ANALYSIS OF β-SITOSTEROL IN SUPPLEMENT USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY: DEVELOPMENT AND VALIDATION

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ABSTRACT

β-sitosterol is one phytosterol commonly found in plant membranes. It has been reported to have many potential benefits such as antioxidant, antimicrobial, antiviral, anti-diabetic, anticancer, anti-inflammatory, and antipyretic activities. Various nutraceutical supplements containing β-sitosterol are marketed worldwide. The claim regarding health benefits is used on the label of the supplement, so quality control is needed to ensure the safety, quality, and efficacy of these products. Hence, this study aimed to develop and validate a simple and reliable HPLC method for the determination of β-sitosterol in supplements. The method utilizes an LC Column 150 x 4.6 mm Phenomenex with isocratic elution of methanol and acetonitrile (90: 10 v/v). An effluent flow rate of 1.5 mL/min and UV detection at 202 nm was used for the analysis of β-sitosterol. A validated HPLC method has been developed and used for the quantification of β-sitosterol. The developed method has high precision and accuracy, with a rapid sample preparation procedure and short analysis time, which is suitable for quality control of β-sitosterol in various supplements.

Keywords: Supplement, β-sitosterol, Validation, Method Development, High-Performance Liquid Chromatography (HPLC).

INTRODUCTION

As one of the most abundant phytosterols widely distributed in the plant kingdom, β-sitosterol is structurally similar to cholesterol but contains an additional ethyl group at C-24.¹ It is used as a safe and effective nutraceutical supplement, reported having cell membrane stabilizing effect² and found not to cause genotoxicity and cytotoxicity in rats.³ β-sitosterol also has many potential benefits such as antioxidant, antiviral, antimicrobial, anthelmintic, immunomodulatory, anti-diabetic, anticancer, antifertility, anti-inflammatory, antipyretic, anti-arthritis, antinociceptive, anti-mutagenic, and angiogenic activities.²,⁴,⁶ β-sitosterol combined with plant extract and other plant sterol used as nutraceutical supplements and have been marketed worldwide. The European Foods Safety Authority (EFSA) recommends consuming 1.5-3 g per day of phytosterol and/or stanol for 2-3 weeks for reducing LDL cholesterol and the risk of coronary heart disease (CHD).¹¹ In 2000, FDA claimed the relation between plant sterol/stanol esters consumption and reduced risk of CHD (65 FR 41 54686). Among the requirements, based on the weight of non-esterified phytosterols, consumption of 2 g phytosterol per day has been associated with a reduced risk of CHD. According to the provisional final rule (65 FR 54686), phytosterol mixtures must consist of at least 80% combined weight of β-sitosterol, campesterol, stigmasterol, sitosterol, and/or campestanol.¹² The increased trend toward self-medication, health awareness, and disease prevention leads to the rapid growth of the food and personal care product industry, especially in the supplement and functional food sector. Supplements often inhabit a gray area between food or pharmaceuticals, which makes it difficult the regulation. The claim regarding health benefits is used on the label of the supplement, so quality control is needed to ensure the safety, quality, and efficacy of this product.¹³ Therefore, a simple, fast, and accurate analysis method for supplement and herbal extract quality control is needed. Numerous chromatographic


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methods have been used to isolate and purify β-sitosterol from various plant families. Some methods have been already reported for the identification and determination of β-sitosterol in herbal extracts samples such as HPLC, GC/MS, 1H- and 13C-NMR, MS, IR, and UV-Vis. HPLC with the reverse stationary phase (RP-18) is one of the most applied chromatographic techniques for the identification of β-sitosterol. A variety of HPLC techniques for the quantification of β-sitosterol in herbal extract and vegetable oil has been reported. However, no study has been reported to estimate β-sitosterol in supplement products using the HPLC method. Thus, the aim of this study was to develop and validate a reliable HPLC method of analysis for the estimation of β-sitosterol from supplements. The method was applied to analyze supplement samples from various market origins.

**EXPERIMENTAL**

**Material and Samples**
The standard β-sitosterol used as reference was acquired from MarkHerb (School of Pharmacy, Bandung Institute of Technology, Indonesia). The solvent methanol (HPLC-grade), acetonitrile (HPLC-grade), and chloroform (analytical grade) were achieved from Merck. A 0.45 mm membrane from Sartorius was used to purify all the eluents. Product samples were obtained from different markets in Indonesia.

**Instrumentations**
Hewlett-Packard 8453 diode array UV-Vis spectrophotometer was used for the determination of β-sitosterol absorption maximum wavelength. The HPLC instrument Waters e2695 included a detector of UV–Vis Waters 2489 was used for analysis. The separation was carried out on a reversed-phase C18 column using Phenomenex® Luna C18 (250 x 4.6 mm i.d.; 5 µm). Empower 3 software was used to process and display the results of the separation data.

**Mobile Phase Preparation**
Various compositions of mobile phase (Table-1) were prepared to get the optimal mobile phase system for analyzing β-sitosterol in the sample. Solvents were mixed outside the HPLC system then filtered with 0.45 µm micropore membrane and sonicated for 15 minutes.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Methanol</th>
<th>Acetonitrile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Composition</td>
<td>70%</td>
<td>30%</td>
</tr>
<tr>
<td></td>
<td>80%</td>
<td>20%</td>
</tr>
<tr>
<td></td>
<td>90%</td>
<td>10%</td>
</tr>
</tbody>
</table>

**Preparation for β-sitosterol Standard Solution**
200 μg/mL β-sitosterol standard solution was prepared for a stock standard solution. The 10 mg β-sitosterol standard was dissolved with a few drops of chloroform and then added with mobile phase until 50 mL.

**Determination of β-sitosterol Absorption Maximum Wavelength**
1 μg/mL standard solution was prepared from 200 μg/mL stock standard solution then absorption maximum wavelength was measured with Diode Array and Spectrophotometer Hewlett Packard 8453.

**Optimization of HPLC System**
B-sitosterol standard solution was injected into the HPLC system with various flow rates, injection volumes, and mobile phase composition. Optimization of the HPLC system was done by determining the flow rate, injection volume, and mobile phase composition which resulted in optimum retention time and good chromatogram.

**System Suitability Test**
30 μg/mL standard solution was injected into the optimum HPLC system with six replications. The parameters of the SST include the percent relative standard deviation of time retention and peak area (%RSD), resolution (Rs), tailing factor (t), capacity factor (k’), and plate number (N) were determined.

**Validation Method of HPLC**
Validation of method analysis includes selectivity, linearity, intraday precision, interday precision, and accuracy according to the International Conference on Harmonization (ICH) guidelines Q2 (R1) and AOAC 1998.
Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals.\textsuperscript{17,18}

\textbf{Selectivity}

The selectivity test was determined by analyzing the standard $\beta$-sitosterol solution, sample, and solvent. When the resolution value ($Rs$) $\geq 1.5$ with no peaks interferes with the analyte peak in the chromatogram, then it is considered to have a good selectivity.

\textbf{Linearity}

Standard solutions with 15, 30, 45, 60, 75, and 90 $\mu$g/mL were prepared from 200 $\mu$g/mL stock standard solutions. The peak area was measured with three replications for each concentration. The calibration curves of the standards were acquired by plotting graphs of mean peak areas of each standard versus corresponding. It was then evaluated by the coefficient relation ($R^2$) and the coefficient regression function ($V_{X0}$).

\textbf{Limit of Detection (LOD) and Limit of Quantification (LOQ)}

The limit of detection (LOD) is defined as the lowest concentration of an analyte that can be detected but not necessarily quantified. A signal-to-noise (S/N) ratio of 3:1 is generally considered acceptable for estimating the LOD. The limit of quantification (LOQ) is defined as the lowest concentration of an analyte that can be quantitatively determined under the stated condition with acceptable precision and accuracy. The typical signal-to-noise (S/N) ratio is 10:1. The LOD value follows the equation $3.3 \times (S_{y/x}/b)$, and LOQ is $10 \times (S_{y/x}/b)$.

\textbf{Precision}

Precision refers to how close the measured values are to each other. Validation precision of single-laboratory should include intraday and interday components. Intraday and interday precision were determined by measuring absorbance with 2 different time intervals in a day, for three consecutive days. The precision test was done using 30 $\mu$g/mL standard solution and a supplement sample that has been spiked with 30 $\mu$g/mL of standard solution. Six replicates were done for each concentration. Percentage coefficient variation ($%CV$) was also calculated to evaluate method precision.

\textbf{Accuracy}

Accuracy refers to how close the true values and the mean result are obtained by applying the developed method. It was determined by the percent recovery of the analyte based on a regression equation. The accuracy test was done on the standard solution at a concentration of 24, 30, and 36 $\mu$g/mL and a sample that has been spiked with a standard solution of 24, 30, and 36 $\mu$g/mL. Three replicates were done for each concentration.

\textbf{Method Applicability to Real Samples}

Samples of supplements containing $\beta$-sitosterol that originated from different manufacturers were collected. The supplement tablets were crushed to form a fine powder. Each of the samples was dissolved with 1 mL chloroform, added with mobile phase, then sonicated for 10 minutes. Dilution with mobile phase was done to get peak area within the linear range. A 0.45 $\mu$m membrane was used to purify the sample solution. The solution was then analyzed using a validated HPLC system and a validated calibration curve was used to calculate $\beta$-sitosterol concentration in the sample.

\textbf{RESULTS AND DISCUSSION}

\textbf{Optimization of HPLC System}

In this study, a reversed-phase HPLC method combined with UV-Vis detection has been developed for the analysis of $\beta$-sitosterol in supplements. The C-18 column was preferred because it is one of the most applied chromatographic techniques for the identification of $\beta$-sitosterol that can result in better peak shape and resolution. Isocratic elution was chosen considering its simplicity which requires only one pump and is able to minimize the variation of baseline and ghost peaks.\textsuperscript{19} Based on the result of spectrophotometer analysis, the absorption maximum wavelength of $\beta$-sitosterol is 202 nm. Table-2 shows various flow rates and mobile phase compositions to optimize the chromatographic conditions. Among those variations, a mobile phase...
of methanol and acetonitrile (9:1 v/v) was found to be suitable for the analysis of β-sitosterol. Additionally, a flow rate of 1.5 mL/min with an injection volume of 20 μL provided optimal conditions for analysis of β-sitosterol at UV detection of 202 nm.

Table-2: Optimization of HPLC Condition with Various Flow Rate (mL/min)

<table>
<thead>
<tr>
<th>MeOH - ACN (v/v)</th>
<th>t retention (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7:3</td>
<td>20.74 22.91 28.56</td>
</tr>
<tr>
<td>8:2</td>
<td>16.33 18.81 22.76</td>
</tr>
<tr>
<td>9:1</td>
<td>13.53 15.68 17.95</td>
</tr>
</tbody>
</table>

System Suitability Test
The system suitability parameters were investigated for optimum HPLC conditions. United States Pharmacopeia (USP) established criteria of system suitability parameters for HPLC methods of pharmaceutical analysis. Therefore, analyte percent relative standard deviation (%RSD) of time retention and peak area, resolution (Rs), tailing factor (τ), capacity factor (k'), and theoretical plates (N) were determined. Table-3 shows that the developed method meets the acceptance criteria for all the system suitability parameters.

Table-3: Results from System Suitability Study

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Results</th>
<th>Acceptance Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>%RSD: - t ret - AUC</td>
<td>1.1%</td>
<td>≤2%</td>
</tr>
<tr>
<td>Resolution (Rs)</td>
<td>2.55</td>
<td>≥1.5</td>
</tr>
<tr>
<td>Tailing factor (τ)</td>
<td>0.984</td>
<td>≤2</td>
</tr>
<tr>
<td>Capacity factor (k')</td>
<td>6.85</td>
<td>1&lt;k'&lt;10</td>
</tr>
<tr>
<td>Theoretical plates (N)</td>
<td>1.24 x 10⁴</td>
<td>&gt;2000</td>
</tr>
</tbody>
</table>

Validation Method of HPLC
The specificity test allows one to verify if the method is selective enough to quantify the analyte in the presence of other components which may be expected to be present in the sample. Figure-1 shows the retention time of β-sitosterol standard and sample solutions. Similar retention times (~13 min) were observed in all the chromatograms of standard and the sample solution with good resolution at each peak, indicating that the matrices did not interfere with the analysis.

Fig-1: Chromatogram of Blank (A), β-sitosterol Standard (B), Samples of Supplement Containing β-sitosterol (C-E)

Standard solution series within a range of 15-90 μg/mL was analyzed in triplicate using the HPLC method. The linearity of the developed method was then evaluated. From the data obtained, a calibration curve was plotted from the standard mean peak area to the β-sitosterol standard solution concentration (Fig.-2). The $R^2$ (square of the correlation coefficient) value was found to be >0.999, indicating that the developed method is linear within the studied range. The linear equation obtained was $y = 5384.56x + 5555.18$ with
R² and Vₓ₀ of 0.99901 and 1.88, respectively, demonstrating the suitability of the developed method was established over the considered range (15-90 μg/mL).

![Fig.-2: The Calibration Curve of β-sitosterol Standard Solution](image)

The residual standard deviation (Sₓ/y) and slope (b) of the calibration curves were used to calculate LOD and LOQ using the equations, LOD = 3.3×(Sₓ/y/b) and LOQ = 10×(Sₓ/y/b). According to the obtained results, the LOD was 3.26 μg/mL and the LOQ was 9.89 μg/mL, respectively. The precision of the developed method was tested using the β-sitosterol standard solution and a sample that has been spiked with a β-sitosterol standard solution. β-sitosterol standard solution concentration that has been used for this purpose is 30 μg/mL which lies within the linearity range. For each of the intraday and interday testing periods, the analysis was done in six replicates. In accordance with the AOAC Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals, the requirement for %CV should be less than 6%. As shown in Table-3, the highest %CV value was found to be 3.92% with both intraday and interday precision being less than 6%. Therefore, the developed method was confirmed to have good precision. The Horwitz ratio (HorRat), the ratio of the observed relative standard among laboratories to the corresponding predicted relative standard, was used to evaluate the precision of the developed methods, HorRat value ≤ 2 indicated good precision. Accuracy test was used to measure the suitability of the system to recover β-sitosterol concentrations following the analysis. β-sitosterol standard solution with concentrations of 24, 30, and 36 μg/mL was used as representative of 80, 100, and 120 % of the β-sitosterol actual amount. An accuracy test was also conducted on samples spiked with the same concentration of β-the sitosterol standard solution. The analysis was done in triplicates for each β-sitosterol concentration. The results were then expressed in terms of percentage recovery with the acceptable recovery percentage of 80-115 % in accordance with AOAC Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals. The result of the accuracy test is shown in Table-5. The percentage recovery for the standard solution was in the range of 96.12-105.73 % while for the sample spiked percentage recovery was in between the range of 91.61-99.60 %. The developed method has fulfilled the acceptance criteria for validation parameters.

| Table-3: Precision Test for Sample Spiked and β-sitosterol Standard Solution |
|----------------|------------------|-----------------|-----------------|-----------------|----------------|
| Precision   |
| Test       | Period          | Standard        | %CVb            | HorRat Value   | Sample Spikedc | %CVd           | HorRat Value   |
| Intraday    | Morning         | 29.73±3.95      | 3.92            | 0.47            | 27.75±3.44     | 2.36           | 0.28           |
|             | Afternoon       | 28.99±3.88      |                 |                 | 27.46±1.27     |               |               |
| Interday    | Day-1           | 29.36±3.92      | 2.84            | 0.34            | 27.60±2.36     | 2.02           | 0.27           |
|             | Day-2           | 29.46±2.93      |                 |                 | 27.39±1.67     |               |               |
|             | Day-3           | 31.23±1.67      |                 |                 | 30.17±2.72     |               |               |

Notes: a-c Average of concentration (μg/mL) ± %RSD of each period; (n = 6).

b-d Average of %RSD for intraday and interday.
Table-4: Result of Accuracy Test on Sample Spiked and β-sitosterol Standard Solution

<table>
<thead>
<tr>
<th>Concentration (μg/mL)</th>
<th>Standard Solutions\textsuperscript{a}</th>
<th>%Recovery\textsuperscript{b}</th>
<th>Sample Spiked\textsuperscript{c}</th>
<th>%Recovery\textsuperscript{d}</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>25.37±2.16</td>
<td>105.73±2.16</td>
<td>23.90±3.66</td>
<td>99.60±3.66</td>
</tr>
<tr>
<td>30</td>
<td>30.78±0.30</td>
<td>102.60±0.30</td>
<td>27.48±1.59</td>
<td>91.61±1.59</td>
</tr>
<tr>
<td>36</td>
<td>34.60±1.49</td>
<td>96.12±1.49</td>
<td>34.70±1.71</td>
<td>99.14±1.71</td>
</tr>
</tbody>
</table>

Notes: \textsuperscript{a,c} Average of concentration (μg/mL) ± %RSD; (n = 3).
\textsuperscript{b,d} Average of %recovery ±%RSD.

Assay of β-Sitosterol in Supplement
The developed method has been applied to determine the β-sitosterol concentration in tablet and capsule supplements that were commercially available in the local market. The amounts of β-sitosterol determined in various samples are given in Table-5. Compared with other chromatography methods, our approach has high accuracy and precision with a rapid sample preparation procedure and short analysis time. The developed method is suitable for the quality control of β-sitosterol in various supplements.

Table-5: Total Percentage of β-sitosterol Content in the Sample

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Concentration of β-sitosterol (%)\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supplement 1 Capsules</td>
<td>15.97±0.01</td>
</tr>
<tr>
<td>Supplement 2 Capsules</td>
<td>3.47±0.01</td>
</tr>
<tr>
<td>Supplement 3 Tablets</td>
<td>38.15±0.01</td>
</tr>
</tbody>
</table>

Notes: \textsuperscript{a} Average of concentration (μg/mL) ± %RSD; (n = 3).

CONCLUSION
A validated HPLC method has been developed and used for the quantification of β-sitosterol. The proposed method has high precision and accuracy with a rapid sample preparation procedure and short analysis time which is suitable for quality control of β-sitosterol content in various supplements. In general, the method showed good linearity with $R^2$ and $V_x0$ of 0.99901 and 1.88. The LOD and LOQ were 2.92 and 8.84 μg/mL, respectively. From the result of the analysis, β-sitosterol concentration in the supplement products was determined in the range of 3.47-38.15%.

ACKNOWLEDGEMENT
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REFERENCES
12. Food and Drug Administration (FDA), Food Labeling; Health Claim; Phytosterols and Risk of Coronary Heart Disease, 75 FR 76525 (2010).