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# Validation of an RP-HPLC Method for Quantitative Analysis of Phikud Navakot Extract using the Standard Addition Method

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**ABSTRACT**

The aim of the study was to develop a reversed phase-high performance liquid chromatography-diode array detector (RP-HPLC-DAD) analytical method for monitoring three analytical markers, gallic acid, vanillic acid and ferulic acid, in Phikud Navakot herbal extract (NVK-E), using the standard addition method. Method validation of RP-HPLC-DAD method was performed under gradient conditions of methanol and 1% v/v acetic acid (pH 2.7) at a flow rate of 1.0 ml/min with a total run time of 70 min. A C18 (4.6 x 250 mm, 5 µm) HPLC column was used. The column oven was controlled at 27ºC and DAD was adjusted from 190-400 nm with a monitoring wavelength of 270 nm. The results for all three analytical markers showed good linearity with correlation coefficients of >0.99. The percentage recovery of each analytical marker was between 92-105%. The precision of the system, presented in terms of HorRatr values, were <2 for all three markers. The linearity, accuracy and precision of the system complied with AOAC Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals (2013). These results indicated that the developed RP-HPLC-DAD analytical method can be utilized for monitoring the quality of NVK-E.



## 1. Introduction

Phikud Navakot is a traditional Thai herbal medicine that has been used in Thailand for more than 100 years and consists of equal quantities of 9 herbs: *Angelica sinensis*, *Angelica dahurica*, *Ligusticum sinense*, *Atractylodes lancea*, *Artemisia annua*, *Picrorhiza kurrooa*, *Saussurea lappa*, *Nardostachys grandiflora* and *Terminalia chebula*. Phikud Navakot is used for the treatment of hiccups and circulation disorder, and as an anti-helmintic and analgesic agent [1]. Phikud Navakot herbal extract (NVK-E) decreases vasorelaxation due to carbachol in the rat aorta, which suggests an effect on muscarinic receptors and supports the use of Phikud Navakot

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against dizziness and fainting [2]. Supapornhemin *et al*. (2013) showed that ethanolic NVK-E also has a lipid lowering effect that occurs through enhancement of LDL-R expression and inhibition of HMG-CoA reductase expression [3]. A recent study also suggested that Phikud Navakot and its 1:1 ethanol to water extract has an antioxidant activity against DPPH, $O_2$•-, OH•, NO• and $H_2O_2$, which in turn affects the cardiovascular system [4]. These results and the increasing popularity of herbal and traditional medicines suggest that the pharmacological activities and standardization of Phikud Navakot require further in-depth evaluation.

Quality control of NVK-E is an important part of pharmaceutical product development to ensure the safety, quality and efficacy of the herbal product. Many analytical techniques have been utilized to control the quality of herbal products, including HPLC, TLC, GC and capillary electrophoresis, depending on the nature of the active constituents to be analyzed [5]. These methods are often unsuccessful due to the complex nature of herbal products. However, Nalinratana *et al*. (2014) was successful in developing a gradient HPLC method using acetonitrile and 1% v/v acetic acid as solvent to monitor NVK-E using three phenolic compounds as analytical markers: gallic acid, vanillic acid and ferulic acid (Fig. 1) [4].

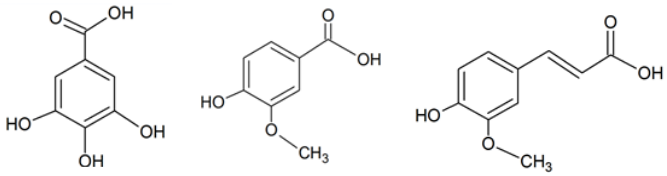


**Figure 1** Chemical structures of (A) gallic acid (B) vanillic acid, and (C) ferulic acid

NVK-E is a highly viscous liquid with a complex matrix composition; hence, a standard addition method was chosen to analyze the three analytical markers in NVK-E. In this study, a standard mixture solution was added into the analyte to compensate for the matrix effect of the sample. The standard addition method is particularly useful for analysis of analytes when the matrix effect within the sample is unknown or variable [6]. The HPLC system was validated in compliance with AOAC Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals (2013) (henceforth, referred as AOAC Guidelines).

## 2. Materials and Methods

### *2.1 Materials*

Crude Phikud Navakot herbal materials were purchased from Vejpong Pharmacy Co., Ltd., Thailand and extracted using a well-controlled process at Thai-China Flavours and Fragrances Industry Co., Ltd., Thailand. Standards of gallic acid (gallic acid monohydrate, 99.4% purity, Fluka), vanillic acid (98.7% purity, Fluka), ferulic acid (99.8% purity, Sigma-Aldrich) and glacial acetic acid (Sigma-Aldrich) were purchased from Chemical Express Co., Ltd., Thailand. Methanol (HPLC grade) was purchased from Merck Ltd., Thailand.

#### *2.1.1 Extraction method of Phikud Navakot*

Equal quantities of 9 herbs in Phikud Navakot dried crude materials were ground and macerated for 24 hours in 80% methanol using 10 times the total weight of crude materials. Extraction proceed at 100°C for the duration of 3 hours and filtered. Finally, the filtrate was evaporated to obtain the final extract with herb to extract ratio (HER) of (3.3:1).

#### *2.1.2 Instrumentation and chromatographic conditions*

Chromatographic analysis was performed using a Shimadzu high performance liquid chromatographic system with a quaternary pump (LC-20AD). An auto-sampler (Shimadzu SIL-20AC) and column oven (CTO-20A) were used with a photodiode array detector (SPD-M20A). Results were acquired and evaluated by LC Solution software. Chromatography was performed under gradient conditions with a total run time of 70 min. The mobile phase was filtered through a 0.22-µm membrane filter before use. The mobile phase consisted of solvent A (methanol) and solvent B (1% v/v acetic acid, *pH* 2.7). The gradient started with 100% solvent B for 45 min, was adjusted to 40% solvent A and 60% solvent B for 10 min, to 80% solvent A and 20% solvent B for 10 min, and finally to 100% solvent B for 5 min. The flow rate of the system was 1.0 ml/min with the column temperature controlled at 27°C. The injection volume was fixed at 100 µl. DAD was adjusted at 190-400 nm with a monitoring wavelength of 270 nm. The HPLC column used was a Shiseido MGII C18 (4.6 x 250 mm, 5 µm) column. Gallic acid, vanillic acid and ferulic acid were found to elute at retention times of 12.2, 34.8 and 46.0 min, respectively.

#### *2.1.3 Preparation of standard mixture solutions*

Gallic acid, vanillic acid and ferulic acid were individually dissolved in methanol to prepare a stock solution for each standard. Each stock solution was used to prepare standard mixture solution of gallic acid, vanillic acid and ferulic acid by diluting with water to concentrations of 43, 35 and 10 µg/ml, respectively. The standard mixture solution was further diluted with water to obtain final concentrations of 1.60, 1.40 and 0.04 µg/ml, for gallic acid, vanillic acid and ferulic acid, respectively. The standard mixture solution was filtered through a 0.22-µm membrane filter prior to use.

#### *2.1.4 Preparation of sample solution*

NVK-E (50 mg) was accurately weighed and placed in a well-sealed glass container. Water (80 ml) was added and the solution was sonicated for 1 hour. The solution was further diluted with water to 100 ml and filtered

through a 0.22-µm membrane filter before use.

### *2.2 Method Validation*

**System suitability** : System suitability was performed by RP-HPLC-DAD by injecting six replicates of NVK-E with standard mixture solution to obtain HPLC peak responses.

**Selectivity** : Selectivity was examined by separately injecting standard mixture solutions of the three analytical markers, NVK-E, and NVK-E with standard mixture solution using RP-HPLC-DAD to obtain HPLC peak response.

**Linearity** : Linearity of the system was examined using the standard addition method. Five different concentrations of standard mixture solution were used to construct the calibration curve. NVK-E (50 mg) was accurately weighed and placed in well-sealed glass containers. Water (80 ml) was added and the solution was sonicated for 1 h. A standard mixture solution was added to each NVK-E solution to obtain final concentrations of 2.40, 2.80, 3.20, 3.60 and 4.00 µg/ml for gallic acid; 2.10, 2.45, 2.80, 3.15 and 3.50 µg/ml for vanillic acid; and 0.60, 0.70, 0.80, 0.90 and 1.00 µg/ml for ferulic acid. Each solution was then diluted with water to a volume of 100 ml. Each concentration was analysed in triplicate. A calibration curve was constructed by plotting peak areas against concentrations. The slope, y-intercept and R2 were calculated using the least squares regression method.

**Accuracy and Precision** : Accuracy and precision of the system were evaluated using the standard addition method. Three different concentrations were prepared by accurately weighing 50 mg of NVK-E into well-sealed glass containers, after which 80 ml of water was added and the solution was sonicated for 1 h. A standard mixture solution was added to each NVK-E sample to obtain final concentrations of 2.40, 3.20 and 3.60 µg/ml for gallic acid; 2.10, 2.80 and 3.15 µg/ml for vanillic acid; and 0.60, 0.80 and 1.00 µg/ml for ferulic acid. Water was then added to obtain a volume of 100 ml. Each concentration was analysed in triplicate. Inter-day precision and accuracy were performed on three different days. Average % recovery, % relative standard deviation (%RSD), and Horwitz Ratio (HorRatr) values were calculated to determine the accuracy and precision of the system. HorRatr and RSDr (calculated, %) were calculated from the following formulas:

HorRatr = RSDr (found, %) / RSDr (calculated, %) (equation 1)

RSDr (calculated, %) = C-0.15 (equation 2)

C expressed as a mass fraction of gallic acid , vanillic acid or ferulic acid in NVK-E.

## 3. Results and Discussion

Control of consistent quality of NVK-E after each production batch was of great concern. Although the origin of NVK-E derived from nine different herbs, this study proposed to treat NVK-E as if it came from a single plant source. The justification for this decision was based on the evaluation of two large production batches of Phikud Navakot extract within the past two years. The results showed similar total phenolic content (%GAE) and EC50(µg/ml) when evaluated with DPPH, superoxide, hydrogen peroxide, hydroxyl and nitric oxide. This consistent comparable standard quality between batches was due to the stringent control of the extraction method and the high quality of raw materials used.

In this study, gallic acid, vanillic acid and ferulic acid were 3 phenolic analytical markers in NVK-E. These 3 analytical markers were the main identifiable compositions in total phenolic content, which was found to be related to the cardiovascular activity of NVK-E[4]. Two production batches of NVK-E also exhibited comparable amounts and ratios of gallic acid, vanillic acid and ferulic acid. Thus, these markers were justified to be appropriate in controlling the quality of NVK-E and future pharmaceutical formulations despite composition complexity or its nine herbal origin.

Once the 3 analytical markers (i.e. gallic, vanillic and ferulic acids) were appropriately identified and justified according to previous rationale, analytical method validation for these markers in NVK-E must be done. This was to ensure that the proposed analytical method was valid to be used to control the quality of each production batch of NVK-E for future pharmaceutical product development and registration.

### *3.1 Method Validation*

Method validation was performed in accordance with the AOAC Guidelines. System suitability, selectivity, linearity, accuracy and precision were determined.

System suitability : The system suitability was evaluated in six successive replicates. The %RSD for peak areas of all three markers were <2.0. Resolution, tailing factor and theoretical plates were also evaluated (Table 1). System suitability was found to complied to the AOAC Guidelines.

**Table 1** System suitability of the RP-HPLC-DAD system

| Analytical markers | Concentration (µg/ml) | % RSD[a] | Tailing factor ($T^f$) | Resolution ($R_s$) | Number of theoretical plates (N) |
|---|---|---|---|---|---|
| Gallic acid | 3.6 | 0.6 | 1.1 | - | 13866 |
| Vanillic acid | 2.8 | 1.1 | 1.0 | 56 | 124578 |
| Ferulic acid | 0.8 | 1.4 | 1.1 | 29 | 249135 |
| [a] n=6 | | | | | |

**Selectivity** : Peak response of the three markers in standard mixture solution were clearly separated from each other, with retention times of 12.2, 34.8 and 46.0 for gallic acid, vanillic acid and ferulic acid, respectively (Fig. 2). Peak responses of the three markers in NVK-E were also well separated from each other and exhibited retention times identical to the results for standard solution (Fig. 3). For NVK-E with standard mixture solution, peak response areas of the three markers corresponding to the retention times of gallic acid, vanillic acid and ferulic acid

increased in direct correlation with concentrations of the added standard mixture solution. The results suggested that the matrix of NVK-E did not interfere with the peak responses of all three analytical markers (Fig. 4).

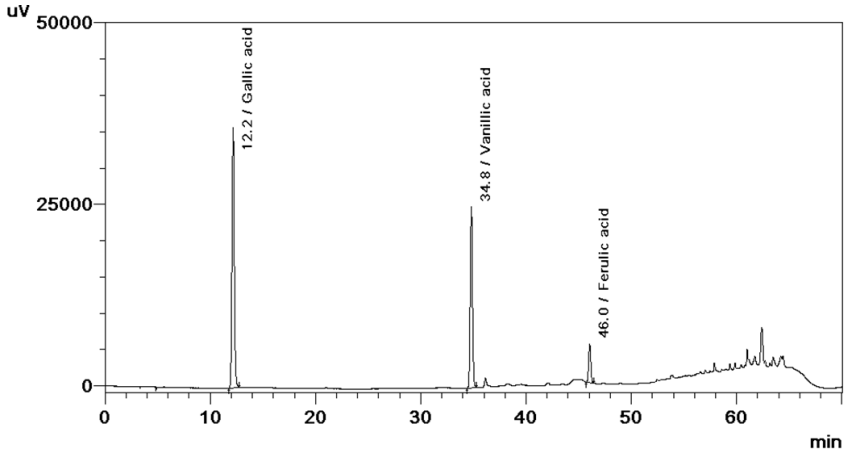


**Figure 2** Chromatogram of a standard solution of 1.60 µg/ml gallic acid (12.2 min), 1.40 µg/ml vanillic acid (34.8 min) and 0.40 µg/ml ferulic acid (46.0 min).

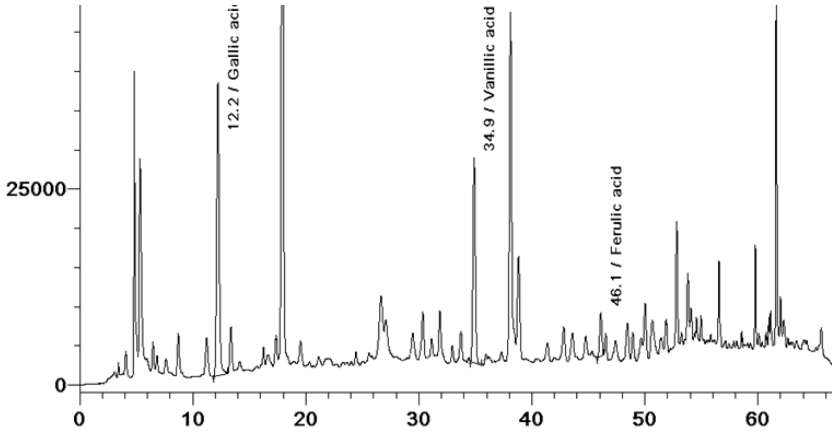


**Figure 3** Retention times of gallic acid, vanillic acid and ferulic acid in 50 mg of NVK-E are seen at 12.2, 34.9 and 46.1 min, respectively.

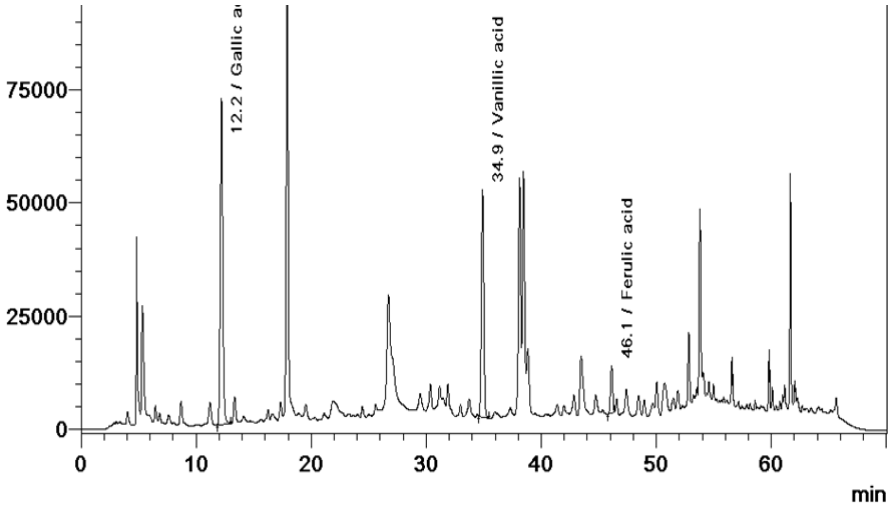


**Figure 4** Chromatogram of 50 mg NVK-E with standard mixture solutions at concentrations of 1.60 µg/ml gallic acid (12.2 min), 1.40 µg/ml vanillic acid (34.9 min), and 0.40 µg/ml ferulic acid (46.1 min).

**Linearity** : Linearity parameters of the system are shown in Table 2. The results showed that all three analytical markers exhibited good linearity within specified ranges with coefficients of determination ($r^2$) of 0.9995, 0.9999 and 0.9983 for gallic acid, vanillic acid and ferulic acid, respectively. These results complied with the AOAC Guidelines, which stated that the correlation coefficient should be >0.99 for acceptable linearity

**Table 2** Linearity parameters of the RP-HPLC-DAD system

| Analytical markers | Linearity range (µg/ml) | Slope | Y-intercept | $r^2$ |
|---|---|---|---|---|
| Gallic acid | 2.40-4.00 | 329847.54 | -1247.9003 | 0.9995 |
| Vanillic acid | 2.10-3.50 | 271915.75 | 15220.3255 | 0.9999 |
| Ferulic acid | 0.60-1.00 | 187276.71 | -5495.9840 | 0.9983 |

**Accuracy and Precision** : The accuracy of the system is shown in Table 3 as the average %recovery. The results were found to be 98-102, 99-102 and 98-101 for gallic acid, vanillic acid and ferulic acid, respectively. The average %recovery of all three markers complied with the AOAC Guidelines, which stated that this value should be between 92-105%. The precision of the system is shown as a HorRatr values in Table 3. The AOAC Guidelines suggested that HorRatr values <2 indicated good repeatability. The repeatability results for all three markers showed HorRatr values of <2, which complied with the AOAC Guidelines.

**Table 3** Accuracy and Precision of the RP-HPLC-DAD system

| | Average Recovery (%) ± SDa | Amount added (µg/ml) | Amount found (µg/ml) ± SD[b] | %RSD (found) | $HorRat_r$ |
|---|---|---|---|---|---|
| Gallic acid 50% Day1 | 102.11 ± 0.90 | 2.51 | 2.54 ± 0.02 | 0.77 | 0.34 |
| Day2 | 101.64 ± 0.69 | 2.72 | 2.77 ± 0.02 | 0.59 | 0.26 |
| Day3 | 100.52 ± 0.31 | 2.82 | 2.84 ± 0.01 | 0.20 | 0.09 |
| Gallic acid 100% Day1 | 99.56 ± 0.65 | 3.37 | 3.36 ± 0.01 | 0.44 | 0.19 |
| Day2 | 100.21 ± 0.35 | 3.58 | 3.59 ± 0.00 | 0.13 | 0.06 |
| Day3 | 101.39 ± 1.86 | 3.69 | 3.74 ± 0.06 | 1.70 | 0.75 |
| Gallic acid 150% Day1 | 98.41 ± 0.52 | 4.28 | 4.21 ± 0.04 | 1.02 | 0.45 |
| Day2 | 100.41 ± 1.74 | 4.44 | 4.50 ± 0.01 | 2.22 | 0.97 |
| Day3 | 100.38 ± 1.45 | 4.57 | 4.58 ± 0.06 | 1.28 | 0.56 |
| Vanillic acid acid 50% Day1 | 102.11 ± 0.90 | 1.94 | 1.98 ± 0.02 | 1.08 | 0.45 |
| Day2 | 101.64 ± 0.69 | 2.10 | 2.13 ± 0.01 | 0.44 | 0.18 |
| Day3 | 100.52 ± 0.31 | 2.24 | 2.25 ± 0.01 | 0.27 | 0.11 |
| Vanillic acid 100% Day1 | 99.73 ± 0.96 | 2.61 | 2.60 ± 0.02 | 0.76 | 0.32 |
| Day2 | 100.56 ± 1.15 | 2.78 | 2.80 ± 0.03 | 1.02 | 0.43 |
| Day3 | 100.40 ± 1.24 | 2.93 | 2.94 ± 0.03 | 1.09 | 0.46 |
| Vanillic acid 150% Day1 | 98.99 ± 0.48 | 3.32 | 3.28 ± 0.03 | 0.95 | 0.40 |
| Day2 | 101.58 ± 1.41 | 3.46 | 3.51 ± 0.06 | 1.81 | 0.76 |
| Day3 | 100.76 ± 1.09 | 3.62 | 3.65 ± 0.04 | 1.23 | 0.51 |
| Ferulic acid 50% Day1 | 101.36 ± 0.86 | 0.59 | 0.60 ± 0.01 | 1.10 | 0.39 |
| Day2 | 99.29 ± 1.12 | 0.59 | 0.59 ± 0.01 | 0.99 | 0.35 |
| Day3 | 98.90 ± 0.63 | 0.75 | 0.74 ± 0.00 | 0.63 | 0.22 |
| Ferulic acid 100% Day1 | 98.30 ± 0.80 | 0.79 | 0.77 ± 0.00 | 0.50 | 0.18 |
| Day2 | 98.59 ± 0.91 | 0.79 | 0.78 ± 0.01 | 1.11 | 0.40 |
| Day3 | 99.95 ± 1.05 | 0.96 | 0.96 ± 0.01 | 1.06 | 0.38 |
| Ferulic acid 150% Day1 | 98.39 ± 0.98 | 0.99 | 0.98 ± 0.01 | 1.43 | 0.51 |
| Day2 | 99.28 ± 1.02 | 0.98 | 0.98 ± 0.01 | 1.43 | 0.51 |
| Day3 | 99.80 ± 2.01 | 1.17 | 1.17 ± 0.03 | 2.18 | 0.78 |

[a] n=3 [b] n=3

### *3.2 Analysis of NVK-E by RP-HPLC-DAD method*

In the analysis of a single lot extraction of NVK-E, the amounts of analytical markers found were 3.4, 2.9 and 0.8% w/w for gallic acid, vanillic acid and ferulic acid, respectively.

## 4. Conclusion

The developed RP-HPLC-DAD technique using standard addition method for monitoring gallic acid, vanillic

acid and ferulic acid as analytical markers of NVK-E showed good linearity, precision and accuracy, which agreed well with AOAC Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals (2013). The results confirmed that this method could be utilized for monitoring the quality of NVK-E and may also be modified to analyse NVK-E in future formulations.

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