

A novel reverse phase high-performance liquid chromatography method for standardization of *Orthosiphon stamineus* leaf extracts

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ABSTRACT

Background: *Orthosiphon stamineus* Benth. (Lamiaceae) is a traditional medicinal plant which has been used in treating various ailments such as kidney diseases, bladder inflammation, arthritis and diabetes. The leaves contain high concentration of phenolic compounds, thus, rosmarinic acid (RA), 3'-hydroxy-5, 6, 7, 4'-tetramethoxyflavone (TMF), sinensetin (SIN) and eupatorin (EUP) were chosen as a marker compounds for standardization of various *O. stamineus* leaf extracts. **Objective:** The aim was to develop and validate a new high-performance liquid chromatography (HPLC) method for quantification of 4 marker compounds (RA, TMF, SIN, EUP) in various *O. stamineus* leaf extracts. **Materials and Methods:** The method was developed and validated using RP-HPLC-diode-array detection at 320 nm for accuracy, precision and limits of detection and was applied for quantification of it markers in five different extracts prepared in solvents with increasing polarity, using a gradient mobile phase 0.1% formic acid: Acetonitrile at a flow rate of 1 ml/min on reverse phase acclaim polar advantage II C18 column (3 μ m, 3 $\times$ 150 mm) with 18 min separation time. **Results:** The developed method provided satisfactory precision, and the accuracy of this method was in the range of 90.2% to 105.5%. All of 4 compounds showed good linearity at $R^2 > 0.999$. **Conclusion:** The developed method is a simple, cost effective with shorter run time (18 min) in comparison to previous methods (30 min) and utilization of environmental-friendly solvents system. Therefore, this method has the potential to replace currently used methods in the routine standardization work of *O. stamineus* extracts, raw materials and its commercial products.

Key words: 3'-hydroxy-5, 6, 7, 4'-tetramethoxyflavone, eupatorin, *Orthosiphon stamineus*, rosmarinic acid, sinensetin

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INTRODUCTION

Orthosiphon stamineus Benth. (Lamiaceae) is locally known as "Misai Kucing", and has been traditionally used in the treatment of various ailments including eruptive fever, epilepsy, gallstones, hepatitis, rheumatism, hypertension, syphilis and renal calculus. In Malaysia, the leaves are usually prepared as tea and consumed to improve health, and for treatment of kidney and bladder inflammation, gout and diabetes.^[1,2] This herb has gained a great interest

nowadays due to its wide range of pharmacological effects such as antioxidant activity,^[3-6] antiangiogenic activity,^[7-10] diuretic and hypoglycemic effects.^[11-13] *O. stamineus* was listed in some pharmacopoeias including The Malaysian Herbal Monograph,^[14] British Pharmacopoeia,^[15] and Materia Medika Indonesia.^[16] Previous phytochemical studies on *O. stamineus* have reported the leaves contain a high concentration of phenolic compounds; Sumaryono *et al.*^[17] isolated twenty phenolic compounds including nine lipophilic flavones, two flavonol glycosides, and nine caffeic acid derivatives which includes rosmarinic acid (RA) and 2,3-dicaffeoyltartaric acid. This plant was also found to contain other compounds such as isopimarane-type diterpenes, highly oxygenated stamine-type diterpenes, and the pentacyclic triterpenes such as betulinic acid, oleanolic

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acid, ursolic acid and *b*-sitosterol.^[18] Literature survey reveals few high-performance liquid chromatography (HPLC) methods for the quantification of polyphenols in this plant extracts^[3,17,19-21] but these methods suffered from drawbacks like time consuming for HPLC separation, use of buffer which may deteriorate efficiency of column or HPLC system and use of unfriendly solvent system. Thus, there is a great need for a faster, reproducible and reliable method for routine standardization work^[22] of *O. stamineus* extracts as well as raw leaves and commercial products for quality control purposes. In that sense, this present study has been undertaken to develop and validate a novel RP-HPLC-diode-array detection (DAD) method for the analysis of four marker compounds which include RA, 3'-hydroxy-5, 6, 7, 4'-tetramethoxyflavone (TMF), sinensetin (SIN) and eupatorin (EUP) [Figure 1] with a shorter run time and using environmental-friendly solvent system and to suit to mass spectrometry (MS) analysis as well as application in pharmacokinetic study in future research.

MATERIALS AND METHODS

Chemicals and reagents

High-performance liquid chromatography-grade acetonitrile and formic acid were purchased from Merck (Petaling Jaya, Selangor, Malaysia). The reference compounds RA, TMF, SIN and EUP were purchased from ChromaDex (USA). Deionized water for HPLC was prepared using ultra-pure water purifier system (Thermo Scientific Barnstead). The reverse phase Acclaim Polar Advantage II C18 column used (3 μ m, 3 $\times$ 150 mm) was purchased from Dionex (Thermo Scientific).

Collection and authentication of plant

Orthosiphon stamineus was cultivated and propagated under controlled conditions in a joint venture of Universiti Sains Malaysia (USM) - Universiti Malaysia Perlis (UNIMAP) at Titi Tinggi, Perlis, Malaysia. The plant material was authenticated by the Herbarium, School of Biological Sciences, USM, where a voucher specimen (no. 11009) was deposited. The leaves were oven-dried at 40°C and

powdered using a milling machine (Restch, Germany). The powdered leaves were stored in a tight container at room temperature and in dry area.

Extraction of plant material for analysis

Fine powdered material of 500 g was extracted with methanol, ethanol, methanol: Water (1:1) and ethanol: Water (1:1) using Soxhlet extraction method at 60°C for 8 h, while the water extract was extracted using reflux extractor for 8 h. The process was repeated thrice in both extraction methods, then extracts were bulked, concentrated using a rotary evaporator under vacuum at 60°C (Buchi, Germany), and finally freeze-dried (Scanvac coolsafe, Denmark). The lyophilized extracts were kept in a freezer at -20°C prior to use.

Preparation of the standard mixtures

Stock solutions of RA, TMF, SIN and EUP were prepared at 1 mg in 1 ml of HPLC-grade methanol: Water (1:1). The stock solution was diluted with the same solvent into 10 concentrations range from 0.195 to 100 μ g/ml to make a working standard solution. These standard solutions were stored at 4°C prior to use.

Preparation of the sample solution

The different extracts were prepared by dissolving the freeze dried powder at 10 mg/1 ml in a mixture of methanol: Water (1:1) and sonicated for 30 min as a stock solution. The stock solution was then diluted in the same solvent to 1 mg/ml as a sample solution. The sample solutions were then filtered through 0.45 μ m syringe filters and transferred into HPLC vial.

Instrumentation and chromatographic conditions

The instrument consisted of a Dionex-Ultimate® 3000 Rapid Separation LC system, which was equipped with an auto sampler, a quaternary pump, a degasser, a column oven, and a DAD detector.^[22,23] The chromatographic analysis was carried out using a reverse phase Acclaim Polar Advantage II C18 column. The column temperature was maintained at 40°C. The mobile phase was consisted of 0.1% formic acid solution (A) and acetonitrile (B) with gradient elution system as shown in Table 1 at a flow rate of 1 ml/min while

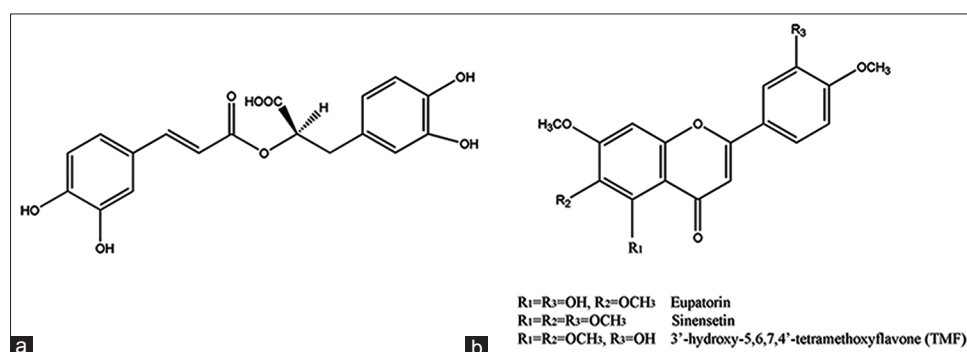


Figure 1: Chemical structure of (a) rosmarinic acid; (b) 3'-hydroxy-5, 6, 7, 4'-tetramethoxyflavone, sinensetin and eupatorin Chemical structure

the separation time was 18 min and the injection volume was 5 µl. The spectral data from the DAD-3000RS DAD was set at 210, 254 and 320 nm. For quantitative analysis, the wavelength was set at 320 nm. The peak identification was based on the retention time and the DAD spectrum against the standard presented in the chromatogram. The data acquisition was performed by Chromeleon software version 6.8 (Dionex, Thermo Fisher Scientific Inc.).

Method validation

The method was validated according to the International Conference on Harmonization guidelines.^[24] The following validation characteristics were evaluated: Selectivity, linearity, precision, accuracy and the limits of detection and quantification (LOD and LOQ).^[24,25]

Selectivity

The selectivity of the method was determined by comparing the retention time and the ultraviolet–visible spectroscopy (UV-Vis spectra) of RA, TMF, SIN and EUP obtained in the sample extracts with those of the reference compounds. The UV-Vis spectra were analyzed at three levels (beginning, middle and end) of each peak investigated.

Linearity

Linearity was determined by injecting 5 µl of the standard mixture in 10 concentrations ranges from 0.195 to 100 µg/ml in triplicate. The calibration curves were obtained for each individual compound by plotting the peak area versus concentration. Regression analysis was performed in order to determine the linearity (R^2) of the calibration graphs.

Precision

Precision was performed according to International Conference on Harmonization guidelines that included repeatability and intermediate precision.^[24] The reproducibility is not normally expected if intermediate precision is performed.^[25] For repeatability (intra-day assay precision), five concentrations of the marker compounds from 6.25 to 100 µg/ml were injected in six replicates on the same day and under the same experimental conditions. The intermediate precision was performed by same analyst using a different HPLC system (Agilent) and five concentrations (6.25-100 µg/ml) of marker compounds were

injected in three different days in triplicate. The precision method was reported as a percentage of relative standard deviation (%RSD) of peak concentration and retention time.

Accuracy

Accuracy was determined as a percentage recovery of RA, TMF, SIN and EUP by the standard addition method. The marker compounds were added at 12.5 and 25 µg/ml to the EtOH extract at 1000 µg/ml and were analyzed by HPLC. The peak area of the compounds in the EtOH extract (B), the individual reference compounds (C) and their combinations (A) was recorded. The percentage recovery was calculated as the following:

Percentage recovery = $([A-B]/C) \times 100$. The results are presented average $\pm$ standard deviation (SD) ($n = 3$).

Limits of detection and quantification

The LOD and LOQ were calculated through the slope and SD method^[24] using the following formula;

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

Where:

δ : Is the SD of the Y intercept of the linear regression equations.

S: Is the slope of the linear regression equations.

Quantification of rosmarinic acid, 3'-hydroxy-5, 6, 7, 4'-tetramethoxyflavone, sinensetin and eupatorin from different *Orthosiphon stamineus* extracts

O. stamineus extracts (5 µl) were injected at 1 mg/ml and the peak area corresponding to RA, TMF, SIN and EUP were recorded. Concentration of the marker compounds in the samples was then calculated by applying the linear regression equations of the standards calibration curves ($n = 3$). The content of these four marker compounds were presented as a w/w percentage (% w/w) of the dried extract.

RESULTS AND DISCUSSIONS

A reverse phase HPLC-DAD method for simultaneously determining of four marker compounds was successfully developed and validated for routine standardization of *O. stamineus* extracts. This method can be applied for herbal industries to detect and quantify all of the markers in raw leaves and commercial products for quality control purposes. All these marker compounds were detectable with a better peak shape and good resolution within 10 min. The scanned UV spectra of all compounds between 200 and 400 nm by DAD were showed in Figure 2. The spectrum showed that RA was absorbed in 195.7 nm and 327.9 nm while TMF and SIN were absorbed at 211.4 nm, 238.9 nm and 329.9 nm. EUP was absorbed in 210 nm,

Table 1: Gradient elution program used in separation of *O. stamineus* marker compounds

Time	Flow rate (ml/min)	Solvent ratio	
		A (0.1% formic acid)	B (ACN)
0	1	85	15
1	1	85	15
12	1	35	65
15	1	85	15
18	1	85	15

O. stamineus: *Orthosiphon stamineus*; ACN: Acetonitrile

274.4 nm and 340 nm. All the components were strongly absorbed in 320 nm and this considering the developed chromatographic condition is suitable to detect and quantify all of the markers with optimized wavelength at 320 nm. The peaks of the 4 marker compounds were scanned at their start, apex and tail.

Method validation

Selectivity

Selectivity of the method was determined by comparing the retention time of compounds obtained in the sample extracts with those of the reference compounds. The retention time of RA, TMF, SIN and EUP were 6.44 ± 0.003 , 8.30 ± 0.002 , 9.09 ± 0.002 and 9.93 ± 0.003 min, respectively. The retention time of the same compounds in *O. stamineus* extracts were 6.45 ± 0.004 , 8.30 ± 0.002 , 9.09 ± 0.000 and 9.93 ± 0.001 min, respectively. This indicates that the present HPLC method is rapid, precise

and accurate. The UV-Vis spectra of the compounds in *O. stamineus* extracts were also compared with the reference compounds [Figure 2]. These spectra were used to check the purity of the peaks in the extracts. The spectra of RA, TMF, SIN and EUP detected in the different extracts [Figure 3] were found to match those of the reference compounds. These findings confirm identity of the compounds, purity of the peaks, and selectivity of the developed method.

Linearity, limit of detection and limit of quantification

Mixtures of standard solutions were diluted in MeOH: Water (1:1) to make a ten series of concentrations range from 0.195 to 100 $\mu\text{g/ml}$ to establish a calibration curve. The calibration curve for four marker compounds was constructed by plotting the individual peak area (Y) versus concentration ($\mu\text{g/ml}$). Linearity of the developed method was presented in terms of correlation coefficient (R^2). The LOD and LOQ in chromatographic condition were

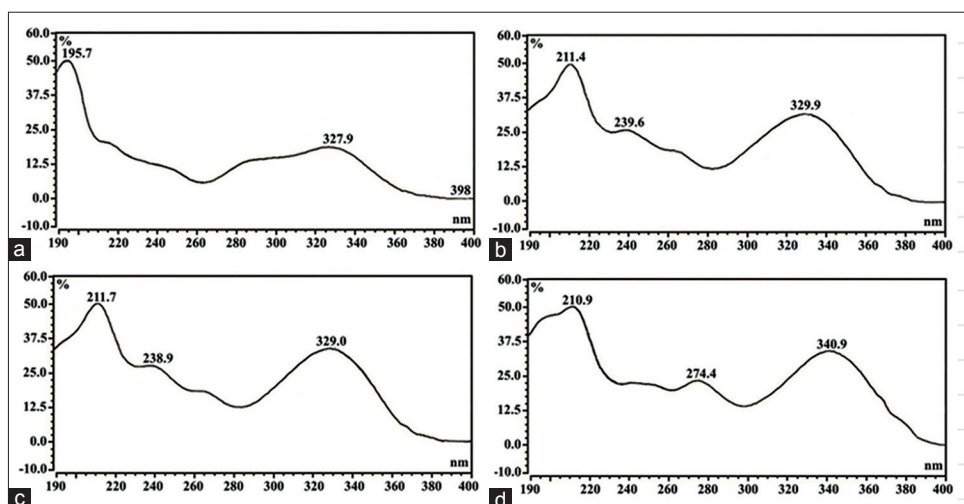


Figure 2: Ultraviolet-visible spectroscopy of the reference compounds: Rosmarinic acid (a), 3'-hydroxy-5, 6, 7, 4'-tetramethoxyflavone (b), sinensetin (c) and eupatorin (d). The spectra were collected by scanning the peaks at their start, apex and end

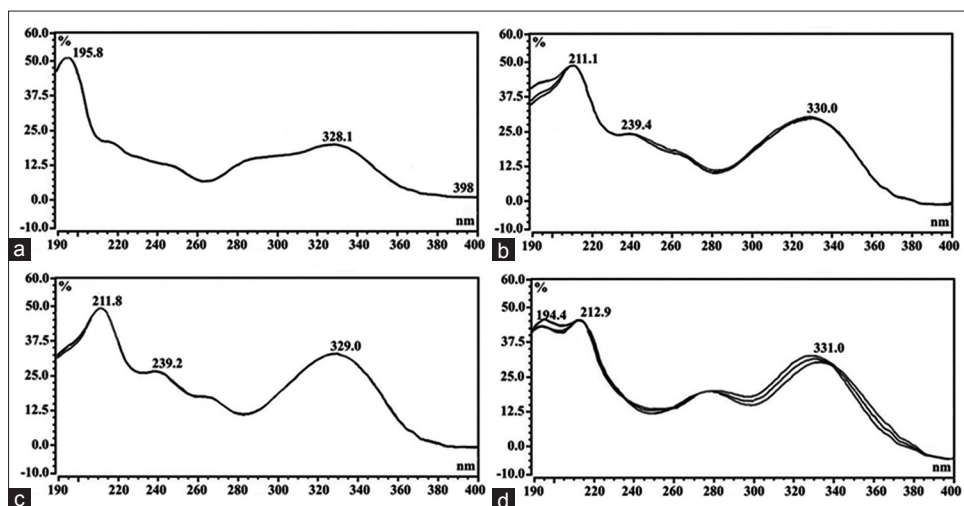


Figure 3: Ultraviolet-visible spectroscopy of the marker compounds in *Orthosiphon stamineus* extracts: Rosmarinic acid (a), 3'-hydroxy-5,6,7,4'-tetramethoxyflavone (b), sinensetin (c) and eupatorin (d). The spectra were collected by scanning the peaks at their start, apex and end

determined at signal-to-noise ratio (S/N) of 3 and 10, respectively. The regression equation with correlation equation (R^2), LOD and LOQ were showed in Table 2. All of the compounds showed good linearity with correlation equation (R^2) range from 0.9990 to 1.000 and RSD of < 0.25%. The LOD was < 0.24 µg/ml and LOQ was < 0.73 µg/ml. Though LOD and LOQ values in this method are slightly higher than in previous methods^[3,21] the compounds can still be detected and quantified at a very low concentration (<1.0 µg/ml), which indicates good sensitivity of the present method.

Precision

Precision was measured in accordance with ICH recommendations.^[24] The RSD values for repeatability (intra-day precision) were investigated to be within the range of 0.099-0.721% for peak concentration and 0.088-0.046% for retention time. An intermediate precision (inter-day precision) was investigated using different HPLC system by same analyst with three different days. The RSD values for HPLC system 1 (Dionex) were

in the range of 0.006-0.882% for peak concentration and 0-0.023% for retention time while for HPLC system 2 (Agilent) showed RSD values for peak concentration and retention time were in the range of 0.007-0.606% and 0-0.329%, respectively. The RSD values for all compounds were within the prescribed limits of the ICH guidelines (RSD < 2%).^[21] These results were confirmed that the present method is precise and reproducible even the method was transferred to other HPLC system with the used of the same column and the same chromatographic condition. Tables 3 and 4 depicts the average %RSD values of the four reference compounds.

Accuracy

Accuracy of the method was evaluated by performing a recovery study at concentrations of 12.5 and 25 µg/ml. The results are presented as average percentage recovery $\pm$ SD [Table 5]. The results showed a good accuracy, which indicates that the method is reliable and reproducible for determination of the marker compounds in *O. stamineus* leaf extracts.

Table 2: The linearity, correlation coefficient (R^2), LOD and LOQ of the compounds studied

Markers	Linearity range (µg/ml)	Regression equation ^a	R^2 (n=3)	LOD (µg/ml) Average $\pm$ SD	LOQ (µg/ml) AV $\pm$ SD
RA	0.195-100	Y=0.2123x-0.0587	0.9999	0.23 $\pm$ 0.08	0.69 $\pm$ 0.25
TMF	0.195-100	Y=0.3979x+0.0571	0.9999	0.17 $\pm$ 0.01	0.53 $\pm$ 0.03
SIN	0.195-100	Y=0.2671x-0.0111	1.0000	0.24 $\pm$ 0.03	0.73 $\pm$ 0.09
EUP	0.195-100	Y=0.2395x+0.0545	0.9990	0.17 $\pm$ 0.03	0.53 $\pm$ 0.10

^aY=Peak area; x=concentration (µg/ml); LOD=Limit of detection; LOQ=Limit of quantification; SD=Standard deviation; RA=Rosmarinic acid; TMF=3'-hydroxy-5, 6, 7, 4'-tetramethoxyflavone; SIN=Sinensetin; EUP=Eupatorin

Table 3: Analytical results for repeatability (intra-day precision) of compounds studied

Compounds	Concentration (µg/ml)	Repeatability (intra-day precision) (n=6)			
		Mean $\pm$ SD (µg/ml)	%RSD	RT $\pm$ SD	%RSD
RA	100	103.10 $\pm$ 0.225	0.219	6.43 $\pm$ 0.001	0.015
	50	47.42 $\pm$ 0.189	0.399	6.44 $\pm$ 0.001	0.016
	25	23.14 $\pm$ 0.062	0.269	6.44 $\pm$ 0.001	0.040
	12.5	12.12 $\pm$ 0.012	0.099	6.45 $\pm$ 0.005	0.015
	6.25	6.58 $\pm$ 0.042	0.632	6.45 $\pm$ 0.001	0.022
TMF	100	99.85 $\pm$ 0.353	0.354	8.30 $\pm$ 0.001	0.015
	50	49.97 $\pm$ 0.345	0.690	8.30 $\pm$ 0.001	0.010
	25	24.53 $\pm$ 0.157	0.638	8.30 $\pm$ 0.001	0.014
	12.5	12.81 $\pm$ 0.063	0.490	8.30 $\pm$ 0.004	0.046
	6.25	6.171 $\pm$ 0.038	0.614	8.30 $\pm$ 0.001	0.018
SIN	100	100.51 $\pm$ 0.340	0.338	9.09 $\pm$ 0.001	0.015
	50	50.38 $\pm$ 0.342	0.678	9.09 $\pm$ 0.009	0.009
	25	24.91 $\pm$ 0.150	0.602	9.09 $\pm$ 0.001	0.016
	12.5	12.5 $\pm$ 0.061	0.488	9.09 $\pm$ 0.004	0.040
	6.25	6.25 $\pm$ 0.038	0.602	9.09 $\pm$ 0.001	0.015
EUP	100	100.25 $\pm$ 0.155	0.154	9.93 $\pm$ 0.002	0.020
	50	50.72 $\pm$ 0.366	0.721	9.93 $\pm$ 0.001	0.008
	25	25.14 $\pm$ 0.165	0.655	9.93 $\pm$ 0.002	0.021
	12.5	12.26 $\pm$ 0.060	0.493	9.93 $\pm$ 0.004	0.040
	6.25	5.94 $\pm$ 0.008	0.142	9.93 $\pm$ 0.001	0.008

Results are shown as %RSD of peak concentration $\pm$ SD, and %RSD of RT $\pm$ SD. SD=Standard deviation; %RSD=Percentage of relative standard deviation; RA=Rosmarinic acid; TMF=3'-hydroxy-5, 6, 7, 4'-tetramethoxyflavone; SIN=Sinensetin; EUP=Eupatorin; RT=Retention time

Quantification of rosmarinic acid, 3'-hydroxy-5, 6, 7, 4'-tetramethoxyflavone, sinensetin and Eupatorin in *Orthosiphon stamineus* extracts

The developed method was applied to a simultaneously determination of four marker compounds in five different extracts of *O. stamineus*. The result showed good separation of the 4 marker compounds in 5 extracts as shown in Figure 4. RA, TMF, SIN and EUP in *O. stamineus* extracts were then quantified by applying the regression equations of the reference compounds. The results are presented as average w/w% $\pm$ SD in Table 6. Methanolic extract contained the highest amount of SIN ($1.80 \pm 0.003\%$) and TMF ($0.28 \pm 0.005\%$), while the RA content is the highest ($4.78 \pm 0.219\%$) in methanol: Water extract. The methanolic and ethanolic extracts were found to contain high EUP of $5.27 \pm 0.321\%$ and $5.36 \pm 0.215\%$, respectively. Water extract however, showed the lowest content of all marker compounds. The results showed that the content of marker compounds is varying from different extracts of the same plant.

CONCLUSIONS

Rosmarinic acid, TMF, SIN and EUP were selected as reference compounds for standardization of the *O.*

stamineus extracts due to their various pharmacological effects reported. Several methods have been reported for quantification of these markers in *O. stamineus* leaf extracts;^[3,20,21] however these methods are either time consuming or require the use of complex mobile phase, which may limit their routine application in standardization of *O. stamineus* extracts, raw leaves and commercial products. Therefore, still there is a need for easier, faster, reliable and versatile methods for the qualitative and quantitative evaluation of the *O. stamineus* extracts. In this work, an improved method was developed for simultaneous detection of four marker compounds including RA, TMF, SIN and EUP. The method was validated based on ICH guidelines^[24] and all of the parameters used were compatible to the guidelines. The present method has several advantages over the existing methods such as shorter run time, use of an environmental friendly mobile phase and the method is reproducible when applied to other HPLC system. Previous methods reported a total run time of 30 min to separate the marker compounds (RA, TMF, SIN, and EUP).^[3,20] In another method the run time was improved to < 20 min,^[21] but all of these studies have used unfriendly solvent systems which consist of phosphate buffer and tetrahydrofuran that cannot be used directly in the MS analysis. The use of phosphate buffer is not encouraged because it may lead to salt precipitation

Table 4: Analytical results for intermediate (inter-day precision) of compounds studied

Compounds/ concentration ($\mu\text{g/ml}$)	Intermediate precision (inter-day) (n=3)							
	HPLC sytem 1				HPLC sytem 2			
	Mean $\pm$ SD ($\mu\text{g/ml}$)	%RSD	RT $\pm$ SD	%RSD	Mean $\pm$ SD ($\mu\text{g/ml}$)	%RSD	RT $\pm$ SD	%RSD
RA								
100	101.51 $\pm$ 0.227	0.224	6.43 $\pm$ 0.001	0.018	100.60 $\pm$ 0.831	0.066	6.86 $\pm$ 0.023	0.329
50	46.55 $\pm$ 0.216	0.463	6.44 $\pm$ 0.000	0.006	48.90 $\pm$ 0.360	0.058	6.88 $\pm$ 0.001	0.015
25	24.16 $\pm$ 0.077	0.317	6.45 $\pm$ 0.001	0.013	24.63 $\pm$ 0.222	0.072	6.88 $\pm$ 0.002	0.025
12.5	11.80 $\pm$ 0.033	0.278	6.45 $\pm$ 0.000	0.019	12.51 $\pm$ 0.305	0.195	6.89 $\pm$ 0.002	0.025
6.25	6.540 $\pm$ 0.020	0.305	6.45 $\pm$ 0.001	0.003	6.50 $\pm$ 0.491	0.606	6.89 $\pm$ 0.001	0.017
TMF								
100	99.96 $\pm$ 0.224	0.224	8.30 $\pm$ 0.002	0.023	100.35 $\pm$ 0.102	0.007	8.64 $\pm$ 0.008	0.090
50	49.81 $\pm$ 0.439	0.882	8.30 $\pm$ 0.001	0.007	49.27 $\pm$ 0.868	0.128	8.65 $\pm$ 0.002	0.018
25	24.80 $\pm$ 0.149	0.602	8.30 $\pm$ 0.000	0.000	24.99 $\pm$ 0.574	0.167	8.65 $\pm$ 0.002	0.018
12.5	12.17 $\pm$ 0.061	0.491	8.30 $\pm$ 0.001	0.007	12.71 $\pm$ 0.253	0.143	8.65 $\pm$ 0.001	0.012
6.25	6.26 $\pm$ 0.010	0.159	8.30 $\pm$ 0.001	0.013	6.57 $\pm$ 0.387	0.418	8.65 $\pm$ 0.001	0.012
SIN								
100	100.57 $\pm$ 0.315	0.313	9.09 $\pm$ 0.001	0.013	100.29 $\pm$ 0.490	0.024	9.42 $\pm$ 0.006	0.062
50	50.80 $\pm$ 0.307	0.604	9.09 $\pm$ 0.001	0.013	49.20 $\pm$ 0.624	0.061	9.42 $\pm$ 0.001	0.012
25	25.20 $\pm$ 0.143	0.568	9.09 $\pm$ 0.001	0.006	25.05 $\pm$ 0.416	0.08	9.42 $\pm$ 0.002	0.021
12.5	12.70 $\pm$ 0.033	0.257	9.09 $\pm$ 0.001	0.006	12.71 $\pm$ 0.752	0.281	9.42 $\pm$ 0.002	0.022
6.25	6.34 $\pm$ 0.010	0.164	9.09 $\pm$ 0.001	0.006	6.53 $\pm$ 0.086	0.061	9.42 $\pm$ 0.001	0.011
EUP								
100	100.41 $\pm$ 0.666	0.038	9.93 $\pm$ 0.001	0.013	100.41 $\pm$ 0.471	0.027	10.28 $\pm$ 0.003	0.034
50	48.98 $\pm$ 0.064	0.008	9.93 $\pm$ 0.001	0.006	49.04 $\pm$ 0.954	0.111	10.28 $\pm$ 0.001	0.006
25	25.16 $\pm$ 0.025	0.006	9.93 $\pm$ 0.001	0.008	25.16 $\pm$ 0.018	0.004	10.28 $\pm$ 0.001	0.011
12.5	12.55 $\pm$ 0.034	0.015	9.93 $\pm$ 0.001	0.010	12.61 $\pm$ 0.976	0.438	10.28 $\pm$ 0.000	0.000
6.25	6.52 $\pm$ 0.075	0.064	9.93 $\pm$ 0.001	0.006	6.52 $\pm$ 0.053	0.045	10.28 $\pm$ 0.001	0.006

Results are shown as %RSD of peak concentration $\pm$ SD, and %RSD of RT $\pm$ SD. SD=Standard deviation; RA=Rosmarinic acid; TMF=3'-hydroxy-5, 6, 7, 4'-tetramethoxyflavone; SIN=Sinensetin; EUP=Eupatorin; *O. stamineus*=*Orthosiphon stamineus*; %RSD=Percentage of relative standard deviation; HPLC=High-performance liquid chromatography; RT=Retention time

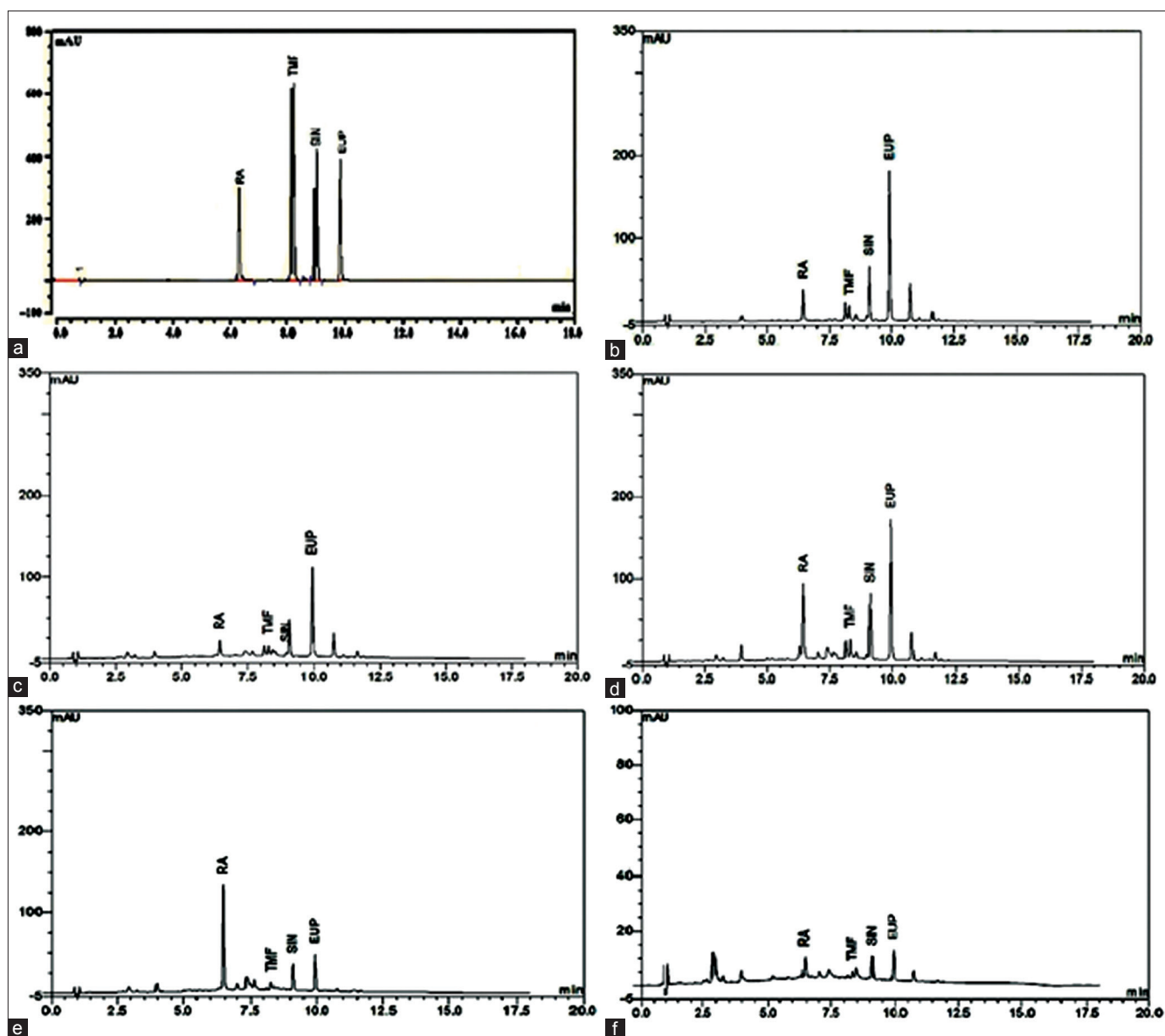


Figure 4: High-performance liquid chromatography chromatograms of *Orthosiphon stamineus* extracts at 320 nm. Standard mixture of rosmarinic acid, 3'-hydroxy-5, 6, 7, 4'-tetramethoxyflavone, sinensetin and eupatorin (a), methanolic extract (b), methanol:water extract (1:1) (c), ethanolic extract (d), ethanol:water extract (1:1) (e) and water extract (f)

Table 5: Analytical results for accuracy tests

Markers	Concentrations (µg/ml)	Recovery (average±SD)
RA	25	90.2±1.30
	12.5	98.4±0.83
TMF	25	96.2±1.56
	12.5	104.0±1.10
SIN	25	91.7±1.39
	12.5	99.6±1.15
EUP	25	99.8±0.11
	12.5	105.5±0.20

Results are shown as average percentage recovery of the marker compounds±SD. SD=Standard deviation; RA=Rosmarinic acid; TMF=3'-hydroxy-5, 6, 7, 4'-tetramethoxyflavone; SIN=Sinensetin; EUP=Eupatorin; *O. stamineus*=*Orthosiphon stamineus*

at the interface, contamination of the needle electrode for atmospheric pressure ionization or interference with creation of fine charged droplets and reduced sensitivity for electrospray ionization. Comprehensive information can be found at website cited below.^[26] Our improved method uses 0.1% formic acid as a buffer system which is friendly for MS analysis, because it improves ionization of the compounds. This makes our method suitable for MS analysis of unknown compounds as well, besides the reported marker compounds. We believe that this method is suitable for routine standardization of *O. stamineus* extracts, raw leaves and commercial products for herbal industries

Table 6: Concentration of RA, TMF, EUP and SIN marker compounds in various leaf extracts of *O. stamineus*

Extracts	Method of extraction	Content (w/w)%±SD (n=3)			
		RA	TMF	SIN	EUP
Meoh	Soxhlet	2.86±0.620	0.28±0.005	1.80±0.003	5.27±0.321
MW	Soxhlet	4.78±0.219	0.10±0.005	0.82±0.033	1.35±0.051
EtOH	Soxhlet	1.61±0.050	0.21±0.009	1.42±0.059	5.36±0.215
EW	Soxhlet	1.03±0.039	0.13±0.006	1.04±0.036	3.32±0.134
H ₂ O	Reflux	0.71±0.008	0.01±0.001	0.22±0.008	0.33±0.037

The results are presented as average w/w%±SD. SD=Standard deviation; RA=Rosmarinic acid; TMF=3'-hydroxy-5, 6, 7, 4'-tetramethoxyflavone; SIN=Sinensetin; EUP=Eupatorin; *O. stamineus*=*Orthosiphon stamineus*

due to its short running time, low cost, high precision, accuracy, sensitivity and hope this method can be applied for future research in pharmacokinetic study.

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