A validated RP-HPLC Method for Quantification of Alpha-tocopherol in Elaeis guineensis Leaf Extracts

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Abstract

Background: Elaeis guineensis (oil palm) is a tropical tree from the family Arecaceae. The tree is rich in vitamin E such as alpha-tocopherol, an essential antioxidant in the lipid phase of the human body.

Objective: This study aims to develop a reverse phase HPLC method for determination of alpha-tocopherol in E. guineensis leaf extracts.

Methods: This method was validated at 220 nm for linearity, precision, accuracy and the limits of detection and quantification. Linearity was in the range 0.2–100 µg/mL at R² = 0.9999. Results: Precision was determined as relative standard deviation by considering the retention time and the peak area of the reference compound, and was found to be <0.1%, and <3.4%, respectively. Accuracy was determined as the percentage recovery of the reference compound at two levels and was in the range 96–105%. The limits of detection and quantification were found to be 0.56±0.03 µg/mL and 1.65±0.03 µg/mL, respectively. The method was successfully applied for quantification of alpha-tocopherol in the E. guineensis leaf extracts; the concentration in the ethanolic extract was 1.65% and in water extract was 0.01%.

Conclusion: The developed method showed good linearity, precision, accuracy and high sensitivity. Hence, it may be applied in routine quantification of alpha-tocopherol in E. guineensis.

Key words: Elaeis guineensis, alpha-tocopherol, RP-HPLC

Introduction

Elaeis guineensis is commonly known as African oil palm. It consists of pinnate-leaved palms and having dense clusters of crowded flowers with bright red fruit and yielding high quality palm oil [1]. It is native to west Africa (Madagascar) [2, 3] and Southeast Asia such as Sumatera Indonesia, Sri Lanka and Malaysia [3]. It was first introduced in Malaysia during the British colonization in 1870’s. It is widely cultivated in tropics, and the most productive parts of the industry at present are located in Malaysia and Indonesia, which provides most of the oil entering international trade [3, 4].

E. guineensis leaf was traditionally used for the treatment wounds [5-7], headache, cancer and rheumatism and also used as an aphrodisiac, diuretic and liniment [1, 8]. Recent studies reported various pharmacological effects of leaf including antioxidant, antimicrobial [6-8], antihypertensive [9, 10], antihyperglycaemic [11] and wound healing properties [6, 12].

Alpha-tocopherol (Figure 1) is the fat soluble vitamin richly found in E. guineensis and present in the oil at high concentration. Alpha-tocopherol is the most active component of vitamin E complex and has potent antioxidant activity. It acts to protect against free radical damage that cause arthritis, heart problems, aging, many types of cancer, cataract, senile dementia (Alzheimer type), atherosclerosis and other circulatory diseases [13]. Alpha-tocopherol cannot be synthesized by our body and is found in small amount in most food. Unlike the other fat soluble antioxidants that can be stored in the liver for long time, alpha tocopherol is stored throughout body fats and is used up very quickly.

E. guineensis fruit has been reported to contain high concentration of alpha-tocopherol. However, there is no simple method to determine the amount of alpha-tocopherol from leaves. Thus, this study aims to develop and validate a reverse phase high performance liquid chromatography (RP-HPLC) method for determination of alpha-tocopherol in oil palm leaf extracts.

Materials And Methods

Chemicals

HPLC and analytical grade solvents were acquired from Merck Sdn. Bhd. (46150 Petaling Jaya, Selangor, Malaysia) and standard alpha-tocopherol was purchased from Sigma-Aldrich (Kuala Lumpur, Malaysia). The reverse phase Acclaim Polar Advantage II C18 column (3 × 150 mm × 3 µm) was purchased from Dionex (Sunnyvale, California).

Extraction of E. guineensis leaf extracts
The commercial sample of *E. guineensis* leaf was obtained from HERBagus Sdn Bhd. Leaves were cleaned, oven-dried and grinded to powder form. The sample (25 g) was accurately weighed and extracted with 400 mL ethanol using Soxhlet extractor for 24 h. For water extract, 25 g sample was refluxed with 250 mL distilled water for about 24 h. The extracts were concentrated using rotary evaporator and freeze-dried. The freeze-dried extracts were stored in desiccator until analysis.

**Sample Preparation**

Pre-treatment for *E. guineensis* leaf extracts was done to convert to corresponding fatty acid methyl ester (FAME) following the standard procedure by Hammond (1993) with slight modifications. Crude extract (420 mg) was treated with 5 mL mixture of sulphuric acid (H$_2$SO$_4$): toluene: methanol (1: 10: 20) and refluxed for 1 h. After cooling, the mixture was diluted with 6 mL distilled water followed by 10 mL n-hexane. The top n-hexane layer was then isolated using separatory funnel. Sodium sulphate (Na$_2$SO$_4$) was added to absorb water, filtered and centrifuged. The supernatant was transferred into amber vial and kept in refrigerator at 4°C for further analysis [14].

**Standard preparation**

Alpha-tocopherol standard was prepared at 1 mg/mL in HPLC grade methanol. The standard was further diluted to produce 100, 50, 25, 10, 5, 3, 1, and 0.2 µg/mL. The pre-treated sample extracts (20 mg/mL) were further diluted with n-hexane (HPLC grade) to produce a concentration of 500 µg/mL. The stock solutions were filtered through 0.45-µm syringe filters.

**Reverse phase HPLC method**

The concentration of alpha-tocopherol in the extracts was determined by Dionex-Ultimate® 3000 Rapid Separation Liquid Chromatography (RSLC) system, equipped with auto sampler, quaternary pump, degasser, column oven, and a DAD detector. The spectral data was collected at multiple UV detection (220 nm, 292 nm and 360 nm). A gradient mobile phase (Table 1) was used that consists of acetonitrile and water (95:5) on Acclaim Polar Advantage II C18 column (3 × 150 mm × 3 µm) at a flow rate of 1.0 mL/min, 10 µL injection volume and detection was optimized at 220 nm with 10 min separation time.

**Method validation**

The proposed method was validated according to the ICH guidelines [15]. The following validation characteristics were evaluated: linearity, precision, accuracy and the limits of detection and quantification (LOD and LOQ).

**Linearity**

Linearity (as the regression coefficient $R^2$) was determined by injecting 10 µL of the standard compound in a concentration range 0.2–100 µg/mL. The calibration curves were obtained by plotting the peak area versus concentration, and regression analysis was performed in order to determine the linearity.

**Precision**

The peak area and the retention time were evaluated for precision analysis. The standard was analyzed at 8 concentration points in the range 0.2–100 µg/mL. The intraday and interday precision was determined in terms of the relative standard deviation (%RSD), ($n = 5$).

**Accuracy**

Accuracy was determined as a percentage recovery of alpha-tocopherol, added to the ethanol extract at 900 µg/mL. The recovery was studied at 12.5 µg/mL and 25 µg/mL of the reference compound. The peak area of the compound in the extract (B), the reference compound (C) and their combinations (A) was recorded. The percentage recovery was calculated as the following [16]:

$$ \text{Percentage recovery} = \left( \frac{(A - B)}{C} \right) \times 100$$

The results are presented average ± SD ($n = 3$).

**Limits of detection and quantification (LOD & LOQ)**

The LOD and LOQ were calculated through the slope and standard deviation method (ICH, 1997), using the following formula;

$$ \text{LOD} = \left( \frac{3.3 \times \delta}{S} \right) / S, \quad \text{and} \quad \text{LOQ} = \left( \frac{10 \times \delta}{S} \right) / S,$$

Where:

$\delta$: is the standard deviation of the Y intercept of the linear regression equations.

$S$: is the slope of the linear regression equations.

**Quantification of alpha-tocopherol concentration in *E. guineensis* extracts**

*E. guineensis* extracts at 500 µg/mL were injected (10 µL), and the peak area corresponding to alpha-tocopherol was recorded. The concentration of the compound in the samples was then calculated by applying the linear regression equation of alpha-tocopherol calibration curve. The results are presented as a %w/w using the formula:

$$ \% \text{w/w} = \left( \text{the found concentration} / 500 \, \mu\text{g/mL} \right) \times 100 \quad (n = 3).$$

**Results and Discussion**
Alpha-tocopherol was detected at 6.05±0.03 min. The applied chromatographic conditions produced good separation of alpha-tocopherol in the plant extract; the peaks were sharp and have a clear baseline separation (Figure 2). The calibration data are presented in Table 2.

Good linearity of peak area versus concentration was observed within the range 0.2–100 µg/mL ($R^2 = 0.9999$). The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample that can be detected but not necessarily quantitated as an exact value. The quantification limit of an individual analytical procedure is the lowest amount of analyte that can be quantitatively determined with suitable precision and accuracy. The detection and quantification limits of alpha-tocopherol obtained were 0.56±0.03 µg/mL and 1.65±0.03 µg/mL, respectively. The quantification of the standard compound was determined using the calibration equation of alpha-tocopherol standard, and the results are presented as average %w/w ±SD. The concentration of alpha-tocopherol in ethanol and water extract was 1.65±0.02% and 0.01±0.00%, respectively.

Accuracy of the method was established by mean of a recovery experiment. It was studied at two levels of standard compound, 12.5 and 25 µg/mL based on external standard addition method. The percentage recovery at 12.5 µg/mL was 96±2.3%, and at 25 µg/mL was 105±8.9%, which indicates high accuracy of the method. The precision was presented in terms of %RSD of the retention time and peak area of the standard compound. The %RSD for retention time was <0.1% and that of the peak area was <3.4%. The %RSD values indicate high precision and good reproducibility of the method.

By applying the proposed method, it was possible to determine the alpha-tocopherol concentration in the leaf extracts. The previous studies are mainly applicable for detection of alpha-tocopherol in oil based products such as vegetable oils [17], virgin olive oil [18], rice bran oil [19, 20] and cow’s milk [21].

Converting alpha-tocopherol to fatty acid methyl ester (FAME) in the extracts provides more effective and straightforward procedure for extracting the fat soluble compound. This is an alternative to saponification method because heat and alkaline conditions used in saponification can result in substantial degradation of alpha-tocopherol [22]. Stability of alpha-tocopherol in the plant extract was reported to be more than 6 h, suggested low probability of losing the compound due to degradation [22].

Recent studies for quantification of alpha-tocopherol involved complex combination of solvent systems and required a long running time [17-19, 23]. The proposed method was developed to improve detection of alpha-tocopherol in *E. guineensis* extracts. The peaks were separated smoothly following the gradient flow of acetonitrile and water as the solvent system. The retention time was improved to almost 6.0 min, compared to 25 min in the previous methods. The results showed the amount of alpha-tocopherol in ethanol extract was higher compared to water extract, which reflects the fact that alpha-tocopherol is a fat soluble vitamin and insoluble in water-based extracts.

**Conclusion**

The developed method showed high precision with coefficient of variation values of <0.1% (retention time) and <3.4% (peak area). The method also showed high accuracy with average % recovery of 96 ± 2.3%. The method is specific and can be used for routine quantification of α-tocopherol in *Elaeis Guineensis* leaf extracts.

**References**

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Illustrations

Illustration 1

Table 1: The gradient mobile phase applied for elution of alpha-tocopherol.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>% A (Water)</th>
<th>% B (Acetonitrile)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>5</td>
<td>95</td>
</tr>
<tr>
<td>8.0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>15.5</td>
<td>5</td>
<td>95</td>
</tr>
</tbody>
</table>
Illustration 2

Table 2: Calibration data of the developed HPLC method. Results are shown as average ±SD. (n=10)

<table>
<thead>
<tr>
<th>Retention time (AV±SD)</th>
<th>Regression Equation</th>
<th>$R^2$</th>
<th>Linearity (µg/mL)</th>
<th>LOD (µg/mL)</th>
<th>LOQ (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.05±0.03</td>
<td>$Y = 0.2715x + 0.0312$</td>
<td>0.9999</td>
<td>0.2-100</td>
<td>0.56±0.03</td>
<td>1.65±0.03</td>
</tr>
</tbody>
</table>
Illustration 3

Figure 1: Chemical structure of alpha-tocopherol
Illustration 4

Figure 2: HPLC chromatograms of (a) alpha-tocopherol standard; (b) ethanol extract; and (c) water extract of E. guineensis leaf at retention time 6.08 min, 6.14 min and 6.12 min respectively.
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