Journal of Medicinal Materials, 2022, Vol. 27, No. 2 (pp. 107 - 111)

DEVELOPMENT AND VALIDATION OF FLAME ATOMIC ABSORPTION SPECTROMETRY (FAAS) FOR THE DETERMINATION OF POTASSIUM IN *STACHYS AFFINIS* ROOTS

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(Received March 29th, 2022)

Summary

Development and Validation of Flame Atomic Absorption Spectrometry (FAAS) for the Determination of Potassium in *Stachys affinis* Roots

Stachys affinis was considered a food and a medicinal plant with the s of anti-oxidation, antibacteria, anti-inflammation... and contains high content of potassium in roots. In this study, for development of potassium in *Stachys affinis*, Box-Behnken model was applied to optimize the sample digestion followed by FAAS. The experimental variables chosen were ashing time (270 - 390 min), furnace temperature (450 - 650°C), and nitric acid concentration (16 - 64%). Concentration values of potassium were used as a response. The potassium concentration can reach as high as 17.74 ± 0.26 mg/g at the ashing time of 340 min, furnace temperature of 461°C, and nitric acid concentration 38%. The analytical method developed and validated according to ICH and AOAC guidelines met the requirements of selectivity, precision (RSD = 1.44%), accuracy (recovery rate ranged from 95.6% to 107.8%). The calibration curve was obtained using linear regression y = 0.1049x - 0.0102 (R^2 = 0.9991) in the range of potassium concentration from 0.2 ppm - 5 ppm. The LOD and LOQ values for potassium were 0.046 ppm and 0.138 ppm, respectively.

Keywords: *Box-Behnken design, FAAS, Stachys affinis.*

1. Introduction

In China and Japan, *Stachys affinis* was considered a food and a medicinal plant with effects of anti-oxidation, antibacterial, anti-inflammatory... Besides the main active ingredient, Stachyose, roots of *Stachys affinis* were shown that there are many elements such as K, P, Ca, Mg, Fe... and potassium (2.36%) was the most abundant micro-nutrient [1].

There are various analytical methods for the determination of potassium such as gravimetry, atomic emission spectrophotometry, atomic absorption spectrophotometry, ... Among these analytical methods, the AAS technique is the most used method because it reached the best results in a shorter time and with minimal contamination and reagent consumption [2]. However, the sample preparation is the critical part of this technique because of high errors. Therefore, a good choice of sample treatment becomes a key ensuring to obtaining reliable results [2],[3].

Sample preparations for potassium determination in this study were studied based on guidelines of TCVN 10916:2015 and AOAC 985.35 as follows the sample was heated with nitric acid on an electric stove until the sample is dry and then ashed in the furnace at 525°C until a white or light grey ash residue is formed (< 8 h) [4],[5]. The different matrices might cause different results in the digestion process, which will result in different mineral analysis results. Therefore, it is necessary to re-investigate the optimum conditions of the sample treatment process for the specific sample. The main factors affecting the recovery of sample treatment were the volume of nitric acid, temperature, and the digestion time [6]. Typical ashing temperatures were chosen in the range of 450 to 550 °C [2],[6],[7],[8], and the taken time of digestion was 15 minutes to 8 hours [4],[5],[6] depending on the sample matrix.

To investigate the optimum conditions for digestion process, design of experiments is considered as a useful methodology that examines the simultaneous influences of the factors in this study, Box-Behnken design (BBD) was used to construct second order models that could predict how variables (ashing time, furnace temperature, and nitric acid concentration) affected the digestion process of *Stachys affinis* powder. The method validation was carried out according to ICH and AOAC guidelines.

2. Materials and methods

2.1. Materials

Plant materials: The roots of *Stachys affinis* Bunge Lamiaceae were provided by Tipharco Pharmaceutical Joint Stock Company (Ward 9, My Tho City, Tien Giang Province, Vietnam) collected in May 2019 then washed, dried at 60°C until the constant weight. The moisture content of the powder (5.74%) was carried out according to Appendix 9.6 of Vietnamese Pharmacopoeia V [9]. The sample was ground to a fine powder and stored at room temperature.

Chemicals and reagents: All chemicals and reagents were of analytical grade. Potassium standard solution 1000 µg/mL (≥ 99.0% purity, lot: HC85201630) was purchased from Merck

(Germany), nitric acid (65%) was purchased from Xilong (China).

The instrument blank: A solution of nitric acid 1%.

Standard solutions: The working solutions in the concentration range of 0.2 - 5 ppm for potassium were prepared by diluting 1000 µg/mL potassium in 1% nitric acid.

Apparatus: Spectrometric analyses were performed using the atomic absorption spectrometer (iCE 3300 Thermo Scientific, USA); Analytical balance (Practum 124-1S 0.1 mg, Sartorius, Germany); Furnace (Lenton, UK). The statistical software MODDE 12.1 (Sartorius, Germany) was used for estimating the responses of experimental variables. The statistical analysis for the analytical responses and validation data were evaluated with Microsoft Excel 2016 software.

2.2. Sample preparation

Sample solutions: Accurately weigh 0.5000 g powder of *Stachys affinis*, add 2 mL concentrated nitric acid and heat on an electric stove at 200°C until the sample is black. Ash the sample in the furnace until a white or light grey ash residue is formed. Dissolve the residue with nitric acid 1% in a 50 mL volumetric flask, filter and dilute 1 mL of the filtrate solution into a 100 mL volumetric flask with nitric acid 1% prior to FAAS analysis.

Spiked samples solutions: Accurately weigh 0.5000 g powder of *Stachys affinis*, add the reference standards (potassium standard solution 1000 µg/mL) at three different volume levels (4, 5, 6 mL) to obtain the spike concentrations corresponding to 80, 100 and 120%. Add 2 mL concentrated nitric acid and continue processing as the sample solution.

2.3. FAAS procedure

A Thermo Scientific iCE 3300 atomic absorption spectrometer with an air-acetylene flame (with a fuel flow of 0.9 L/min) was used to perform the measurements of potassium absorbance. A Potassium Hollow Cathode Lamp at a wavelength of 766.5 nm with a bandpass of 0.5 nm was used. Burner height was 7.0 mm. Background correction was achieved with a deuterium lamp.

2.4. Optimization of sample treatment procedure

A Box-Behnken design (BBD) with three independent variables was used for the optimization of sample treatment before the determination of potassium by FAAS. The variables were coded at three levels: −1, 0, and +1. The whole design consisted of 15 experimental points, including three replications of the center points and 12 factorial points. The independent variables and their ranges were as follows: ashing time (X_1) ranged from 270 to 390 minutes, furnace temperature (X_2) ranged from 450 to 650°C, and nitric acid concentration (X_3) ranged from 16 to 64% (Table 1). 15 experimental runs were performed at random and the potassium content (Y mg/g) in powder of *Stachys affinis* roots was chosen as the response for having the highest concentration of the samples. Analysis of variance was used to identify the factors which significantly influence the response. A second-order polynomial regression equation was used to predict the response and the process parameters are optimized for obtaining a specific objective function. [10]

The experimental design and statistical analysis select the condition so that the potassium concentration is maximum were performed using MODDE 12.1 software. Quantifying potassium according to the conditions predicted by the software. If the results are reproducible, these optimized conditions were used to validate the analytical procedure.

Table 1. The experimental variables and levels of BBD

Independent variables	Level		
	-1	0	+1
X_1 - Ashing time (min)	270	330	390
X_2 - Furnace temperature (°C)	450	550	650
X_3 - Nitric acid concentration (%)	16	40	64

2.5. Validation of analytical procedures

Validation of the method to assay potassium content in the roots of *Stachys affinis* was performed according to International Conference on Harmonization guidelines (ICH) and AOAC Guidelines for Single Laboratory. Evaluated parameters are specificity, linearity of the calibration curve, LOD, LOQ, precision and accuracy. Specificity was evaluated by measuring the absorbance of the blank solution, standard solution, sample solution and spiked samples solution. The linearity of the FAAS method for the determination of potassium was evaluated in a concentration range of 0.2 - 5 ppm. The sensitivity of the method was estimated by finding the limit of detection (LOD) and the limit of quantification (LOQ) from the regression data. The precision of the method was evaluated by estimating its repeatability, intermediate precision. Repeatability is determined by measuring the concentration of 6 sample solutions and calculating the RSD value. For intermediate precision, sample solutions were prepared and measured on two

different days by two different analysts. Accuracy was determined by calculating percent recovery after adding a known amount of reference standards at three different concentration levels to the samples to obtain the concentrations corresponding to 80, 100 and 120%. [11],[12]

3. Results and discussion

3.1. Optimization of sample treatment procedure

The experimental design matrix used, and the results obtained by BBD were listed in Table 2. From the results in Table 2, using MODDE 12.1 software to analyze the influence of independent variables on quantified potassium concentration. According to the ANOVA results (Table 3), the coefficient of determination (R^2 = 0.967) and adjusted coefficient of determination (R^2 adj = 0.906) indicated a high dependence and correlation between the observed and the predicted values of the response. The model P-value of 0.004 < 0.05 implies the model is significant. the lack of fit P-value of 0.841 > 0.05 implies the lack of fit is not significant relative to the pure error and proved that the model was suitable for the experiment.

Table 2. Experimental design matrix and the responses for BBD.

Run	X_1 (min)	X_2 (°C)	X_3 (%)	Y (mg/g)
1	270	450	40	16.6
2	390	450	40	17.6
3	270	650	40	12.0
4	390	650	40	12.8
5	270	550	16	15.5
6	390	550	16	15.2
7	270	550	64	15.5
8	390	550	64	15.9
9	330	450	16	17.5
10	330	650	16	12.6
11	330	450	64	17.0
12	330	650	64	13.1
13	330	550	40	17.8
14	330	550	40	16.3
15	330	550	40	16.6

Table 3. ANOVA results for optimization by BBD.

	DF	SS	MS	F	p
Regression	9	51.6567	5,76235	16.0459	**0.004**
Residual	5	1.79558	0,359117		
Lack of fit	3	0.527269	0,175756	0.277149	**0.841**
Pure Error	2	1.26831	0,634157		
Total Corrected	14	53.6567	3,83262		
R^2 = 0.967; Q^2 = 0.790; R^2 adj = 0.906					

The values of p less than 0.05 indicated model terms are significant. From the results of Table 4, it can be deduced that the linear contribution of furnace temperature (X_2), the quadratic contribution of furnace temperature (X_2^2) and the quadratic contribution of ashing time (X_1^2) are significant model terms. Interactions of the individual variables in this study were not significant to potassium concentration in the selected range. The effective order of test variables on the potassium concentration was as follows: $X_2 > X_1 > X_3$.

The regression equation is set up as follows:

$$Y\ (mg/g) = 16.9257 + 0.244126X_1 - 2.28762X_2 - 0.869084X_1^2 - 1.31008X_2$$

Where, Y: potassium content (mg/g), X_1: ashing time (min), and X_2: furnace temperature (°C).

Or: The potassium content = 16.9257 + 0.244126 * Ashing time - 2.28762 * Furnace temperature - 0.869084 * Ashing time^2 - 1.31008 * Furnace temperature^2.

From Fig. 1, it can be seen how the value of the potassium concentration may decrease if we take a higher furnace temperature (X_2). Also, we can infer that although ashing time (X_1) and concentration of nitric acid (X_3) do not greatly influence the resolution, better resolutions were obtained for medium values of ashing time and concentration of nitric acid.

Table 4. Coefficients in terms of coded factors

	Coeff. SC	Std. Error	P
Constant	16.9257	0.345985	$6.74401*10^{-8}$
X_1	0.244126	0.211872	0.301321

X_2	-2.28762	0.211872	0.000118205
X_3	0.0815007	0.211872	0.7163
X_1^2	-0.869084	0.311867	0.0385942
X_2^2	-1.31008	0.311867	0.00848313
X_3^2	-0.541834	0.311867	0.142825
X_1X_2	-0.0525007	0.299632	0.867784
X_1X_3	0.17125	0.299632	0.59237
X_2X_3	0.25025	0.299632	0.441677

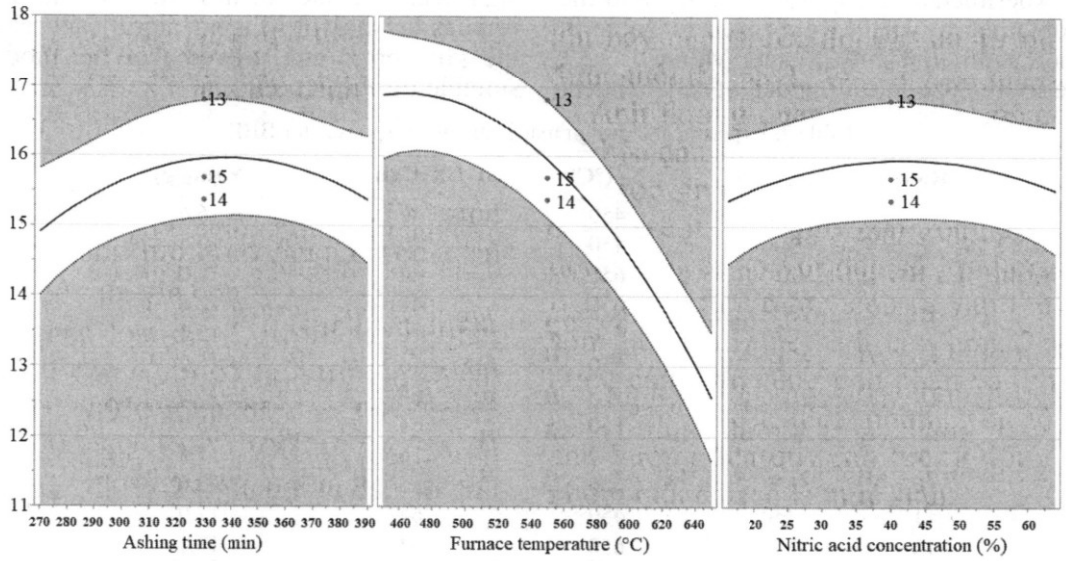

Fig. 1. Analysis of the individual variables in BBD

According to the results of the prediction optimizer tool on MODDE 12.1, the predicted optimal conditions for maximum potassium concentration were as follow: The ashing time of 340 min, furnace temperature of 461°C and nitric acid 38%.

At these optimal conditions, the predicted potassium concentration was 17.95 (mg/g). Three replicates of verification experiments were undertaken, and the outcome was 17.78 (mg/g), which was very close to the predicted value of MODDE 12.1 software.

3.2. Validation of analytical procedures

Specificity

The type of acid used in the sample preparation procedure may strongly affect the measurement result. It is commonly known that in all atomic spectrometric techniques nitric acid is the most desirable reagent [7],[9]. This study used acid nitric for the digestion process and dilution of ash residue. The results of specificity (Table 5) show that: The absorbance of the blank solution (nitric acid 1%) closes to zero. Thus, nitric acid does not affect the absorption of potassium. The standard solution and sample solution both have absorbance at the maximum wavelength of potassium. The spiked samples solution has higher absorbance than the standard solution and sample solution. So, the analytical procedure is good specificity.

Linearity and sensitivity

The results of the linearity are summarized in Table 5. The correlation coefficient $R^2 = 0.9991 > 0.995$ displays that there was a good linear relationship between the concentration and the absorption intensity of the potassium solution according to linear equations y = 0,1049x - 0.0102, in the potassium concentration range from 0.2 ppm - 5 ppm, LOD and LOQ for potassium were 0.046 ppm and 0.138 ppm, respectively. This shows that the method was linear within the established range and the proposed analytical method was sufficiently sensitive.

Table 5. Results of specificity and linearity

Specificity	Solution	Blank	Standard	Sample	Spiked samples standard	
	Absorbance	-0.0032	0.0863	0.1695	0.2672	
Linearity	**Concentration (ppm)**	0.2	0.5	1	2	5
	Absorbance	0.0151	0.0481	0.0863	0.1954	0.5172
	y = 0,1049x - 0.0102, $R^2 = 0.9991$ σ = 0.0015, LOD = 0.046 ppm, LOQ = 0.138 ppm					

System suitability

The results of system suitability are summarized in Table 6. The RSD values of analyzing six replicates of a standard solution at 1 ppm concentration of potassium were 0.49% < 2%. So, the reproducibility of the FAAS system is adequate for the analysis to be done.

Precision

A summary of repeatability and intermediate precision is listed in Table 6. The RSD values were 0.91% and 1.93%, respectively. %RSD < 2% as suggested by ICH [5]. The results of ANOVA analysis showed that the value of F = 0.0126 < Fcrit = 4.9646 means that there was no difference between the two data sets. These results show that the proposed method is precise for the determination of the potassium concentration in *Stachys affinis*.

Table 6. Results of system suitability, repeatability and intermediate precision

	1	2	3	4	5	6	Average	RSD %
System suitability	0.0863	0.0859	0.0869	0.0868	0.0867	0.0860	**0.0864**	**0.49**
Analyst 1	17.61	17.49	17.88	17.78	17.71	17.91	**17.73 (mg/g)**	**0.92**
Analyst 2	17.83	17.87	17.40	17.38	17.69	18.30	**17.75 (mg/g)**	**1.93**
Intermediate precision: Average = 17.74 mg/g; SD = 0.26 mg/g; RSD = 1.44%								

Accuracy

The recoveries that determine the accuracy of the method are summarized in Table 7. The proposed method resulted in satisfactory recoveries ranging from 95.6% to 107.8%. The recoveries demonstrated that the matrixes have a negligible effect on the quantification of these compounds and the method was accurate within the desired range (80 - 115.0% as suggested by AOAC [6]).

Table 7. Results of accuracy studies

Samples	Concentration (ppm)		Recovery (%)	Average %	RSD %
	Spiked	**Detected**			
80%	0.80	0.82	102.5	102.7	0.31
		0.82	103.1		
		0.82	102.6		
100%	1.00	0.99	98.8	97.1	0.63
		0.96	95.6		
		0.97	96.8		
120%	1.20	1.24	103.7	105.4	2.00
		1.29	107.8		
		1.26	104.9		

These results showed that the method for assay potassium by FAAS met requirements of system suitability, specificity, linearity, sensitivity, precision, and accuracy for the determination of the potassium concentration in the roots of *Stachys affinis*.

4. Conclusions

Box-Behnken design was applied to the optimization of the digestion process conditions. The factors including the ashing time, furnace temperature and nitric acid concentration were investigated here. All the results indicate that BBD was successfully applied to determine the optimum factors for the determination of potassium content. The experimental values match well with the predicted data.

These optimized conditions were used to validate the analytical procedure. The method for determination of potassium in *Stachys affinis* met the requirements of system suitability, specificity, linearity, LOD, LOQ, precision and accuracy.

This study will provide an efficient, accurate and reliable method for the determination of Potassium contents in *Stachys affinis* roots by using FAAS.

Acknowledgment: *The roots of Stachys affinis were supported by Tipharco Pharmaceutical Joint Stock Company (Head office located at No. 15, Doc Binh Kieu Street, Ward 2, My Tho City, Tien Giang) (Project no. 2010-01-01-GEP04). The authors are grateful to M. Pham Quang Binh (Chairman of the Board) and Mrs Pham thi Xuan Quyen (CEO of Tipharco company) for providing the material plants.*

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Journal of Medicinal Materials, 2022, Vol. 27, No. 2 (pp. 112 - 116)

STANDARDIZED FLAVONOID EXTRACT FROM *DIOSPYROS KAKI* L.F LEAVES IMPROVES DYSLIPIDEMIA IN HIGH-CHOLESTEROL DIET FED RATS

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(Received March 29th, 2022)

Summary

Standardized Flavonoid Extract from *Diospyros kaki* l.f Leaves Improves Dyslipidemia in High-Cholesterol Diet Fed Rats

The current study designed to investigate the anti-dyslipidemia effects of the standardized flavonoid extract from *Diospyros kaki* L.f leaves (DK extract) in chronic high-cholesterol diet fed rats. Wistar rats were orally administered oil-cholesterol mixture. Rats were daily treated with DK extract (50 and 100 mg/kg of body weight; p.o.) or atorvastatin (10 mg/kg of body weight; p.o.) for 4 consecutive weeks. Body weight, serum lipid profiles, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were evaluated for every 2 weeks. Body weight was significantly increased in the DK extract-treated group compared to the vehicle-treated group. Treatment of DK extract decreased the serum total cholesterol, triglycerides, and non-high-density lipoprotein cholesterol levels, and increased the serum high-density lipoprotein cholesterol. Additionally, DK extract substantially diminished enzymatic activity serum AST and ALT which were increased in the hypercholesterolemia rats. Our finding suggested that DK extract improves dyslipidemia and liver function in high-cholesterol diet fed rats.

Keywords: *Diospyros kaki* L.*f leaves, Flavonoid, High-cholesterol diet, Dyslipidemia.*

1. Introduction

Dyslipidemia is a major contribution to the onset of cardiovascular diseases such as atherosclerosis, coronary heart disease, and cerebrovascular disease, which are the main causes of the global burden of diseases [1]. Dyslipidemia is defined as elevation of serum total cholesterol and/or triglyceride or reduced high-density lipoprotein cholesterol. Treatments of these disorders include diet control, physical exercise, surgery, and medications [2]. Currently, although statins have been widely used to reduce plasma lipids, their usage may be limited due to their side effects such as hepatotoxicity, rhabdomyolysis, or skeletal muscle injury [3]. Thus, alternative therapeutics using herbs and/or natural products have been proposed to control lipid metabolism [4].

Diospyros kaki L.f., called persimmon, belongs to the family Ebenaceae. This plant is widely distributed in China, India, Japan, Korea, and Vietnam. Persimmon leaves were traditionally utilized as a medicine, health beverage, and cosmetic [5]. Evidence showed that the powdered whole persimmon leaf improved plasma and hepatic lipid levels profile in high-fat fed rats [6]. However, the constituents of persimmon leaves are resposible for producing these effects remain unclear. We recently demonstrated that standardized flavonoid extract from *Diospyros kaki* leaves (DK extract) exhibited the hypolipidemic effects using tyloxapol-injected mice, an animal model of endogenous dyslipidemia [7]. The effects of flavonoid extract from persimmon leaves in the exogenous dyslipidemic animals remain not adequately clear.

Diet-induced hyperlipemia is the most relevant stimulus for the induction of atherosclerotic lesions in humans. Thus, diet-induced hypercholesterolemia is almost always useful for the assessment of agents that interfere with absorption, degradation, and excretion of cholesterol, with minimal effects on cholesterol biosynthesis. Thus, elucidation of the anti-dyslipidemic effects of flavonoids extracted from persimmon leaves using diet-induced a