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## Microwave-assisted extraction of $\beta$ -sitosterol from cocoa shell waste

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# Microwave-assisted extraction of $\beta$ -sitosterol from cocoa shell waste

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**Abstract.** Phytosterol from cocoa shell can be reused in food industries in order to add value of the agricultural waste. Its extraction from the cocoa shell using ethanol can be assisted by using microwave for effective heating. This study was carried out to delineate the effect of temperature, power and radiation time of the microwave onto the extraction of  $\beta$ -sitosterol, as the key phytosterol, from the cocoa shell of *Theobroma cacao* L. species using absolute ethanol. Salkowski test, IR spectra and GC-MS analyses confirmed the presence of  $\beta$ -sitosterol and a flame-ionization-detector gas chromatography was employed to measure its concentration. Based on the one-factor-at-a-time (OFAT) approach, the maximum yield was obtained 13% higher than the yield of conventional maceration, i.e. 3546.1 mg/100g, at the optimum values of 70°C, 500 W and 10 min. Solubility and boiling point of ethanol onto extraction at various extraction temperatures probably caused the differences.

## 1. Introduction

Phytosterol, such as  $\beta$ -sitosterol, stigmasterol and campesterol [1], is a naturally occurring substance found in plants – plant sterol that has similar structure to cholesterol. Awad and Monie [2] found that  $\beta$ -sitosterol could be possible to inhibit the growth of colon and prostate cancer as it has the properties of antidiabetic [3], antioxidant, anticancer, anti-inflammatory, anti-pyretic and anti-stress agent and cancer preventive [4,5]. According to Martínez-Pinilla, Oñatibia-Astibia, & Franco [6], theobromine may act as antitumoral, anti-inflammatory or cardiovascular protector molecule without the undesirable side effects. Besides, this compound when combined together with caffeine was formed as blockers of adenosine receptors which are G-protein-coupled receptors that sense the presence of extracellular adenosine [6].

In the past decades, phytosterols and their derivatives were widely applied in food and cosmetic industries and, recently, have attracted nutritionists on the benefit of cholesterol diet [7]. The production of phytosterol from edible sources is associated with concerns of food supply shortage, thus unviable economics. The use of inedible resources, on the other hand, as the raw material for phytosterols extraction are not practical because of difficulty in toxic removal since it cannot be consumed by human.

Shell of cocoa bean, also known as hull or husk, is the outer portion of beans that encases the nibs. It is rich in fibres, phenolic compounds, theobromine and having a lipid profile similar to that of cocoa butter. Phytosterol is also found in CSW [8] and can be extracted by using solvent extraction [9,10].



Utilization of waste product like CSW can solve the issue of its accumulation in the agro-food industry and reduce the phytosterol production cost.

Little attention is given to sterol extraction from the waste of CSW due to intensive extraction is required to obtain its traces amount. The limitations associated with conventional extraction of vegetable resources include prolonged extraction time, environmental and health risks associated with hazardous solvent, high solvent consumption and possible changes in the characteristics of the extracted material [11]. Hence, alternative extraction technologies, which are efficient and provide high quality products, but do not require the use of toxic chemicals, need to be developed. Ethanol is toxic free, safe to the environment and mostly use extraction solvent for extracting natural based product and can substitute hexane to extract edible product. Ethanol also has adequate polarity in absorbing phytosterol [12]

Microwave-assisted extraction (MAE) is widely used in the extraction of plant components. The application of this technology allows the extraction of natural products in shorter times with high reproducibility and low solvent consumption in simple procedures compared to the conventional techniques [13,14]. The heating mechanism of microwave is based on the motions of the polar molecules and ions inside the solvent and vegetal matrix. In addition, it protects the constituents of thermolabile compounds. Therefore, microwave is chosen as the method to intensify extraction in this study. The objective of this work was to evaluate the influential parameters of MAE such as temperature, power and time of irradiation by using  $\beta$ -sitosterol yield from the analysis using Fourier transform infrared spectroscopy, Salkowski test and Gas chromatography. Benchmarking against reports from previous works is also presented.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Absolute ethanol and HPLC-grade hexane were supplied by Fisher Scientific (M) Sdn Bhd. Standard beta-sitosterol, ACS grade, 99% purity was purchased from Sigma Aldrich, Malaysia. Cocoa shells waste was kindly given by Lembaga Koko Malaysia, Jengka, Pahang.

### 2.2. Cocoa shell sample preparation

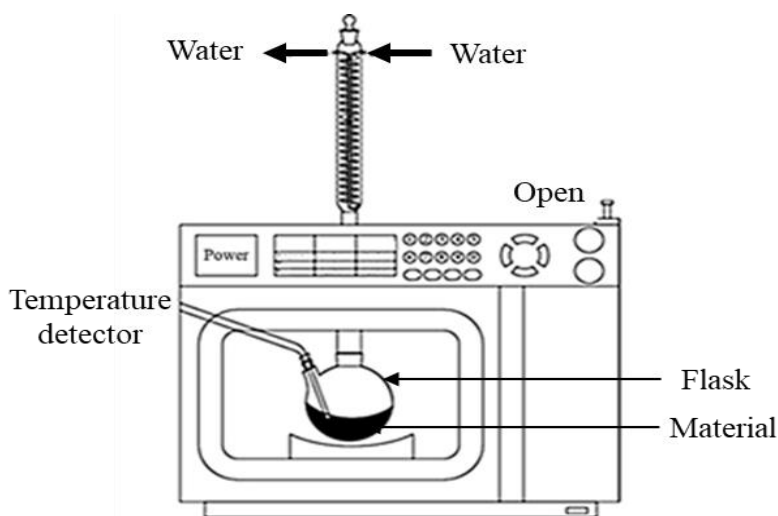
Cocoa shell waste (CSW) used in this study shown in Figure 1(a) were collected from Lembaga Koko Malaysia, Jengka, Pahang, as shown in. The collected sample was cleaned and dried in an oven overnight at 110 °C. Then, the dry cocoa shell was crushed by using a mortar and ground into powder form similar to Figure 1(b) **Figure 2** by using a Retsch grinder with a blade size of 0.08 mm to increase the surface area.



**Figure 1.** Cocoa shell waste in (a) raw form and (b) powder form.

### 2.3. Extraction process

An approximately hundred (100) grams of CSW was extracted in 300 ml of ethanol by using the MAE apparatus shown in **Figure 2**. The extraction was carried out in a closed vessel under ethanolic environment and no evaporation was observed. Extract obtained from each run was filtered with a Whatman N°1 filter paper to remove residue. The filtrate was then dried at 60 °C until the mass remained constant before being kept in a refrigerator at -4 °C for analysis. The extraction of CSW using MAE was done by varying the temperature (50-90 °C), microwave power (400-800 W) and extraction time (6-14 min). The microwave power refers to the maximum power for a particular extraction temperature as the extraction temperature was limited to avoid degradation of product. The sample was then stored at -4 °C to avoid hydrolysis [15] for further analysis.



**Figure 2.** Schematic diagram of the Microwave-Assisted Extraction (MAE) apparatus.

### 2.4. Conventional maceration extraction of $\beta$ -sitosterol

For comparison, conventional maceration extraction was used. In this method, ethanol was added in a volume of about two times than that of the cocoa shells on 100 g of powder dried CSW and allowed to shake on a shaker for three days at room temperature. At the end of three days, the liquid was filtered. The filtrate is dried at 60 °C until its weight remained constant and it was kept in a refrigerator at -20 °C for analysis.

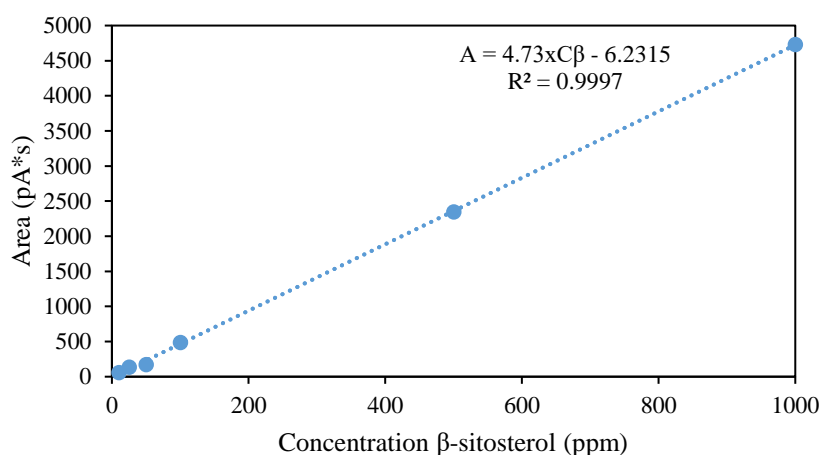
### 2.5. Chromatographic analysis of $\beta$ -sitosterol

The qualitative analysis was carried out using Agilent 6890 SGE BPX5 capillary column (30 m x 0.25 mm inner diameter, 0.25  $\mu$ m film thickness) to identify the significant compounds in the extract. In this analysis, helium was used as carrier gas. The oven temperature was set for the initial temperature at 40 °C (2 min hold) to 300 °C (26 min hold). A split mode of the front inlet was used with a split ratio of 20:1 and the flow rate of helium gas (30 cm/sec) was 1.3 mL/min. Mass Selective Detector (MSD) Transfer Line Heater was 300 °C. The injector temperature was 280 °C, and 1  $\mu$ L of sample extracts was injected.

Quantitative analyses of the  $\beta$ -sitosterol from the cocoa shell were performed by using the Agilent gas chromatography (GC) with a flame-ionization detector (FID) and the HP-5MS column (30m  $\times$  0.32mm i.d.  $\times$  0.25  $\mu$ m film thickness according to the method by Karaoglan and Yilmaz [16] with some modification. The oven of the GC was programmed to hold 2 min at 150 °C firstly, secondly, ramp at 30 °C/min up to 220 °C and hold for 2 min, and finally ramp at 20 °C/min until to 300 °C and hold for 9.7 min. Next, 1  $\mu$ L samples were injected at 250 °C in the splitless mode by using auto-injector and auto-sampler. Helium was used as the carrier gas at a constant flow rate of 1.8 mL/min.

The stock solution of  $\beta$ -sitosterol was prepared by dissolving its exact mass into hexane to give a final concentration of 1000  $\mu\text{g/mL}$ . The solution was then diluted by using the same solvent to prepare six standard solutions at concentrations of 10 to 1000  $\mu\text{g/mL}$  for  $\beta$ -sitosterol, respectively. The experiment was performed in triplicate and the phytosterol ( $\beta$ -sitosterol) yield result was presented as an average value. The data obtained were plotted in a calibration curve as depicted in Figure 3 and fitted to linear trend line as regressed into Equation (1) with  $R^2 = 0.9997$  where  $A$  and  $C_\beta$  are peak area and concentration  $\beta$ , respectively. This calibration curve was used to determine the unknown concentration of  $\beta$ -sitosterol in the tested CSW.

$$A = 4.73C_\beta - 6.2315 \quad (1)$$



**Figure 3.** Calibration curve of  $\beta$ -sitosterol.

## 2.6. Phytosterol characterization

### 2.6.1. Salkowski test

Salkowski test is a type of qualitative analysis to test the presence of steroids and phytosterol in the extracted sample. The concentrated extracted sample was measured for 1 mL and transferred to a small test tube. Then, 2 mL of chloroform and few drops of concentrated sulphuric acid were added to the solution.

### 2.6.2. FTIR analysis

FTIR analysis was performed to examine the presence of a functional group of the  $\beta$ -sitosterol. Two milligrams of sample was applied directly on the diamond and tightened with an FT-IR sample holder as described by Ahmed et al. [17]. For this analysis, the propylene glycol spectrum was used as a background. Sample reading was performed in 32 scans at a resolution of 4  $\text{cm}^{-1}$  at the middle-IR range 4000-600  $\text{cm}^{-1}$ . After that, the results were compared to spectra from several references in order to identify the critical peaks.

## 2.7. Statistical analysis

Triplicate determinations, mean, and standard deviation were calculated. The calibration curve of the standard was obtained for concentration versus absorbance.

## 3. Results and discussion

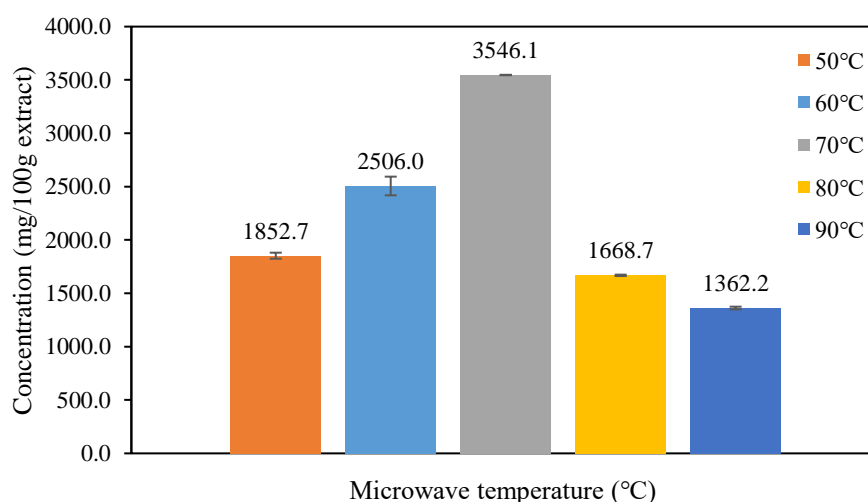
In this work,  $\beta$ -sitosterol from cocoa shell waste was extracted by the MAE technique. Ethanol was used as an environmentally friendly solvent to produce phytosterol compared to other solvents such as

petroleum ether and chloroform [18][12]. The experiment was run at different extraction times and power to get a high yield of phytosterol ( $\beta$ -sitosterol) once at a time (OFAT).

### 3.1. Effect of microwave extraction time

The effect of different microwave extraction time on the concentration of the  $\beta$ -sitosterol was investigated from 6 to 14 min by using 400 W power. Each extraction time was tested using a different experimental set-up. Based on the results shown in Figure , it is clear that the concentration of  $\beta$ -sitosterol increased with the increase of extraction time, and the maximum concentration was revealed at 10 min. The declination after the ultimate peak of  $\beta$ -sitosterol extraction using the same method from *Archindendron pauciflorum*, *Parkia speciosa*, and *Leucaena leucocephala* legume pod was also reported [19].

The initial increment might due to an increase in the penetration of solvent in the sample matrix [13]. The decrease in concentration after 10 min extraction time might be a result of over-exposure or overheating of the sample matrix leading to the thermal degradation of significant chemical constituents in the sample. This data was supported by Gandhi et al. [20] stated that contact time with the extraction system might compromise the chemical structure of the metabolite, resulting in a more significant degradation effect on polar compounds due to the microwave energy effect. It shows that shorter extraction times are required when the microwave technique is employed. Based on these results, an extraction time of 10 min was identified as the optimum extraction of  $\beta$ -sitosterol from cocoa shell waste and was used for the other sets of the experiment.



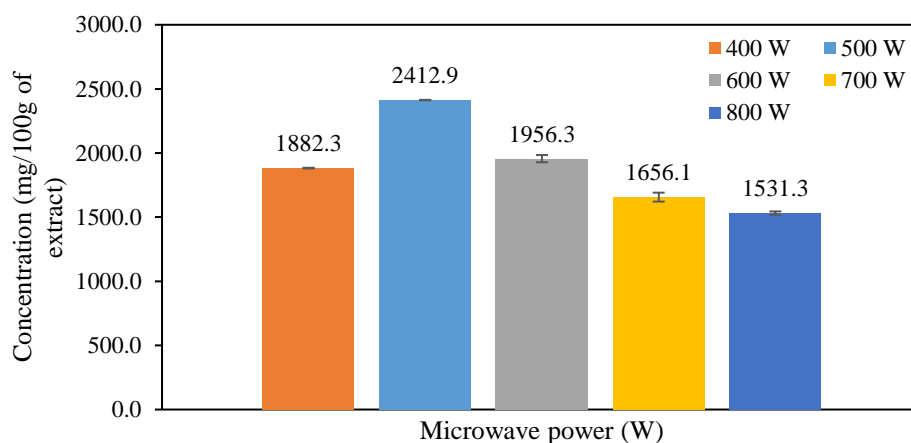
**Figure 4.** Effect of extraction time on the  $\beta$ -sitosterol concentration in cocoa shell extract.

### 3.2. Effect of microwave power

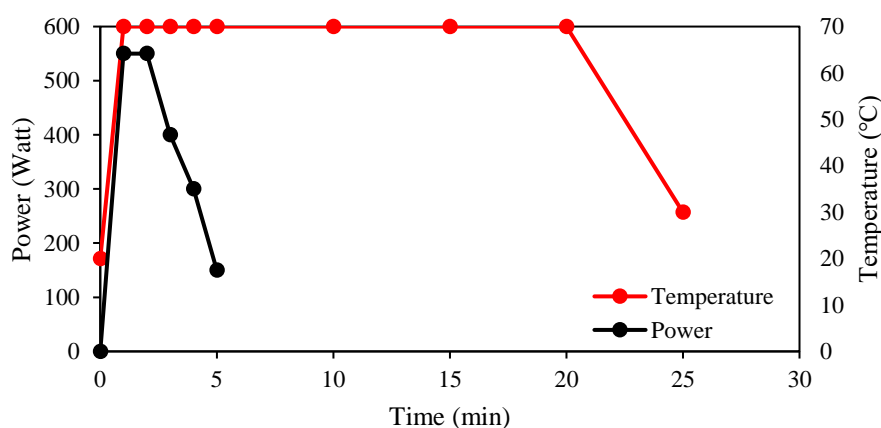
Figure 5 shows the effect of maximum microwave power on the  $\beta$ -sitosterol concentration of cocoa shell from 400 W to 800 W under a fix microwave time of 10 min at a microwave temperature of 60 °C. An increase in microwave power from 400 W to 500 W causes an increase to the concentration of  $\beta$ -sitosterol. Afterward, the concentration declined as the microwave power increased and 500 W is the optimum power.

The microwave setting limited the power to maintain the desired temperature. Previous studies revealed that generally when microwave power was increased, the yield of an extract increased [11]. The reason for this could be associated with the rapid generation of heat inside the immersed CSW with the absorption of microwave energy. Then, the subsequent formation of higher pressure gradient which facilitated the solvent to solubilize solutes and improved the matrix wettings and penetration [21]. The reason for the optimum power might be related to efficiency of heating. Figure 6 shows the power and oven temperature against time. The power was set at 550 W but the power changes with time to regulate

the oven temperature. When the power was increased the time elapsed to increase the temperature to the desired value became shorter. Since this extraction did not involve any stirring, the quick heating at higher power for the same temperature setting will deteriorate the efficiency of the heating.



**Figure 5.** Effect of microwave power on the  $\beta$ -sitosterol concentration in CSW extract.



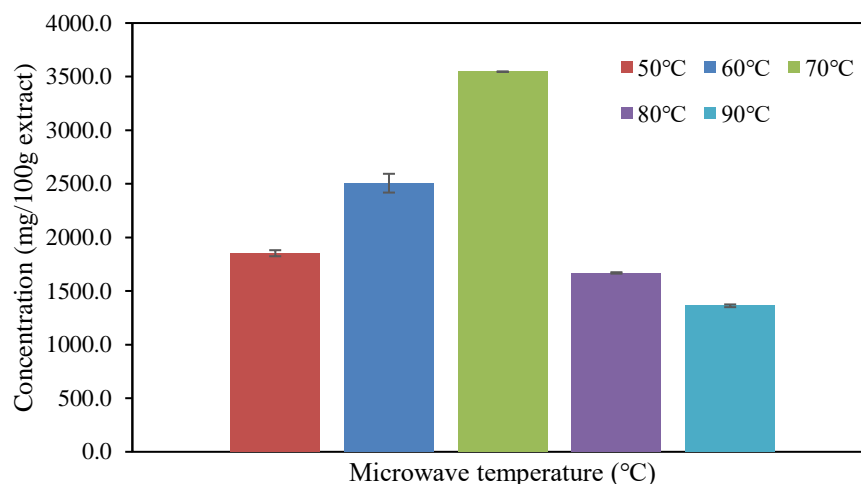
**Figure 6.** Interaction between microwave power and temperature on ethanol extraction.

### 3.3. Effect of microwave temperature

One of the most investigated parameters in microwave-assisted extraction technique is temperature since it is the key factor that contributes to the increase of extract concentration in the process. The optimum extraction time and microwave power from the previous tests were used to study the effect of microwave temperature on the concentration of  $\beta$ -sitosterol from cocoa shell waste as shown in Figure 7. It can be clearly seen that the different microwave temperature ranged from 25 °C to 80 °C changed the sterol extract. The highest concentration of  $\beta$ -sitosterol was obtained at a temperature of 70 °C (3546.1 mg/100 g), followed by 60 °C (2506 mg/100 g) and 50 °C (1852.7 mg/100 g) while the lowest yield was at the temperature of 90 °C (1362.2 mg/100 g). The concentration increased from 1852.7 mg/100 g to 3546.1 mg/100 g when the temperature was raised initially from 50 °C to 70 °C. However, the amount of concentration is reduced drastically when further increase in temperature to 70 °C to 90 °C [13].

In general, the suitable temperature will result in better solute solubility in the extraction solution. The increase in concentration could be associated with the migration of dissolved ions that facilitate

high collection of target compounds [22]. Owing to these observations, the optimum microwave temperature was apparently 70 °C in this study.



**Figure 7.** Effect of microwave temperature on the  $\beta$ -sitosterol concentration in CSW extract.

### 3.4. Comparison of extraction efficiency between MAE and maceration

The optimum values of temperature, power and radiation time of the microwave onto the extraction of  $\beta$ -sitosterol from the CSW using ethanol was compared to extraction by maceration. The results of the concentration of  $\beta$ -sitosterol from both extraction are shown in Table 1. The extraction time of 10 min, 70 °C and 500 W by MAE yielded the highest amount of  $\beta$ -sitosterol, achieving 3546.1 mg/100g. Meanwhile, the quantification of  $\beta$ -sitosterol in the extract obtained by 72 h maceration without heat and without stirrer was 3138.1 mg/100g, which was substantially lower than the amount achieved in the extraction by MAE with a shorter extraction time.

**Table 1.** Concentrations of  $\beta$ -sitosterol with optimum values at 70 °C, 500 W and 10 min of MAE and extraction by maceration of 72 h.

Method	Extraction time (min)	Extraction time (h)	mg of $\beta$ -sitosterol/100g
Microwave-assisted extraction (MAE)	10	-	3546.1 $\pm$ 2.1 <sup>1</sup>
Maceration	-	72	3138.1 $\pm$ 6.5

<sup>1</sup>Each value is the average of three analyses  $\pm$  standard deviation.

Cavdar et al. [23] also stated the mechanism by which MAE uses microwave energy to facilitate the partition of analytes. This caused the  $\beta$ -sitosterol to dilute and dissolve in the solvent in a faster way. MAE method is known as unique effective mechanism as a noncontact energy source to produce heat in the extraction matrix for effective heating, faster thermal energy transfer, less thermal degradation, higher extraction selectivity, a faster start of the extraction process (automated), and a higher yield in a shorter time, compared to conventional extraction methods such as maceration [24]. The maceration method consists of the penetration of the solvent into the cell, causing dehydration or rupture of cell membranes when exposed to a prolonged extraction time [25].

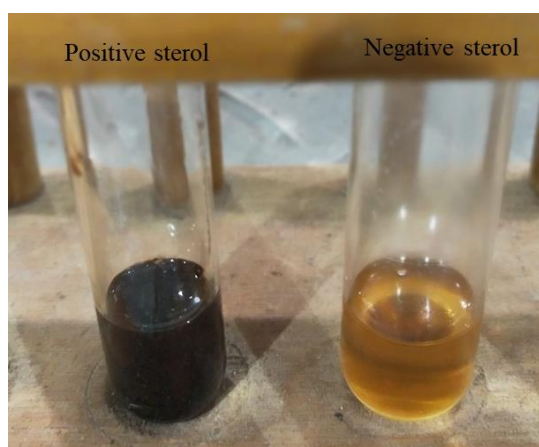


The quantification results obtained can be compared to a research work, in which extraction by phytosterols content in cocoa butter from three different extraction methods. There was reporting cocoa butter extracted using ultrasonic extraction obtained the highest  $\beta$ -sitosterol content (2030 mg/100g of extract), supercritical fluid extraction (2001 mg/100g of extract) and soxhlet extraction (1911 mg/100g of extract) [26], less than the amount obtained in our work by the MAE method as per the maceration technique. Our results are the first work reported in the extraction of  $\beta$ -sitosterol from CSW by the MAE method. From the data of extraction using other methods for different plant sources, the MAE method is a good and safe technique for the extraction of active metabolites from plants.

### 3.5. Characterization of $\beta$ -sitosterol from cocoa shell waste

#### 3.5.1. Salkowski test

In this study, the Salkowski screening test was particularly used to detect the steroid compounds in cocoa shell waste. After the addition of chloroform and sulfuric acid into the extracted sample, chloroform layer appeared dark red and acid layer showed greenish yellow fluorescence indicating the presence of sterols [5,27,28]. Chemical tests were used to identify phytosterols compounds in cocoa shell waste and the results in Figure 8 exhibits Salkowski reaction tests which proved the presence of sterols in ethanolic extract of cocoa shell waste.

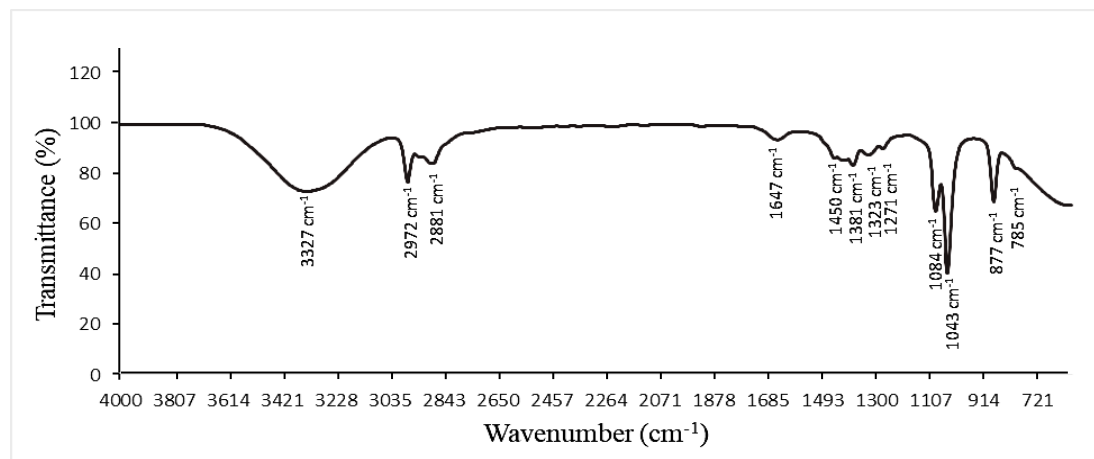


**Figure 8.** Salkowski test on ethanol extract of cocoa shell waste.

#### 3.5.2. Identification by fourier transform infrared spectroscopy (FTIR)

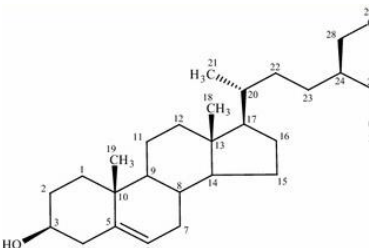
In the FT-IR analysis, samples of the MAE extractions were taken at 10 min, 70 °C and 500 W. The representative FTIR spectra of solution from MAE samples was shown in Figure 9. Sterol nucleus was used as the indicator for the sterol compound from the positive test for sterol given by compound  $\beta$ -sitosterol, it is assumed to be a compound containing sterol nucleus. The light of peak at 3550-3200  $\text{cm}^{-1}$  was excited by intermolecular hydrogen bonding (O-H stretching vibrations). The intensity of this peak range was found to be broad and strong peak. In the range of 2925  $\text{cm}^{-1}$  to 2854  $\text{cm}^{-1}$ , peaks can be seen probably related to C-H stretching vibrations of aromatic ring [29]. Peaks at range 1645  $\text{cm}^{-1}$  to 1544  $\text{cm}^{-1}$  are present in both samples that contributes to C=C stretching vibration of aromatic ring. Besides that, 1450  $\text{cm}^{-1}$  to 1323  $\text{cm}^{-1}$  is from the bending vibration of ethanol. On the other hand, there was one peaks in the range 992  $\text{cm}^{-1}$  to 680  $\text{cm}^{-1}$ , which attributed to angular deformation of C-H of aromatic ring. This data is comparable with the spectral data for  $\beta$ -sitosterol reported by Zeez, Abaas, & Kadhim [30] from Table 2. Kaur et al. [31] reported the presence and characterization of  $\beta$ -sitosterol in *Withania somnifera* L. in which broad peak was obtained in IR Spectra at 3700-3600  $\text{cm}^{-1}$  (OH group), 2790  $\text{cm}^{-1}$  (C-H stretch), 2995  $\text{cm}^{-1}$  show resonance with the C=O group. Similar results were reported by Ododo et al. [32] in which IR peaks were obtained at 3426  $\text{cm}^{-1}$ , 2936  $\text{cm}^{-1}$ , 2832  $\text{cm}^{-1}$ , 1596  $\text{cm}^{-1}$  and 1032  $\text{cm}^{-1}$ .

<sup>1</sup>. From the qualitative point of view, the cocoa shell waste spectra produced by CSW after extract with MAE using ethanol solution from Figure 9 resembles the peak frequencies observed for  $\beta$ -sitosterol. The FT-IR spectra also showed the presence of one C=C in the structure, characteristic of  $\beta$ -sitosterol (Figure in the Table 2).



**Figure 9.** FTIR profile spectra of ethanolic extract from CSW using MAE.

**Table 2.** Characteristic FTIR Absorption of  $\beta$ -sitosterol [33].

$\beta$ -sitosterol	Peak ranges	Functional groups
	3500-3200	Intermolecular hydrogen bonding (O-H stretching vibrations)
	2968	Asymmetrical stretching (CH <sub>3</sub> ) of methyl group
	2926	Asymmetrical stretching (CH <sub>2</sub> ) of methylene group
	2855	Symmetrical stretching of (CH <sub>2</sub> ) of methylene group
	1659-1641	C=C stretching vibration
	1580	Bending vibration of OH
	1461-1374	Bending vibration of isopropyl
	1313	Bending vibration of C-O of 2° alcohol

### 3.5.3. GC-MS analysis

Further analysis by GC-MS has confirmed the identification major peaks representing volatile compounds in sample of MAE onto the extraction of CSW using aqueous ethanol. The assigned peaks were detected by MS detector and the total ion chromatogram of each peak was compared and matched with the library search report based on National Institute of Standards and Technology (NIST) library database (Library ID: NIST02.L). Thereafter, the peaks which matched with NIST database in the quality 80% and above were considered as the particular compounds.

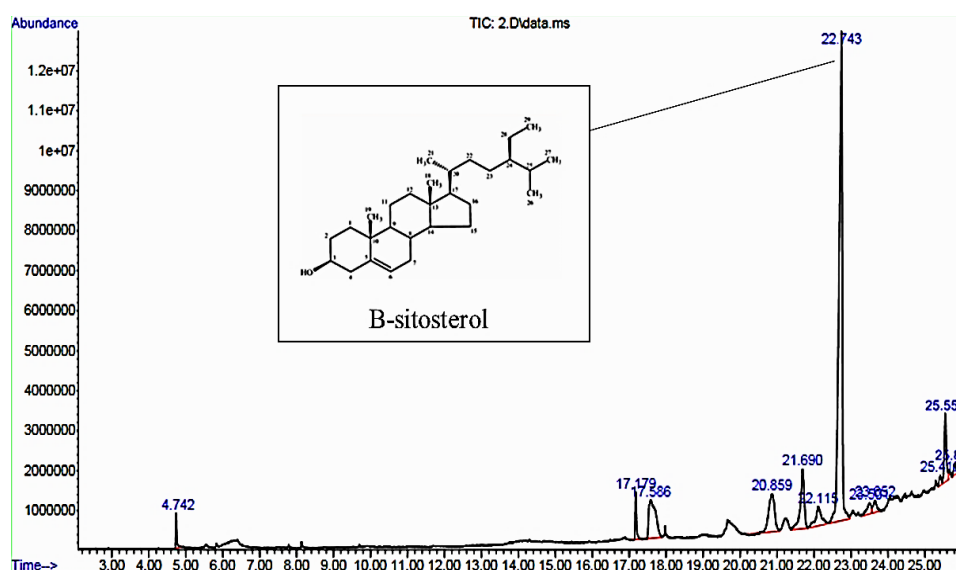
Nine different phytochemicals was identified in ethanol extract as shown in Table 3 and Figure 10. Among the nine identified phytochemicals in the ethanol extract using MAE, there is a  $\beta$ -sitosterol compound which is the major dominant compound that possess antidiabetic (Karan et al., 2012), antioxidant, anticancer, anti-inflammatory, anti-pyretic and anti-stress agent and cancer preventive [4,5]. This compound appears through the peak 6, at a retention time of 22.74 min with the percentage area of 49.54% (Table 3). Meanwhile, theobromine compound appears through peak 2 at a retention time of 17.59 minutes, with area 8.62 which is the predominant compound. Besides, the compound of caffeine

appears through the peak to 1 with a retention time of 17.18 min, with area of 1.88%. According to Martínez-Pinilla, Oñatibia-Astibia, & Franco, theobromine may act as antitumoral, anti-inflammatory or cardiovascular protector molecule without the undesirable side effects. Besides, this compound when combined together with caffeine was formed as blockers of adenosine receptors which are G-protein-coupled receptors that sense the presence of extracellular adenosine [6].

At the peak 4 and 5, stigmasterol which at peak 4 (retention time of 20.86 min), the percentage area was 7.25% while at peak 5 (retention time of 21.69 min) was 7.16%. Stigmasterol is a compound that has a biological activity as anti-osteoarthritic, antioxidant, anti-hypercholesterolemic, cytotoxicity and anti-tumor [31]. It is worth mentioning that the GC-MS spectrum of extracts showed a clear evaluation of the volatile fraction of CSW by gas chromatography coupled to mass-spectrometry (GC-MS), revealed a well diversity composition, with nine compounds detected, where phytosterol such as  $\beta$ -sitosterol and stigmasterol were predominante. Among them,  $\beta$ -sitosterol was found to be major compound while others were found to be present in trace amount. All these advantages demonstrated that MAE was a better method to obtain  $\beta$ -sitosterol from CSW by solid-liquid extraction. The scientific literature reports that one of the benefits of MAE is the greater amount of the compound of interest obtained, compared to conventional extraction methods, such as maceration [18].

**Table 3.** Chemical composition assessed by GC-MS of ethanolic extract from cocoa shell using MAE.

Peak	Retention time (min)	Name of compound	Area (%)
1	17.18	Caffeine	1.88
2	17.59	Theobromine	8.62
3	17.98	Palmitic acid	0.72
4	20.86	Stigmasterol	7.25
5	21.69	Stigmasterol	7.16
6	22.74	$\beta$ -sitosterol	49.54
7	23.20	Docosanoic acid	0.29
8	23.50	Vitamin E	1.54
9	25.82	Oxazolidinone	0.85



**Figure 10.** GC chromatogram by CSW in ethanol extract using MAE method.

#### 4. Conclusion

Cocoa shell waste can be developed as low cost feedstock for  $\beta$ -sitosterol synthesis using Microwave-assisted Extraction (MAE). Based on the one-factor-at-a-time (OFAT), the concentration of  $\beta$ -sitosterol in the cocoa shell waste was 3546.1 mg/100g after extraction. From this study, the most optimum condition for the extraction process of  $\beta$ -sitosterol from cocoa shell waste was 10 min of extraction time using 500 W of microwave power and 70 °C microwave temperature. In this work, we obtained the greater yield of  $\beta$ -sitosterol from MAE from this optimal value, compared to the traditional maceration method. The MAE method can reduced the extraction time using ethanol as an environmentally friendly solvent. This finding may be benefits the healthy food industry to produce phytosterol with high yield and preserve the bioactive and antioxidant content.

#### Acknowledgement

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