and 64.00 μg g^{-1} respectively. The intensity of the ergosterol spots detected on the TLC plates increased proportionately with the increase in mycelial biomass (and hence ergosterol) with the retention factor (R_f value) of 0.68.

3.1.3. Ergosterol from different weights of oil palm

To determine the efficiency of extraction by the microwave method on actual oil palm samples, the amounts of ergosterol from different weights of *Ganoderma* infected oil palm and MAE ergosterol HPLC chromatogram of infected oil palm tissue are provided in Fig. 3. The lowest and the highest biomass of 0.25 g and 4.00 g resulted in the lowest and highest ergosterol concentrations of 2.20 μg g^{-1} and 6.60 μg g^{-1} respectively.

3.1.4. Comparison between different methods of ergosterol extraction

The MAE extraction method was the most efficient compared to the NAE and USE methods. The amounts extracted from 0.50 g fresh weight of *Ganoderma* mycelium with MAE (62.06 μg g^{-1}) were significantly higher than the USE (9.63 μg g^{-1}) and NAE (7.34 μg g^{-1}) methods, whereas the values for USE and NAE were not significantly different. In addition, the TLC analysis also showed the highest intensity of spots produced with MAE and that the size of the spots was proportional to the concentration of ergosterol (Fig. 4). Comparisons of experimental settings between MAE and NAE showed that MAE required shorter time than NAE method and more samples can be extracted within a particular period of time (Table 1).

4. Discussion

Ergosterol is a primary sterol in cell membranes of filamentous fungi and is absent, or present as a minor component, in the majority of higher plants (Madonna et al., 2001). The present work indicated that a microwave extraction of medium high power at 70 °C provided the optimal extraction of ergosterol from *G. boninense* mycelium (Fig. 1). In addition, there was a direct relationship between the weight of mycelium and the concentration of ergosterol (Fig. 2). These results indicated that the method was suitable for analyzing samples of oil palm comparable with that of Mohd Aswad et al. (2011) in the earlier report. There was a direct relationship between the weight of infected oil palm samples and the ergosterol concentration (Fig. 3).

Recent work indicates that ergosterol cannot be detected in healthy oil palms (Mohd Aswad et al., 2011; Toh Choon et al., 2012; Chong, 2012) and that ergosterol analysis is a valid diagnostic method to detect BSR. The methods used for analysis of ergosterol in other systems were frequently based on Seitz et al. (1977, 1979), and involved methanolic extraction, alkaline saponification, pentane extraction and reversed

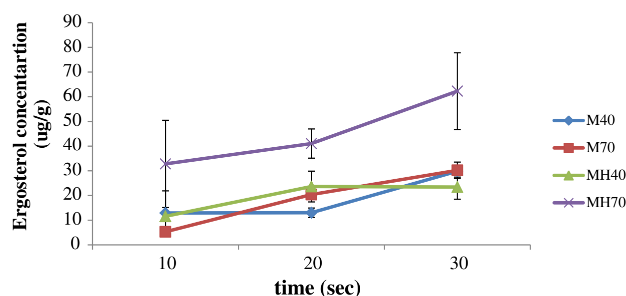

Fig. 1. Ergosterol concentration from 0.5 g fresh weight of *Ganoderma boninense* mycelium with various microwave temperatures, power levels and exposure times (M40 = medium, 40 °C, M70 = medium, 70 °C, MH40 = medium high, 40 °C, MH70 = medium high, 70 °C). Bars represent means ± SD (error bar) of triplicate determinations.

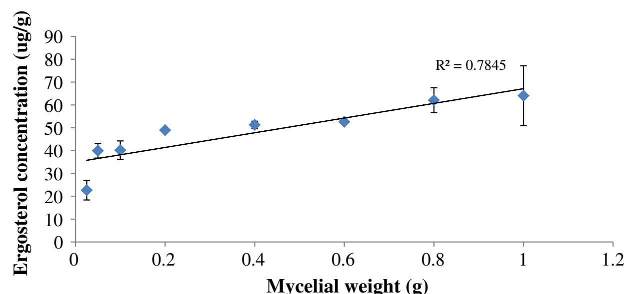

Fig. 2. Effect of sample weights of *Ganoderma boninense* mycelium on ergosterol concentration. Bars represent means ± SD (error bar) of triplicate determinations. As can be determined there was a direct relation between mycelium weight and ergosterol concentration.

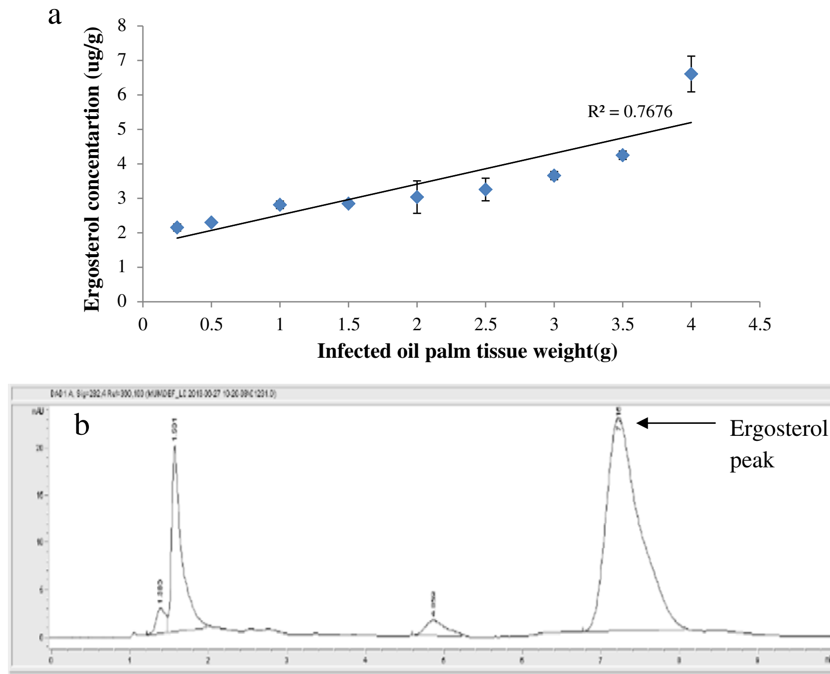

Fig. 3. (a) Effect of infected oil palm tissue sample weight on ergosterol concentration extracted using MAE method with microwave setting of medium high power and 70 °C, for 30 s. Bars represent means ± SD (error bar) of triplicate determinations; (b) MAE ergosterol HPLC chromatogram of infected oil palm tissue indicating the ergosterol peak is well separated from co-extractives and facilitating identification and quantification of ergosterol from infected oil palm. It has been determined previously by various authors that ergosterol is undetected in un-infected oil palm (see Discussion in the present paper).

phase high performance liquid chromatographic (HPLC) separation with ultraviolet (UV) detection at 282 nm. Some modifications reported include combinations of methanolic extraction and saponification (Newell et al., 1988; Zill et al., 1988; Schwadorf and Muller, 1989); the use of CO_2 supercritical fluid extraction (SFE), and supercritical fluid chromatography (Young and Games, 1993). Young (1995) developed the microwave-assisted extraction (MAE) technique for analysis of ergosterol in fungi and samples contaminated by fungi. Ergosterol recoveries using classical extraction and supercritical fluid extraction methods were lower than that of MAE.

The basic principle of the MAE protocol is that ergosterol can be extracted from fungal membranes by the microwave effect which evaporates and generates tremendous pressure on the cell wall due to swelling of the cells (Wang and Weller, 2006). The pressure pushes the cell wall from inside, broadening and eventually breaking it, which aids leaching out of the active constituents from the ruptured cell to the surrounding solvent therefore improving the yield of phytoconstituents. Microwave power and irradiation time were the two factors which influence extraction to a great extent. In this present study, the optimum setting for the MAE technique was 70 °C with medium high power and 30 s exposure time (Fig. 1). A (a) higher and (b) lower microwave temperature and power resulted in (a) the leaking of the solvent from the tube, and (b) less ergosterol respectively. A combination of low or moderate power with longer exposure may be useful, but high power with prolonged exposure involves the risk of thermal degradation (Mandal et al., 2007).

As mentioned in the Introduction, the extraction will include the ergosterol of all the fungi that were present in the initial sample and is a potential limitation. However, it was shown that ergosterol was undetected in healthy oil palms which is an indication of the utility of the method in that any chance contamination will not affect the results. In addition, *Ganoderma* is the only fungus that causes this type of disease and is by far the most prevalent disease of oil palms in SE Asia. Therefore, the level of ergosterol is likely to be related directly to the amount of oil palm rot and not the presence of other fungi. An issue that requires addressing is at what concentration of ergosterol will a decision to cull infected oil palm be made? This will be determined by further studies in the field which correlate ergosterol detection with existing, later parameters used to make the decision to cull (e.g. presence of basidiomata). Furthermore, blind sampling would provide useful information in future studies now that correlations between disease and ergosterol have been made in the current and previous papers. A certain representative number of oil palms from a field could be selected randomly and analysed for ergosterol using drilling of standing trees (Mohd Aswad et al., 2011).

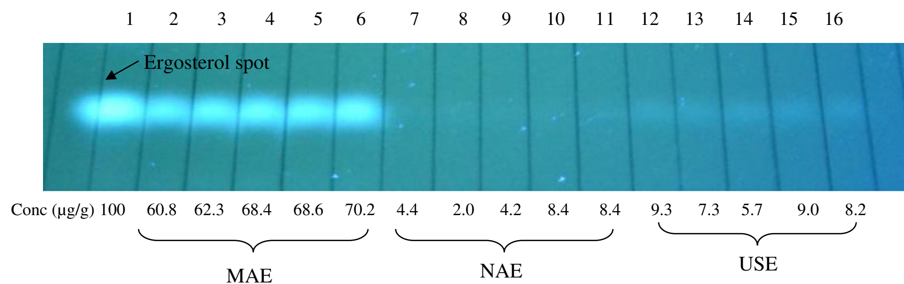

Fig. 4. TLC analysis of different methods of ergosterol extraction. Lane 1; Ergosterol standard. Lanes 2–6; microwave-assisted extraction (MAE) method. Lanes 7–11; non-alkaline extraction (NAE) method. Lanes 12–16; ultra-sonication extraction (USE) method. Actual concentrations were determined by HPLC analyses and an approximate concentration of ergosterol can be obtained from the size of the spots on the TLC plate.

Table 1
Comparisons of efficiency of ergosterol extraction based on MAE and NAE method.

Experimental setting	Microwave-assisted extraction (MAE)	Non-alkaline extraction method (NAE) (Mohd Aswad et al., 2011)
Number of sample/batch	10 samples	1 sample
Volume of solvent used/sample	4 mL	6 mL
Minimum weight of infected tissue/sample	1.0 g	3.0 g
Time required/batch (extraction until TLC/HPLC loading)	25 min	40 min
Recovery rate base on 100 μg ergosterol standard	62%	7%

These palms could then be monitored for subsequent infection to determine the predictive capabilities of the method.

Another important fact about the MAE protocol was that it only involved the use of methanol avoiding other hazardous organic solvents. In addition, only small samples are required which is advantageous. This study also demonstrated a strong correlation between ergosterol concentration and the *G. boninense* mycelial biomass or *Ganoderma* infected palm tissues, indicating that ergosterol concentration increased directly with the increase of mycelial biomass. Thus, the ergosterol method as described herein confirms that it is a major advance in the estimation of fungal biomass (Mille-Lindblom et al., 2004) and applies equally to determining the compound in oil palm. The MAE technique was the most efficient, gave the highest ergosterol yields, provided higher extraction efficiency, took less time and was less labour intensive (Table 1). Finally, the applicability of TLC means that the procedure is effectively portable and could be undertaken on site (e.g. in a motor vehicle situated near a plantation) for particularly rapid results.

5. Conclusion

In conclusion, a novel protocol for ergosterol extraction from *Ganoderma* infected oil palm tissue has been developed. Chromatographic methods are more robust than other biologically-based methods such as PCR. This techniques is more convenient than other ergosterol methods in terms of time for sample preparation, cost, and sample size as only a small sample size was needed for the extraction, more samples can be extracted. This would enable the efficient analysis of a large number of field samples during BSR surveys. Semi-quantitative TLC analysis is recommended as a simple and rapid detection method in the field where an HPLC may not be available. More effective disease management means that existing plantations will be more economic and, for example, reduce the pressure on converting new land to oil palm plantations with potential environmental benefits.

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