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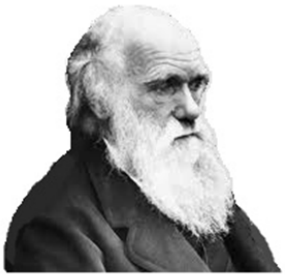

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Development of HPLC method for estimation of β-sitosterol from *Peliosanthes micrantha* hizomes

Do Ngoc Thuy[1], Le Ngoc Hung[1], Nguyen Thi Huong[1,*]

[1] Centre for High Technology Research and Development, Vietnam Academy of Science and Technology, Hoang Quoc Viet 18, 10000, Hanoi, Vietnam

*E-mail address: huong83bm@gmail.com

ABSTRACT

The medicinal plant *Peliosanthes micrantha* of Vietnam has been studied recently. Two components, one of which was β-sitosterol, were isolated. *P. micrantha* rhizomes were first extracted by ethanol (PM-R-ET). β-sitosterol was then isolated as a standard compound. High-performance liquid chromatography method (HPLC) was validated and used for the quantification of β-sitosterol in the extract. We concluded in this study that β-sitosterol content was significant at 0.71 mg/g of fresh *P. micrantha* rhizome extract.

***Keywords*:** *Peliosanthes micrantha*, rhizomes, ethanol, β-sitosterol standard, quantification, HPLC

1. INTRODUCTION

Peliosanthes micrantha Aver.et N.Tanaka, sp. nov. belonging to Asparagaceae was found in central highland Vietnam. According to the indigenous people in Krong No district, Dak Nong province, the rhizomes of *P. micrantha* were traditionally alcohol extracted and has been used to increase physical endurance, and for male impotence (Averyanov *et al.* 2013) [1]. From the ethanol extract of *P. micrantha* rhizomes, Thuy et al tentatively assigned four compounds, namely, pumilaside A, pumilaside C, glycoside J-3, and β-sitosterol by QTOF-MS analysis (Thuy *et al.* 2024) [2]. In one unpublished study, two compounds, pumilaside A and β–sitosterol, were isolated (Thuy *et al.* 2024) [2]. Only a few research of biological activities have been published for the *P. micrantha* species. The *P. micrantha* extract was standardized and showed

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mild DPPH antioxidant activity (Le *et al.* 2024) [3]. This extract was used in a herbal formulation and did not show acute toxicity sign at dose of 17.5 g/kg and sub-chronic toxicity at 400 mg/kg during 28 days on the experimental animals (Le *et al.* 2024) [4].

Pumilaside A was found in the seeds of *Litchi chinensis and shown* to possess potent *in vitro* cytotoxic activity against A549, LAC, Hela, and Hep-G2 cancer cell lines (Xu *et al.* 2010) [5]. The litchi seed's components have been identified to inhibit the growth of breast carcinoma, cervical carcinoma, colorectal carcinoma, lung adenocarcinoma, oral carcinoma, ovarian carcinoma, and pulmonary cells, with the molecular mechanisms acting through decreased levels of cyclin D1, A and B1 and alteration of the Bax: Bcl-2 ratio and activation of caspase 3 (Lin *et al.* 2013) [6]. Pumilaside A was also found in the *Ficus pumila* (Kitajima *et al.* 2000) [7]. The crude extract of *Ficus pumila* showed multiple therapeutic activities, including antioxidant, anti-inflammatory, antibacterial, antitumor, hypoglycemic, and cardiovascular protective effects. For cancer cells, the extract presented an IC50 cytotoxicity value of 131 µg/mL in the human leukemia cancer cell line (MT-4) while its 85% ethanol aqueous solution was more potent against Hela, MCF-7, and A549 cell lines (Qi *et al.* 2021) [8].

β-sitosterol was found in several medicinal plants including *Berberis vulgaris* (Anwar *et al.* 2020) [9], *Solanum xanthocarpum* (Khanam *et al.* 2012) [10], *Stylochiton lancifolius* (Pateh *et al.* 2009) [11], and *Ficus pumila* leaves and stems (Kitajima *et al.* 1998; Xiao *et al.* 2015) [12, 13], etc. β-sitosterol prevents many types of malignancies, lowers levels of low-density lipoprotein, lowers the risk of coronary artery disease, heart attacks, atherosclerosis, and potency to be beneficial against breast, prostate, colon, lung, stomach, and leukemia (Durrani *et al.* 2024) [14]. The β–sitosterol from the hexane extract of the dried roots of *Mondia whitei* showed sexual activities such as the increase of the mounting frequency, penile erection, and ejaculation latency at the dose of 10 mg/kg in rats (Watcho *et al.* 2012) [15].

Chromatographic techniques contribute significantly to the identification, separation, and characterization of bioactive compounds from plant sources. HPLC is a powerful technique for rapid qualitative and qualitative analysis of non-volatile classes of compounds such as phenolics, terpenoids, and alkaloids. A variety of HPLC techniques such as conventional and reverse phase –HPLC has been reported for the quantification of β-sitosterol in herbal extracts (Patidar *et al.* 2024; Lee *et al.* 2018) [16, 17]. Thus, the aim of this study was to develop and validate a reliable HPLC analysis method for the quantitative estimation of β-sitosterol from the *P. micrantha* rhizome extract.

2. MATERIAL AND METHODS

2. 1. Chemicals and reagents

All analytical reagents, methanol, ethanol, n-hexane, ethyl acetate, diclomethane, anhydrous sodium sulfate, and water; and HPLC grade acetonitrile, methanol, and formic acid (≥98%) were obtained from Fisher (USA). Silica gel chromatography columns (40-63 µm and 63-200 µm) were from Fisher (USA), and a reversed-phase YMC-Triart C18 column (5 µm × 250 × 4.6 mm-TA12S05-2546WT) was from YMC (USA). Whatman filter paper was from Sigma Aldrich (USA).

2. 2. Plant material

The rhizomes of *P. micrantha* were collected in Dak Nong Province, Vietnam in October

2021, and identified by Dr. Nguyen Sinh Khang, Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology (VAST). The voucher specimen (PM-R) has been lodged at the Center for High Technology Development, VAST. The fresh rhizomes of *P. micrantha* were washed, sliced, dried in an oven at 70 °C, and ground into fine powder using an agate mortar. The dried powder was finally sifted using sieves with mesh sizes ranging between no.100 and no. 120, aimed at isolating particles with dimensions falling within the 0.125-0.150 mm range.

2. 3. Extract preparation

Dried powder of the rhizomes of *P. micrantha* (14.000 kg) was extracted with 95% ethanol (90.0 L × 3 times) at room temperature in a 150L ultrasonic extractor (20 kHz, 1 kW) as described previously giving PM-R-ET extract (Thuy *et al*. 2024) [2]. The combined extract was concentrated to dryness under reduced pressure, yielding a residue of PM-R-ET (617.25g, brown solid). The extraction was performed 10 times to obtain 6151.24 g of the extract. The sample was stored at -20 °C for future use.

2. 4. Isolation of pure β–sitosterol as standard

β–sitosterol isolation was carried out as described in unpublished data. The first PM-R-ET extract was reconstituted in ethanol 96% and sequentially partitioned with n-hexane, ethyl acetate (EtOAc) to give the n-hexane portion H0 (116.2g), EtOAc portion EA0 (195.4g) and ethanolic portion E0 (213.6g). For pure β–sitosterol isolation as standard, the EtOAc-soluble EA0 (195.4g) portion was further fractionated on silica gel chromatography column (CC 63–200 μm) eluting with gradient elution of increasing polarity solvent mixture of n-hexane–EtOAc (i.e. 100:1→ 90:10 → 80:20 → 60:40 → 40:60 → 20:80 → 0:100, 10.0 L each) to obtain 7 fractions (EA1 → EA7). The EA6 fraction obtained from the solvent mixture of n-hexane–EtOAc of 20:80 was separated on the CC column (40-63 μm) and then eluted by n-hexane–EtOAc (5: 1, v/v) to give 5 subfractions (EA 6.1 → EA 6.5). Then, the EA6.2 subfraction was sequentially eluted by diclomethane-methanol (10:1, v/v) to give 4 subfractions (EA6.2.1 → EA6.2.4). Finally, the EA6.2.2 subfraction was purified on a reversed-phase YMC-Triart C18 column (5 μm × 250 × 4.6 mm-TA12S05-2546WT) by methanol–water (4: 1, v/v) to obtain a colorless solid (E2) (0.031g) after concentration under vacuum. Other extracts were used to isolate the E2 in the same manner, giving 0.305g in total.

2. 5. Qualitative UHPLC-QTOF analysis of β–sitosterol

Sample analysis by the UHPLC-QTOF instrument was investigated with the procedure described elsewhere (Thuy *et al*. 2024) [2]. 100.0 mg of each sample (β-sitosterol and extract PM-R-ET) was accurately weighed into a tube with a cover, and 2.0 mL methanol-water (8:2, v/v) solvent was added. The sample was ultrasonicated for 10 min, then filtrated through a 0.45 (μm) filter membrane before injecting for analysis. Sample analysis was performed on an ExionLCTM UHPLC system (AB SCIEX, USA) consisting of ExionLC degasser, AC pumps, AC autosampler, controller, and AC column oven. Samples were analyzed on a Hypersil GOLD C18 column (150 × 2.1 mm, 3μ) (Thermo Fisher Scientific, USA). The mobile phase, water containing 0.1% formic acid (A) and acetonitrile containing 0.1% formic acid (B), was run at a flow rate of 0.4 (mL/min) at room temperature. The gradient programming was as follows: 0–4 min, 2-20% B; 4-30 min, 20-68% B; 30-32 min, 68-98% B; 32-40 min, 98% B. Sample

injection volume was 5.0 (µL). An X500R QTOF mass spectrometer (AB SCIEX, USA) with a Turbo V ion source was coupled with the UHPLC system. Mass data were acquired in both negative (NEG) and positive (POS) Electrospray Ionization (ESI) modes. The ESI-MS conditions were set as follows: the ion source temperature, 500 °C; curtain gas, 30 psi; nebulizer gas (GS 1), 45 psi; heater gas (GS 2), 45 psi. For the TOF MS scan, the mass range was set at m/z 70–2000. For the TOF MS/MS scan, the mass range was set at m/z 50–1500. For the NEG mode, the ion spray voltage was set at −4.5 kV, the declustering potential (DP) was −70 V, the collision energy (CE) was performed at −20 eV and the collision energy spread (CES) was 10 eV. For the POS mode, the ion spray voltage was set at 5.5 kV, the DP was 80 V, the CE was 20 eV and the CES was 10 eV. All the obtained data were processed by SCIEX OS software version 1.2.0.4122 (AB SCIEX, USA).

2. 6. Quantitative HPLC-DAD analysis of β–sitosterol

Preparation of stock and working standard solution: 10 mg of β-sitosterol standard was weighed and transferred into a volumetric flask of 10 ml, and the volume of the solvent was adjusted with the methanol up to the mark to obtain a standard stock solution of 1000 ppm. Then 1 ml of β–sitosterol was taken from the stock solution and diluted up to 10 ml to prepare working standard solutions. Different concentrations of the solutions (0.5, 1.0, 1.5, 2.0, 2.5 ml) from standard were transferred into 10 ml volumetric flasks and made up the volume to 10 ml with the solvent. Standard drug solutions of 5, 10, 15, 20, 25 µg/ml concentration were used.

Preparation of plant samples: 2 mg of plant extract PM-R-ET was taken in a volumetric flask of 10ml and diluted with methanol up to marks. This solution was filtered with the help of Whatman filter paper and again added with a similar solvent up to marks to obtain 1000 µg/ml concentration. Finally, the resulting solution was filtered again through Whatman filter paper no. 41 and sonicated for 10min.

Chromatographic analysis of β–sitosterol: The HPLC-DAD method was developed for the quantification of β–sitosterol in ethanol extract of *P. micrantha* using the same protocol as described for UHPLC-QTOF analysis (Thuy *et al.* 2024) [2]. Sample analysis was performed on an ExionLCTM HPLC system (AB SCIEX, USA) consisting of ExionLC degasser, AC pumps, AC autosampler, controller, AC column oven, and UV detector. Samples were analyzed on a Symmetry C18 Column (5 µm, 3.9×150 mm) from Waters. The column temperature was 30 °C. Mobile phase: acetonitrile and methanol (50:50 v/v). The flow rate was 1.0 ml/min. In this method, a sample was introduced into the HPLC system in a small volume of 20 µl and developed a chromatogram. Lambda max (λmax) referred to as the highest wavelength in the absorption spectrum was determined, where the absorbance was high. For the measurement of λmax, 50 mg of β–sitosterol was taken and dissolved into 50 ml of methanol to prepare a single aliquot of 10 µg/ml in methanol for measuring the λmax. By this method, we can determine the highest absorbance (λmax) of β–sitosterol in different wavelengths. Identification of β–sitosterol component in each sample was determined by its peak value and retention time under a similar condition with β–sitosterol standard. The calibration curve has been plotted between the peak area and concentration of the respective working standard solutions. The quantity of β–sitosterol was calculated by using the calibration curve through the peak area.

Method validation. Following ICH criteria, the analytical procedure was validated (Nittaya *et al.* 2022) [18]. The validation of the HPLC analytical method encompasses several parameters,

including specificity and sensitivity, linearity, precision, accuracy, range, limit of detection (LOD), and limit of quantification (LOQ).

Specificity. The method's specificity was assessed by introducing 20 µl solutions of β-sitosterol standard, sample, and blank into the HPLC system for the selection of measurement wavelength.

Accuracy. The method accuracy was assessed by carrying out successive measurements (n = 5) of three different concentrations of β-sitosterol standard (5, 10, and 20 µg/ml) using the developed method. The accuracy was determined by calculating the percent recovery (% recovery) of the β-sitosterol standard. To be considered acceptable, the mean recovery should fall within the range of 97.0-103.0 %.

Precision. The precision of the HPLC analytical method was evaluated through two measures: repeatability (within-day) and intermediate precision (between-day). Within-day precision was determined by calculating the percent relative standard deviation (%RSD) for β-sitosterol standard measurements conducted on the same day. On the other hand, between-day precision was assessed by comparing the measurements conducted on five different days, and the standard deviation (SD) and %RSD were calculated for this comparison.

Linearity. Linearity was confirmed by forming standard solutions at five different concentrations. Working standards were created for β-sitosterol within a range of 5 to 25 µg/ml. In the HPLC system, 20 µl of each concentration were injected. Regression analysis was then performed on the peak area plotted against the concentration to determine the calibration equations and coefficient of determination.

Limit of detection (LOD) and limit of quantification (LOQ). LOD is the smallest concentration of β-sitosterol standard in a sample that can be reliably detected. In this study, the LOD was determined by achieving a signal-to-noise ratio of 1:3. On the other hand, LOQ refers to the lowest concentration of β-sitosterol standard that can be determined with acceptable precision and accuracy. In our study, the LOQ was determined based on a signal-to-noise ratio of 1:10.

3. RESULT AND DISCUSSION

3. 1. Preparation and quantitative analysis of standard compound

Our described standard isolation protocol from PM-R-ET has given a compound in the form of colorless prismatic crystals. The purity of the isolated compound was estimated by HPLC-QTOF analysis in negative mode. In Figure 1, the chromatogram obtained showed that the isolated compound was pure. However, its exact purity was not determined. The structure of the isolated compound was confirmed as β-sitosterol by ESI-HRMS-QTOF spectra displaying an adduct ion $[M-H]^-$ at m/z 413.3247 (calculation 413.3783) on negative mode and an adduct ion $[M+Na]^+$ at m/z 437.2359 (calculation 437.2385) on positive mode (Figure 2). The β-sitosterol obtained was used as standard without further purification.

3. 2. HPLC-DAD method validation for β-sitosterol

Wavelength specificity. The UV spectrum of the standard compound, β-sitosterol, displayed its highest absorbance at 204.15 nm as presented in Figure 3. Then, all the chromatograms were monitored in the UV detector at the chosen wavelength of 204 nm.

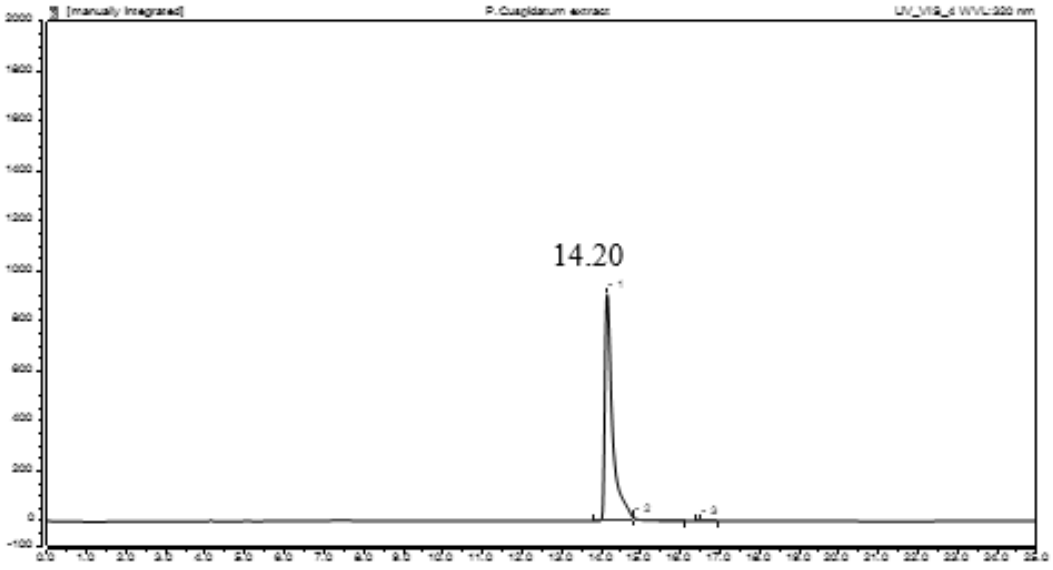

Figure 1. HPLC-QTOF chromatogram of β-sitosterol standard in negative mode.

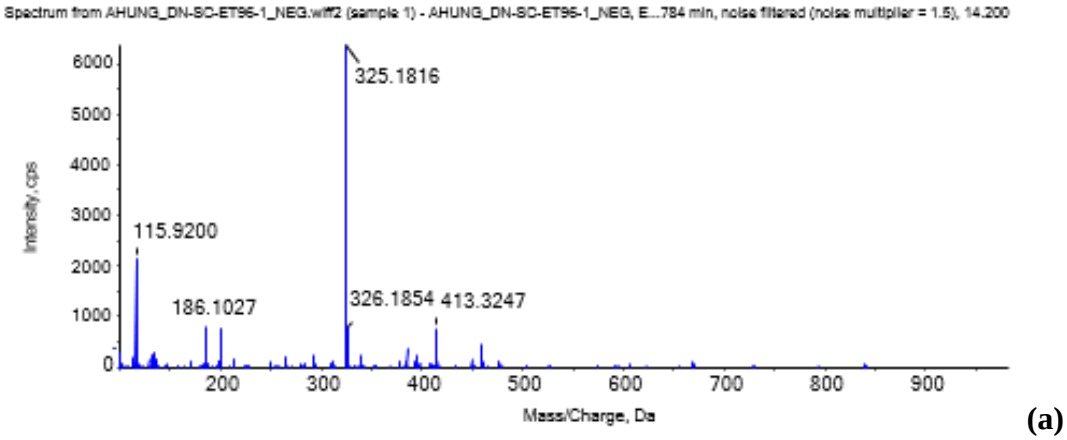

Figure 2. HRMS spectrum of β-sitosterol ion **(a)** $[M-H]^-$, m/z 413.3247, negative mode at 14.20 min. and **(b)** $[M+Na]^+$, m/z 437.2359, positive mode at 10.093 min.

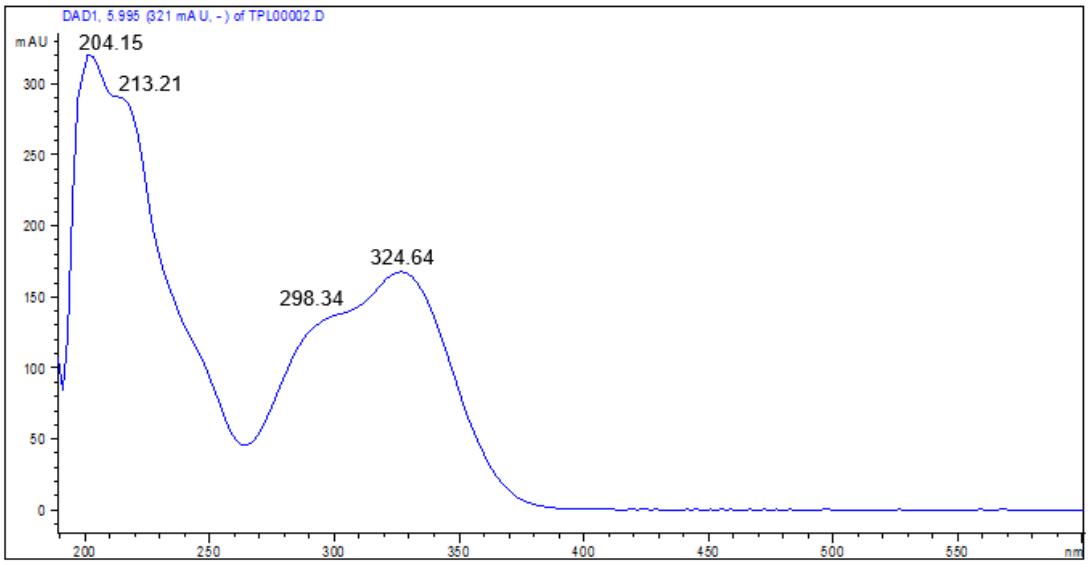

Figure 3. Determination of λmax for β-sitosterol quantification.

***Precision*.** The HPLC peaks of β-sitosterol by 5 measurements of 5 µg/ml standard solutions showed good within-day repeatability (RSD = 0.46%) were presented in Figure 4. All the detailed results are shown in Table 1. The obtained % RSD values for both within-day and between-day measurements of β-sitosterol demonstrated excellent precision, with values below 1%. The acceptable precision threshold was set at 2.0% for the % RSD.

***Accuracy*.** The accuracy of the method was assessed by calculating the % recovery, and the results are summarized in Table 2. The mean percentage recovery of β-sitosterol was observed to range from 99.76% to 100.25%. The percentage recovery results fell within the acceptable limits of 97.0-103.0 %.

***Linearity*.** The linearity of the β-sitosterol analysis was assessed by conducting linear regression analysis. During the analysis, the calibration curve of β-sitosterol was built over the standard concentration range of 5, 10, 15, 20, 25 µg/ml. The linear regression equation for the curve was:

$y = 94.39x - 30.43$

where y is the calculated peak area mean of β–sitosterol and x is the β–sitosterol concentration (µg/ml).

The calibration curve exhibited excellent linearity, with coefficient of determination (R^2=0.99) values exceeding 0.997. The high R^2 value, close to 1.00, indicates a strong linear relationship between the concentration of each compound and its corresponding peak area. The calibration curve for β-sitosterol can be observed in Figure 5.

Limit of Detection (LOD) and Limit of Quantification (LOQ). The results showed the LOD for β-sitosterol was 1.01 µg/ml and the LOQ of these compounds were 0.96 µg/ml.

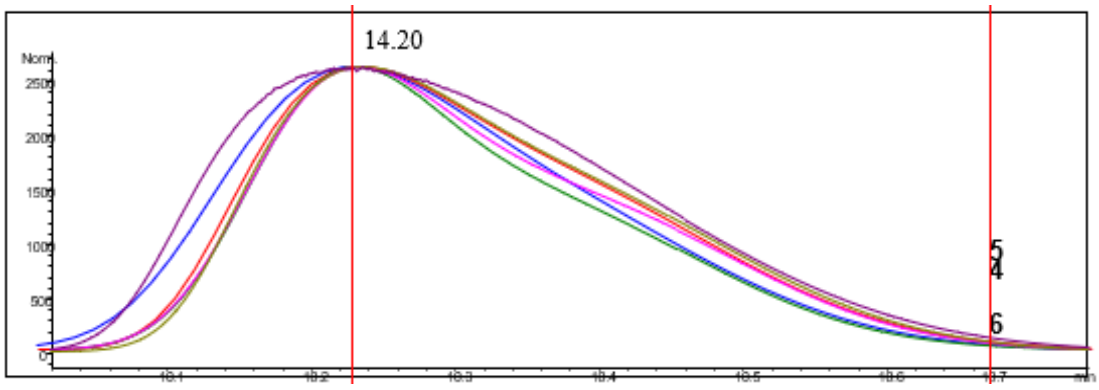

Figure 4. Within-day repeatability at 5 µg/ml (n=5).

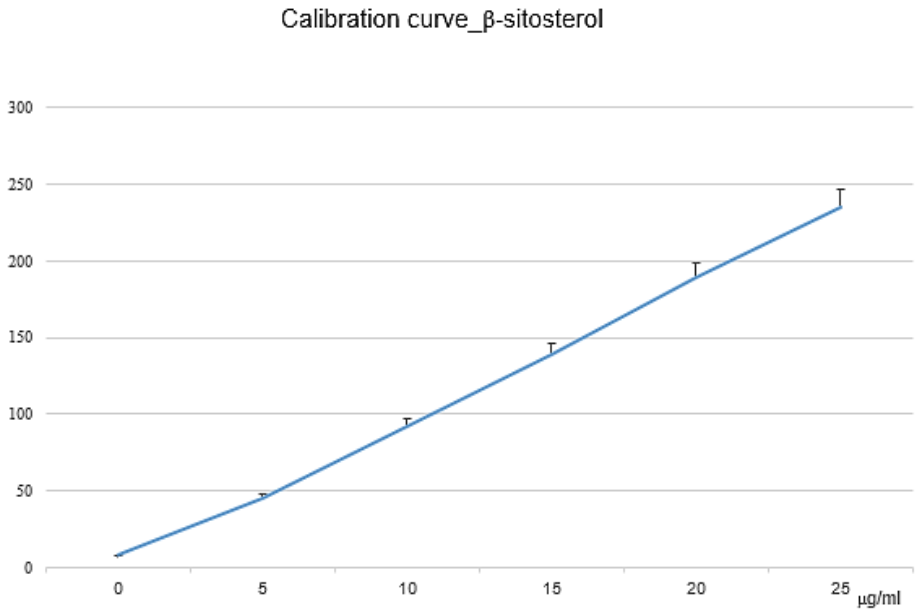

Figure 5. Linear calibration curve for β-sitosterol.

Table 1. Within-day and between-day precision validation of β-sitosterol standard.

Compounds	Concentration (µg/ml)	Within-day repeatability (n=5)		Between-day precision (n=5)	
		Area of peak (mAU)	%RSD	Area of peak (mAU)	%RSD
β-sitosterol	5	292.14 ± 1.34	0.46	294.05 ± 2.19	0.74
	10	587.38 ± 1.66	0.28	588.40 ± 1.71	0.29
	20	1147.19 ± 1.62	0.14	1147.91 ± 2.56	0.22

Table 2. Percentage accuracy validation of β-sitosterol standard

Compounds	Concentration (µg/ml)	Concentration found (µg/ml) (mean ± SD)	Recovery (%) (n=5)
β-sitosterol	5	5.008 ± 0.046	100.14 ± 0.91
	10	9.978 ± 0.048	99.76 ± 0.47
	20	20.051 ± 0.031	100.25 ± 0.20

3. 3. Quantitative analysis of β-sitosterol

The qualitative analysis of β-sitosterol was supportive of its quantitative analysis. The quantitative study of β-sitosterol from the *P. micrantha* extract was performed by the established HPLC method. In Figure 6, the HPLC chromatogram of ethanol *P. micrantha* rhizome extracts gave good separation of β–sitosterol peak at 14.25 minutes for β-sitosterol among all the secondary metabolites. The peak of β–sitosterol in the chromatogram of *P. micrantha* extract was compared with the retention time values with those obtained by the chromatogram of the standard β–sitosterol under similar conditions. The concentration of β-sitosterol in the plant extract sample obtained using the calculated peak area and the regression equation was 10.06 µg/ml, equivalent to 0.71 mg/g of β-sitosterol in fresh *P. micrantha* rzhizomes. β-sitosterol is found in vegetable oils in concentrations of 0.24-8.79 mg/g; specifically, olive oil and olive pomace oil have a high β-sitosterol concentration ranging between 0.91 and 1.52 mg/g. Meanwhile, cereals, vegetables, and fruits contain lower β-sitosterol concentration ranges of 0.24–0.61, 0.02–0.41, and 0.02–0.34 mg/g, respectively (Sofia et al. 2021) [19].

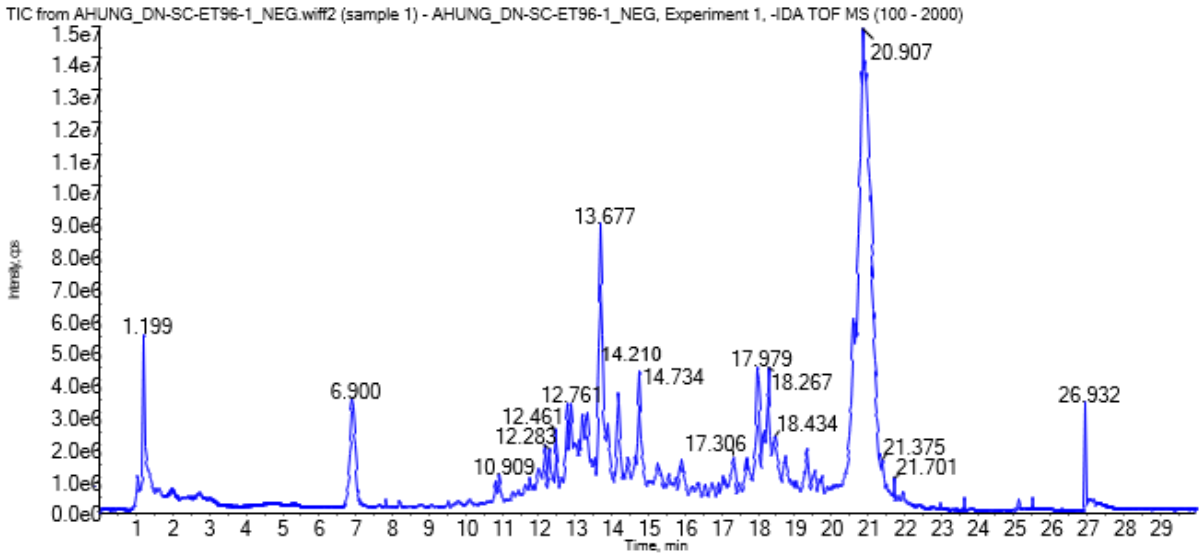

Figure 6. β-sitosterol peak at 14.21 min. in chromatogram of ethanol *P. micrantha* rhizome extract.

4. CONCLUSION

In our study, β-sitosterol is successfully isolated in the big batches, qualitatively determined by HPLC-QTOF method, and used as a standard compound. HPLC method is validated and used for the quantification of β-sitosterol in the ethanol *P. micrantha* rhizomes extract. The validated HPLC analysis reveals that the fresh *P. micrantha* rhizomes contain a significant amount of β-sitosterol at 0.71 mg/g. This validated HPLC method can be useful for future β-sitosterol analysis of different β-sitosterol contained products.

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