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# HPLC method development and validation for quantitation of CBD and THC in Suk-SaiYas Pills

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## Abstract

The Suk-SaiYas recipe is one of the oldest Thai medicinal recipes recorded for insomnia treatment. This recipe comprises 12 herbal medicinal plants, of which cannabis is the major active pharmaceutical ingredient. Recently, traditional medicine has been widely used by many patients. To ensure the therapeutic property of Suk-SaiYas pills, a standard analytical method for measuring the active compound should be identified. This research aims to develop and validate the HPLC method following ICH guidelines to quantify CBD and THC in Suk-SaiYas pills. The chromatographic separation was accomplished using step-gradient elution on the ZORBAX Eclipse Plus C18 (4.6 x 100 mm, 3.5 µm) column at 30°C. Acetonitrile and water were used as mobile phases with a 0.5 ml/min flow rate. CBD and THC exhibited a linear relationship in the concentration range of 0.25 - 10.0 µg/ml with $R^2 > 0.9998$. The repeatability was less than 1%; the intermediate precision did not exceed 2% for both constituents. The average % recovery of two analytes ranged between 92 - 106%. The limit of detection and the limit of quantification was found to be 0.03 and 0.10 µg/ml, respectively. Finally, it may be concluded that the developed HPLC method is precise and accurate for the determination of CBD and THC contents and could be utilized for the quality control of Suk-SaiYas pills

***Keywords*****:** *CBD; HPLC; method validation; pills; Suk-SaiYas recipe; THC.*

## 1. Introduction

Insomnia is a common symptom that impacts the quality of life. Many insomnia patients encounter a high risk of hypertension, depression, and post-traumatic stress disorder (PTSD), leading to morbidity and mortality (Dong, & Yang, 2019; Kartal et al., 2021). There are two standard options for insomnia treatment, including psychological therapy and medical therapy. According to Hertenstein et al. (2022), research indicated that cognitive-behavioral therapy significantly reduced depression, post-traumatic stress disorder, and alcohol dependence insomnia symptoms. Therefore, changing sleep behavior and attitude are recommended as the initial treatment for psychological treatment. Insomnia treatment can be more effective by combining cognitive-behavioral therapy and pharmaceutical therapy (Morin et al., 2015). Even though drug administration reveals more therapeutic efficiency, it has many negative consequences on mental health and the side effects of drug use. Benzodiazepines are a group of modern

medicines that are commonly used for insomnia treatment. However, many current studies have reported a highly addictive side effect of benzodiazepines via GABAA receptor subtypes (Tan, Rudolph, & Lüscher, 2011).

Moreover, doxepin and trazodone are tricyclic antidepressant drugs often used in the early stage of insomnia treatment for sleep-inducing. These medicine groups have been shown over-sedative side effects for high doses and long-term treatment (Wichniak, Wierzbicka, Walęcka, & Jernajczyk, 2017). Traditional medicine utilization is considered an alternative treatment to avoid the addictive properties and side effects of modern drugs. The previous study reported that one of the traditional Chinese medicines could improve insomnia by reducing anxiolytic and antidepressant symptoms. The genetic prediction and docking data revealed that this drug could bind to the active side of proteins controlling insomnia disorder (Jin et al., 2021).

Thailand is a famous country for using folk medicines in treatment. One traditional medicine recipe proved and supported by the Thailand government for insomnia therapy is the Suk-SaiYas recipe. The Suk-SaiYas recipe is one of the oldest conventional Thai medicines enrolled for insomnia treatment. The Suk-SaiYas recipe consists of twelve herbal medicinal plants (Table 1), and cannabis is the direct proportion. Earlier studies indicated that active compounds in cannabis, cannabidiol (CBD), and delta-9 tetrahydrocannabinol (THC) enhanced sleep quality (Babson, Sottile, & Morabito, 2017). The research of Mondino et al. (2019) demonstrated inhalation of the low THC content in cannabis could increase the Non-REM sleep cycle in the resting stage of a rat. The evidence from over-the-counter medication market data also supported that cannabis was used more often by Colorado citizens to reduce insomnia problems (Doremus, Stith, & Vigil, 2019). However, high dose and long-term cannabis use will affect mental health and perceived cognitive function. To maintain the therapeutic properties of cannabis in insomnia treatment, a low quantity and short-term usage were recommended for cannabis users. The research data showed that the user of the cannabis-related problem significantly decreased anxiolytic and depressant symptoms after taking a small amount of cannabis less than three days a week and treating it with behavioral therapy. Consequently, the sleep quality of patients will be improved (Mooney et al., 2018; Short, Zvolensky, & Schmidt, 2021).

**Table 1** The composition of herbal medicinal plants in the Suk-SaiYas recipe

| Plant name | Ratio in recipe |
|---|---|
| *Cinnamomum camphora* (L.) J. Presl | 1 |
| *Azadirachta indica* A. Juss. var. siamensis Valeton | 2 |
| *Kleinhovia hospita* L. | 3 |
| *Cinnamomum bejolghota* (Buch.-Ham.) Sweet | 4 |
| *Nigella sativa* L. | 5 |
| *Aucklandia lappa* Decne | 6 |
| *Myristica fragrans* Houtt. | 7 |
| *Mesua ferrea* L. | 8 |
| *Piper nigrum* L. | 9 |
| *Zingiber officinale* Roscoe | 10 |
| *Piper retrofractum* Vahl. | 11 |
| *Cannabis sativa* L. | 12 |

Besides, a lot of research demonstrated not only cannabis but also other medicinal plants in the Suk-SaiYas recipe could be induced drowsiness and sleep. For example, ginger in the Suk-SaiYas recipe displayed the anxiolytic property (Fadaki, Modaresi, & Sajjadian, 2017), and nutmeg exhibited anti-locomotion (Subarnas, Apriyantono, & Mustarichie, 2010). While, the methanolic extract and essential oil of *Piper nigrum* fruits revealed anxiolytic and antidepressant activities (Hritcu et al., 2015; Ghosh, Kumar, Sachan, & Chandra, 2021). These data may imply that the combination of herbal medicines in the Suk-SaiYas recipe could have reduced the adverse effects of some herbal medicinal plants and enhanced brain and body relaxation and fall asleep easily.

Recently, herbal medicines tend to increase among Thai people because they have minor adverse effects. However, most traditional medicines in the local market lack control over the quality. One of the recommended issues for product certification is quantifying an active compound. High-Performance Liquid Chromatography (HPLC) is the most popular analysis method for detecting an active constituent in the sample. HPLC is a more robust method than Gas Chromatography (GC) to determine the original form of cannabinoids in cannabis plant material such as CBD and THC. This technique could not activate the decarboxylation process of acidic conditions by heating (Ciolino, Ranieri, & Taylor, 2018a). So,

naturally occurring cannabinoid types are quantified accurately by using HPLC. Many HPLC systems have been developed to identify and quantify CBD and THC in the cannabis plant. For example, Hädener, König, and Weinmann (2019) succeeded in quantifying the five cannabinoids in the cannabis plant with precise and accurate results. CBD and THC were eluted entirely within 10 minutes using a core-shell C8 column and 0.1% formic acid in the water/acetonitrile system. The same solvent system was modified and used for routine analysis of the cannabis plant in forensic sciences. This fast-HPLC technique could elute CBD and THC from the cannabis sample within 5 minutes. The results confirmed that the HPLC method showed precise and accurate CBD and THC quantification (Burnier, Esseiva, & Roussel, 2019). Besides, McRae and Melanson (2020) developed the LC-MS/MS method using a gradient elution of formic acid in water and formic acid in an acetonitrile solvent system combination to analyze 17 cannabinoids from plant materials. Furthermore, the HPLC was applied for controlling cannabinoid content in a new generation of cannabis products. The research of Yangsud et al. (2021b) indicated that the HPLC method was utilized to measure CBD and THC contents in cannabis sublingual drops. The active ingredients were monitored at 222 nm. Using the ACE 3 C18-PFP column with isocratic elution of methanol and water (83:17) at a flow rate of 0.4 ml/min could completely separate CBD and THC from other impurities. A similar mobile phase system of methanol and water (85:15) was applied together with the Zorbax C18 column (4.6 mm × 100 mm, 3.5 µm) for the oromucosal spray analysis. The result indicated that CBD and THC could discriminate from other mixtures within 10 min (Saingam, & Sakunpak, 2018). However, the HPLC system for quantitation of active cannabinoids in the traditional medicine composing of many herbal plants, Suk-SaiYas recipe, still has insufficient data. To improve the quality of Suk-SaiYas medicine in the market, our research attempts to develop a new HPLC standard method for determining CBD and THC contents in Suk-SaiYas pills. The method validation procedure is followed the ICH guideline. Many parameters were evaluated, including linearity, range, precision, accuracy, the limit of detection (LOD), and limit of quantitation (LOQ), providing the reliable and consistent analytical method for the routine analysis of pharmaceutically active ingredients in Suk-SaiYas pills.

## 2. Objectives

The objective of the study is to develop and validate a new HPLC standard method for the determination of CBD and THC content in Suk-SaiYas pills.

## 3. Materials and methods

### 3.1 Materials

The standards (CBD and THC) and cannabis leaves were kindly supplied by the College of Pharmacy and Faculty of Agricultural Innovation, Rangsit University, Thailand, respectively. Acetonitrile and methanol of HPLC grade were purchased from Fisher Scientific (USA). Other herbal medicinal plants in the Suk-SaiYas recipe were purchased from the herbal store in Bangkok, Thailand.

Suk-SaiYas pills were prepared by combining two main ingredients, liquid and solid phase, with different percentages (Table 2). While the liquid phase was composed of honey and boiled water, the solid phase was Suk-SaiYas mixed powder and sodium starch glycolate. The liquid ingredient was slowly added to concrete mixtures and kneaded by hand until doughy consistency. The appropriated Suk-SaiYas dough was pressed into the mold and controlled the pill size by mold height adjustments. The rough surface of the pill was rolled into pea-sized balls to make a smooth and round surface. After that, the Suk-SaiYas pill was dried in an oven at 50°C for 24 hours and stored in an amber glass bottle at room temperature.

**Table 2** Suk-SaiYas pills ingredients

| Composition | % in formulation |
|---|---|
| **Solid phase** | |
| Suk-SaiYas powder | 97 |
| Sodium starch glycolate | 3 |
| **Liquid phase** | |
| Honey | 35 |
| Water | 65 |

### 3.2 HPLC analysis

*3.2.1 Sample preparation*

Twenty Suk-SaiYas pills were ground to powder and filtered through a 60-mesh sieve. The Suk-SaiYas fine powder was accurately weighed 50 mg into a 5 ml volumetric flask. Methanol was added to a sample flask to get the final volume. After that, the sample flask was sonicated for 30 min. The supernatant solution was filtered through

a 0.45 µm nylon membrane and transferred into an HPLC vial before analysis.

*3.2.2 Preparation of standard solutions*

CBD and THC standards were accurately weighed and dissolved in methanol at a 1,000 µg/ml concentration for stock solutions. The working solutions were prepared by diluting the stock solution with methanol to get a final concentration.

*3.2.3 HPLC system*

Chromatographic experiments were carried out on Agilent Technologies 1260 Infinity HPLC system (USA), comprising quaternary pumps and a photodiode array detector. The detection wavelength was monitored at 220 nm. Chromatographic separations were achieved on a ZORBAX Eclipse Plus C18 (4.6 x 100 mm, 3.5 µm) column at 30°C with an injection volume of 10 µl. Acetonitrile and water were used as mobile phases for step-gradient elution shown in Table 3 with a 0.5 ml/min flow rate.

**Table 3** HPLC condition for determination of CBD and THC contents in Suk-SaiYas pills

| Time (min) | Acetonitrile (%) | Water (%) |
|---|---|---|
| 0 | 73.5 | 26.5 |
| 9.0 | 73.5 | 26.5 |
| 9.5 | 77.0 | 23.0 |
| 17.5 | 77.0 | 23.0 |
| 18.0 | 90.0 | 10.0 |
| 23.0 | 90.0 | 10.0 |
| 23.5 | 73.5 | 26.5 |
| 32.0 | 73.5 | 26.5 |

*3.2.4 Method validation*

The validation method for the determination of CBD and THC contents in the Suk-SaiYas pills was performed according to the International Council for Harmonization (ICH) Guidelines for validation of analytical procedures Q2 (R1) (European Medicines Agency. (1995).

Specificity

The cannabis powder was prepared using *Cannabis sativa* leaves for the specificity analysis. Briefly, cannabis leaves were dried in the oven at 50°C for 24 hours, ground, and filtered through a 60 mesh sieve to receive the cannabis powder. Then, the cannabis powder, the Suk-SaiYas pill, and mixed standard of CBD and THC were separately prepared in methanol and analyzed using HPLC. After that, the chromatogram of the Suk-SaiYas pill was compared with the standards, cannabis powder, and blank solution. The specificity of the method was evaluated by the discrimination of target analytes, CBD and THC, from other components in the sample.

Range and Linearity

The calibration curve of CBD and THC were established in the range of 0.25 to 10 µg/ml. Seven concentrations of the CBD and THC mixture were prepared in methanol and repeated in triplicates in each concentration. After that, the calibration curve was constructed by plotting a graph between the peak area and standard concentration. Regression equations were calculated to confirm good linearity within the determination of range. The acceptance criteria were determined at $R^2 \geq 0.995$ (AOAC Official Methods of Analysis, 2019).

Precision

The method's repeatability has been confirmed by six determinations of the CBD and THC mixture at 100% of test concentrations (2.5 µg/ml). While the intermediate precisions studied were performed by six independent injections on three consecutive days. After that, the percent relative standard deviation (% RSD) was calculated with the acceptance criteria of not more than 5% and 10% for repeatability and intermediate precisions, respectively (AOAC International, 2020).

Accuracy

The accuracy was studied using the standard addition method at five different concentration levels. Each concentration level was analyzed in three replications. Shortly, the standard of CBD and THC were spiked to samples at 0.5, 1.0, 2.5, 5.0, and 10 µg/ml. The method's accuracy was expressed as % recovery of CBD and THC from the spiked sample. The acceptance criteria were 85 - 118% for cannabinoids content between 0.05 - 0.5% dry weight of plant material (AOAC International, 2020).

Detection limit and Quantitation limit

Limits of detection (LOD) and quantitation (LOQ) were estimated based on the signal-to-noise ratio (S/N). The LOD and LOQ

were specified using instrument reports at S/N of 3:1 and 10:1, respectively.

## 4. Results and discussion

### 4.1 Development of HPLC method

In this research, the authors aimed to optimize the HPLC method to quantify the CBD and THC content in the Suk-SaiYas pills. Previous studies indicated that the type and concentration of an acidic solvent in the mobile phase were affected by cannabinoid separation. Patel, Wene and Fan (2017) found that a lower concentration of an acid solution in the mobile phase improves the cannabinoids separation system. Besides, the temperature and pH condition affected the stability of CBD and THC. These active compounds were unstable in strong pH conditions and temperatures higher than 40°C (Flemming, Muntendam, Steup, & Kayser, 2007; Fraguas-Sánchez, Fernández-Carballido, Martin-Sabroso, & Torres-Suárez, 2020; Yangsud et al., 2021a). Thus, acetonitrile, methanol, and water were chosen for HPLC analysis. The different columns and mobile phase systems were investigated with gradient elution. The HPLC system was achieved using acetonitrile and water as mobile phases. The temperature of the separation was kept constant at 30°C.

The system suitability results in Table 4 indicated that both peaks of analytes, CBD and THC, were separated from other peaks completely and eluted at the same retention time in each sample with less % RSD values. The tailing factor demonstrated minimum values of less than 1.5 for both analytes. Moreover, the asymmetry factor values represented an excellent peak shape of CBD and THC. At the same time, theoretical plates were greater than 2,000.

**Table 4** System suitability parameters

| | **Retention time** %RSD | **Resolution** *Rs* | **Tailing factor** *T* | **Asymmetry** *As* | **Number of theoretical plates** *N* |
|---|---|---|---|---|---|
| CBD | 0.3 | 2.0 | 1.0 | 1.0 | 7983 |
| THC | 0.1 | 4.7 | 1.1 | 1.0 | 13010 |

### 4.2 Method validation

#### Specificity

The HPLC method's specificity was identified by comparing the chromatographic pattern between blank, mixed standard, and samples. The chromatographic results suggested that CBD and THC were eluted at 10.11 ± 0.02 min and 19.27 ± 0.03 min, respectively (Figure 1C). The overlay chromatogram of cannabis powder and Suk-SaiYas pill solution in Figures 1B and 1A displayed the peak area of CBD and THC at the same retention time of mixed standard solution. On the contrary, the blank solution has not detected any signal (Figure 1D).

#### Range and Linearity

The calibration curve of CBD and THC were plotted in the concentration range of 0.25 - 10.0 μg/ml. The results in Figure 2 showed that the peak area signal appeared directly proportional to the standard concentration. It expressed good linearity over the concentration ranges with the coefficient of determination ($R^2$) 0.9998 and 0.9999 for CBD and THC, respectively. The linear regression equation was found to be y = 159,345x + 606 for CBD and y = 133,679x - 2,761 for THC. This result was similar to the research of Patel et al. (2017), which presented that CBD and THC appeared with good linearity in the concentration range of 0.25 – 50 μg/ml with $R^2 > 0.9998$.

#### Precision

The precision of the HPLC method was expressed as repeatability for the same day and intermediate precision for three different days. The data presented less % RSD values of the repeatability range between 0.25 - 0.43% and 0.67 - 0.99% for CDB and THC, respectively (Table 5). Meanwhile, the % RSD of intermediate precision of both analytes was less than 2%. This method appeared to have good precision by presenting a % RSD lower than 5% for repeatability and 10% for intermediate precision.

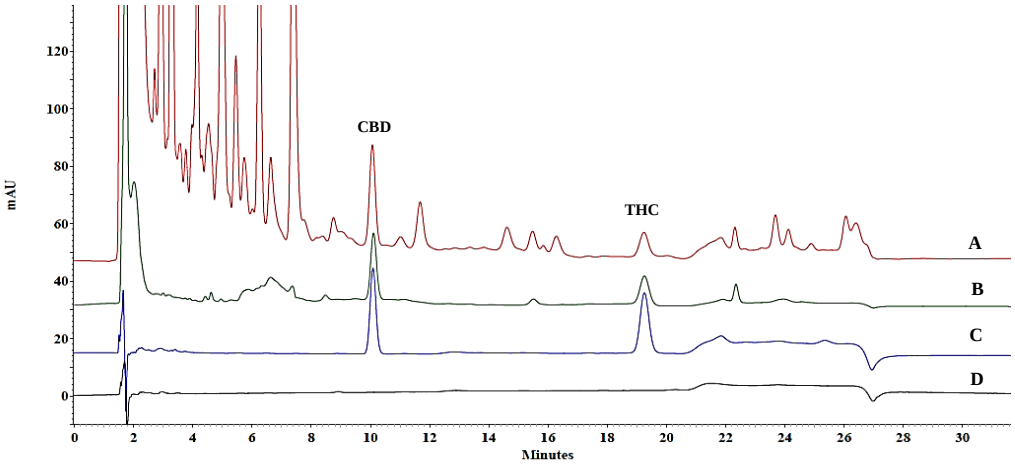


**Figure 1** Overlay chromatograms of the Suk-SaiYas pill (A), cannabis *powder* (B), standards mixture (C), and blank (D)

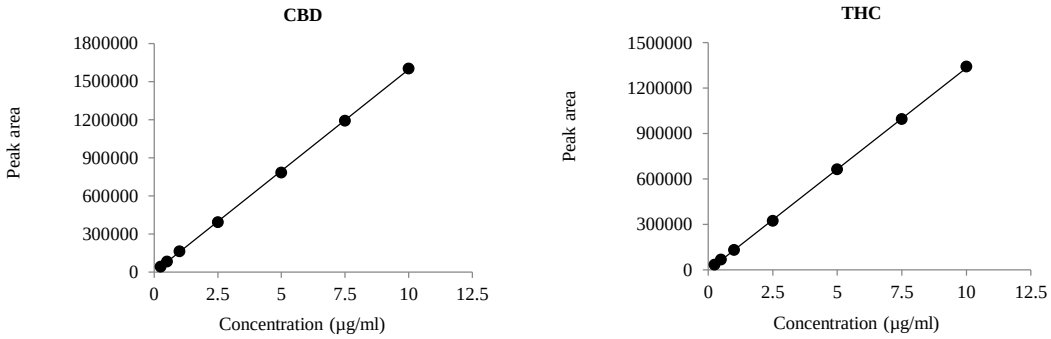


**Figure 2** Calibration curve of standards CBD and THC

**Table 5** Precision data for determination of CBD and THC contents in Suk-SaiYas pills

| | CBD (%RSD) | THC (%RSD) |
|---|---|---|
| Repeatability | | |
| Day 1 | 0.25 | 0.99 |
| Day 2 | 0.38 | 0.67 |
| Day 3 | 0.43 | 0.87 |
| Intermediate precision | 1.83 | 1.22 |

Accuracy

The % recovery value illustrated the method's accuracy. The % recovery of nearly 100% indicated that the method could be analyzed close to the actual value. From these studies, five different concentrations of each compound in Table 6 exhibited the % recovery range between 92 – 107% and 97 – 109% for CBD and THC, respectively. The accuracy values of this system fell within the acceptance criteria for cannabinoid content between 0.05 - 0.5% dry weight (AOAC International, 2020). Thereby, it is concluded the HPLC method provided accurate values for quantitation of CBD and THC in the Suk-SaiYas pills.

**Table 6** Accuracy data from spiked standards CBD and THC in Suk-SaiYas pill sample

| Standard | Concentration (µg/ml) | % Recovery |
|---|---|---|
| CBD | 0.5 | 96.13 ± 3.90 |
| | 1.0 | 105.02 ± 1.79 |
| | 2.5 | 100.73 ± 1.13 |
| | 5.0 | 101.52 ± 0.24 |
| | 7.5 | 99.94 ± 0.40 |
| THC | 0.5 | 104.19 ± 1.60 |
| | 1.0 | 104.60 ± 4.19 |
| | 2.5 | 100.65 ± 3.08 |
| | 5.0 | 102.86 ± 0.24 |
| | 7.5 | 101.14 ± 0.37 |

Detection limit and Quantitation limit

The LOD and LOQ of the HPLC method were calculated based on the signal-to-noise ratio. The quantitation limits of CBD and THC were detected at 100 ng/ml. In comparison, the LOD was found at 30 ng/ml. This result matched the HPLC validation criteria for the cannabis dried plant material detection, which recommended that the LOQ of lower cannabinoids content in cannabis should be less than 0.1% w/w (Vaclavik et al., 2019).

Today, a lot of HPLC research has described quantifying cannabinoids in cannabis plant material. Only some research studies focused on cannabis medicines. The research of Yangsud et al. (2021b) indicated that the HPLC method was utilized to control cannabis sublingual drops in stability testing. Using isocratic elution of water and methanol could completely separate CBD and THC from other impurities. Besides, Saingam and Sakunpak (2018) accomplished monitoring active compounds, CBD and THC, in oromucosal spray from cannabis extract. The developed HPLC method exhibited good linearity with high precision and accuracy for CBD and THC quantitation. Moreover, the HPLC-DAD was employed for quantitative analysis of cannabinoids in commercial products such as cannabis oral supplements, food, and beverages, giving acceptable values of precision and accuracy analysis. The results indicated that 11 cannabinoids were eluted completely within 40 min using acetonitrile and 0.5% acetic acid (66:34) as a mobile phase at a flow rate of 1.0 ml/min. Tablets and capsule powder of cannabis products demonstrated the cannabinoid content between 0.05 – 0.16% w/w with less % RSD at 2.5% and 1.1% for CBD in tablets and capsules, respectively, while the THC presented a % RSD higher than CBD. The accuracy of the method yielded a good % recovery value between 90 -100% for both commercial products (Ciolino, Ranieri, & Taylor, 2018b). Nevertheless, the scientific research for cannabinoid detection in traditional medicines has less supporting data.

## 5. Conclusion

In the current work, the authors developed an HPLC procedure for routine analysis of active compounds in the Suk-SaiYas pills. The developed HPLC method demonstrated precise and accurate values for CBD and THC contents determination. HPLC results confirmed that the analysis method passed the criteria requirement of the AOAC standard method for cannabis quantitation. Consequently, this HPLC method could be applied for quality control of the Suk-SaiYas pills. However, further studies are required to improve the faster HPLC system to reduce analytical time and solvent usage.

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