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Optimizing the method for quantification of apigenin and quercetin in the Thai herbal Sattakavata formula by ultra-performance liquid chromatography coupled with mass spectrometer

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ABSTRACT

Introduction: Sattakavata formula (SVF) is a Thai traditional medicine from Ayurved Siriraj for a relief of joint pain. It comprises six ingredients, namely, *Andrographis paniculata* (Burm. F.) Wall. ex Nees (AP), *Cinnamomum* sp. (CN), *Morus alba* Linn. (MA), *Crateva adansonii* DC. subsp. *trifoliata* (Roxb.) Jacobs (CA), *Moringa oleifera* Lam. (MO), and *Acorus calamus* Linn. (AC). There was no method for identification and quantification of SVF before. **Objective:** The objective of the study was to establish a method for identification and quantification of apigenin and quercetin in SVF and its components using ultra-performance liquid chromatography–mass spectrometry (UPLC-MS). **Materials and Methods:** SVF and six components were extracted with 80% ethanol by ultrasonication. The separation was performed in an RP18 column using a gradient elution with 0.1% formic acid in deionized water and acetonitrile. The method was fully validated in terms of precision, limit of detection, limit of quantification, and recovery. **Results:** Apigenin was found in SVF, AP, and CN, and quercetin was found in SVF, CN, and MA. There was a good linearity ($R^2 > 0.999$) in the range of 100–1400 ng/ml for apigenin and 300–3000 ng/ml for quercetin. The recovery of apigenin and quercetin was in the range of 88.33–111.52% and 90.71–109.17%, respectively. Relative standard deviations of precision in apigenin and quercetin were 0.62–6.02 and 0.65–4.01, respectively. **Conclusion:** A reliable UPLC-MS method for identification and quantification of apigenin and quercetin in SVF was successfully established in this study. The method is useful in the quality control of the herbal medicines and can be used routinely.

Keywords: Apigenin, Ayurved Siriraj recipes, quantification, quercetin, ultra-performance liquid chromatography

INTRODUCTION

Sattakavata formula (SVF) is an herbal mixture unique to Ayurved Siriraj which has been used for over 30 years by Ayurved Thamrong School, now The Center of Applied Thai Traditional Medicine, a department in the Faculty of Medicine Siriraj Hospital, Mahidol University. It is believed to be effective in relieving joint pain, the most common presenting symptom in our clinic. SVF preparation is comprised six components, all in dried powder forms; dried

leave of *Andrographis paniculata* (Burm. F.) Wall. ex Nees (AP), *Cinnamomum* sp. (CN), *Morus alba* Linn. (MA), dried bark of *Crateva adansonii* DC. subsp. *trifoliata* (Roxb.) Jacobs (CA), *Moringa oleifera* Lam. (MO), and dried rhizome of *Acorus calamus* Linn. (AC) [Table 1].[1] Despite its long use, there was no method for identification and quantification of SVF before.

The previous study found that nine flavonoids represent the pharmacological activity that related to the effect of SVF in the part of anti-inflammation.[2-12] Through the screening for nine potential substances, namely, hesperetin, hispidulin, kaempferol, luteolin, naringenin, scutellarein, 6-methoxyluteolin, apigenin, and quercetin using ultra-performance liquid chromatography–mass spectrometry (UPLC-MS) with RP18 column, only apigenin and quercetin were identified and subsequently used as a representative for quality control in this study.

Most herbal mixtures which exhibit anti-inflammatory property are thought to contain flavonoids.[13] Apigenin and quercetin, naturally dietary flavonoids, have potential anti-inflammatory activity as well.[2] While isolated extracts of AP,[14] CN,[15] MA,[16] and MO[17] are shown to have both apigenin and quercetin and AC which contains only apigenin,[18] the flavonoid content of the crude herbal mixture has never been demonstrated. This assessment is crucial in the process of quality control and improvement of the product.

Quantification of flavonoid in herbal admixture presents a particular challenge because the complexity of the mixture may influence the yield of conventional extraction and analysis methods. The previous studies reveal that quercetin and apigenin are detected only in single herb not herbal formula using Fourier-transform infrared spectroscopy,[19,20] thin-layer chromatography (TLC),[21,22] high-performance liquid chromatography (HPLC),[22,23] and gas chromatograph–mass spectrometer (GC–MS).[24,25] The detection of quercetin alone is done using UPLC (a photodiode array detector).[26] While TLC is a simplest technique to use, it has lower resolution than HPLC and UPLC. UPLC with HPLC, on the other hand, can operate at higher pressures and with smaller column particles. As a result, UPLC has better efficiency for chemical components separation than HPLC.[27] In addition, it has closer of retention time (RT), the higher sensitivity, and the shorter analysis time. Other equipment such as GC–MS is more suitable for gas or essential oil while FT-IR is used for identification rather than quantification of the substance in question. In addition, optimizing the mobile and stationary phase based on polarity of each substance of interest will lead to improved accuracy of results. Therefore, this study aims to describe the optimization of UPLC coupled with MS/single quadrupole (SQD) (UPLC-MS) and UPLC coupled with MS quadrupole time of flight (UPLC-MS-Q-TOF) for the quantification and confirmation of flavonoid, respectively, in the crude herbal admixture, SVF.

MATERIALS AND METHODS

Materials

All plant materials were prepared by the good manufacturing practice requirements laid down in accordance with the recommendation of the pharmaceutical inspection cooperation scheme certified unit of Herbal Medicine and Products, Center of Applied Thai Traditional Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand. They were authenticated by experienced Thai traditional practitioners. Then, the powders were stored and preserved at room temperature in dry condition.

Chemicals and Reagent

UPLC quality water was purified using a Milli-Q water system from Millipore (France). The solutions consisted of ethanol; LC–MS grade (Scharlau, Spain). The mobile phase consisted of formic acid; analytical grade (VWR International, England) and acetonitrile; LC–MS grade (Scharlau, Spain). The standard markers were hesperetin ($C_{16}H_{14}O_6$); Mw 302.28 (Sigma, USA), hispidulin ($C_{16}H_{12}O_6$); Mw 300.26 (Sigma, USA), kaempferol ($C_{15}H_{10}O_6$); Mw 286.24 (Sigma, USA), luteolin ($C_{15}H_{10}O_6$); Mw 286.24 (Sigma, USA), naringenin ($C_{17}H_{16}O_5$); Mw 300.31 (Sigma, USA), scutellarein ($C_{21}H_{18}O_{12}$); Mw 462.36 (Sigma, USA), 6-Methoxyluteolin ($C_{16}H_{12}O_7$); Mw 316.265 (Extrasynthese, Malaysia), apigenin ($C_{15}H_{10}O_5$); Mw 270.24 (Sigma, USA), and quercetin ($C_{15}H_{10}O_72H_2O$); Mw 338.27 (Cayman Chemical, USA).

Instrument and Condition

Quantification method using UPLC-MS

The quantification analysis was performed with a Waters ACQUITY UPLC® system (Waters Corp., USA) equipped with a binary solvent delivery system, an online degasser, an auto sampler and a thermostatically controlled column system. The detector was a Waters ACQUITY SQD MS equipped with a Z-spray ESI ion source operating in positive ion mode. Data acquisition and processing were performed using the Empower 2 software.

Table 1: List of unresolved plants mentioned in the case studies, suggested species, and their traditional uses

Local names	Part used	Tentative identification	Collection number	Traditional uses
Fa-Tha-Lai-Chon	Leaves	*Andrographis paniculata* (Burm. f.) Wall. ex Nees	KN-001	Treatment of cough, diarrhea, and fever[1]
Kra-Wan	Leaves	*Cinnamomum* sp.	KN-002	Carminative, expectorate, and treatment of fever[1]
Mon	Leaves	*Morus alba* Linn.	KN-003	Treatment of cough and conjunctivitis[1]
Kum-Bok	Barks	*Crateva adansonii* DC. subsp. *trifoliata* (Roxb.) Jacobs	KN-004	Carminative and treatment of diarrhea, cardiotonic (*Bam-Rung-Hua-Chai*), dermatitis, and gallstones[1]
Ma-Rum	Barks	*Moringa oleifera* Lam.	KN-005	Carminative and regulate the body elements (*Kum-That*)[1]
Wan-Nam	Rhizomes	*Acorus calamus* Linn.	KN-006	Carminative and treatment of pain and bronchitis[1]

The separation was performed on a Waters ACQUITY UPLC® BEH Shield RP18 (100 mm ×2.1 mm; particle size 1.7 μm). Column and auto sampler temperatures were set at 30°C and 20°C, respectively. The mobile phase consisted of 0.1% formic acid in deionized water (A) and acetonitrile (B). The gradient condition was set as the following: The % B was linearly increased from 30% to 33% in 4 min, then to 40% in 6 min, finally to 100% and kept there for another 0.5 min, then linearly ramped down to 30% again in 0.5 min. The total gradient run time was 13 min. The flow rate was set at 300 μL/min. The injection volume was 2 μL for all standards and samples.

Confirmation method using UPLC-MS-Q-TOF

To increase specificity, the Water® Xevo™ QTOF MS (Waters Corp., USA) with AQUITY UPLC system was used to perform the confirmation analysis with the same condition and mobile phase gradient as quantification method using UPLC-MS. However, the collision energy was adjusted to make a suitable pattern of mass spectrum of each standard.

Extraction of samples

Five hundred grams of each sample powder were extracted with 80% ethanol (total volume 5000 mL) 3 times (30 min each time) using ultra-sonication. After that, the extraction solvent was pooled and filtrated through Whatman® glass microfiber filter (Grade GF/A) using a Buchner funnel. Then, the ethanol was separated out of the solvent using rotary evaporator. The remaining residue was lyophilized to dryness and stored in cabinet desiccator until use.

Validation of the UPLC-MS method

Both apigenin and quercetin were accurately weighed and dissolved in methanol to prepare stock solutions at a concentration of 10 μg/mL. Stock solutions were further diluted to construct calibration curves. The calibration curves were the plots of diluted concentrations of compounds against their peak areas. Their linearity was measured from the correlation coefficient.

Limit of detection (LOD) and limit of quantification (LOQ) were analyzed 5 times. The LOD and LOQ were calculated as the concentrations needed to produce signal-to-noise ratios of ≥3 and ≥10, respectively. The method precision was calculated by analyzing of three standard concentration solutions. The precision was represented by the relative standard deviation (RSD), which was calculated using the equation RSD = (standard deviation/mean) ×100. The precision was measured 5 times in a single day (intraday precision) and 3 times a day over 3 consecutive days (interday precision).

The accuracy of this method was evaluated through a recovery test, whereby three concentrations of standard compounds (low, medium, and high) were added to each sample. The recovery was calculated as follows: Recovery (%) = ([Detected concentration−Initial concentration]/Spiked concentration) ×100.

The mass error (in Da) was calculated as follows: Mass error = ([Measurement mass−Exact mass)/Exact mass) $\times 10^6$.

RESULTS

To determine whether positive or negative ion mode should be used, standard apigenin (1400 ng/mL) and quercetin (3000 ng/mL) were selected from the highest concentration of the standard curve. Area under the peak from positive and negative ion mode was compared. The area from the positive ion mode was 2.92 times higher for apigenin and 5 times higher for quercetin when compared to the negative ion mode. Thus, positive ionization mode was selected in this study.

Usually, methanol or acetonitrile in combination with water was employed for the separation of phenolic acids and/

Table 2: Setting of UPLC method for apigenin and quercetin and Rt, LOQ, and LOD (n=6)

Analyte	Cone voltage (V)	Collision energy (V)	Mass (m/z)	Weight of non-linear fit curve	Rt (min)	Linear range (ng/mL)	LOQ (ng/mL)	Average s/no. of LOQ	LOD (ng/mL)	Average s/no. of LOD
Apigenin	60	36	271	$1/x^2$	8.06	100–1400	100	97.36	5	4.73
Quercetin	60	32	303	1/x	5.44	300–3000	300	32.00	100	6.74

LOD: Limit of detection, LOD: Limit of quantification

Table 3: Intraday and interday precisions of apigenin and quercetin

Chemical compound	Concentration (ng/mL)	Intraday		Interday	
		Amount found (ng/ml)		Amount found (ng/ml)	
		Mean	%RSD	Mean	%RSD
Apigenin	300	294.83	1.59	305.52	1.96
	900	809.55	0.96	857.84	3.01
	1300	1142.30	0.62	1192.71	6.02
Quercetin	400	417.01	0.82	409.32	1.25
	900	839.50	0.65	853.55	2.49
	2500	2056.67	1.39	2160.76	4.01

Intraday: n=5, interday: 3 days each day n=3

Table 4: Recovery data of apigenin and quercetin analyzed by the developed UPLC method in SVF, *Andrographis paniculata* (Burm. f.) Wall. ex Nees, *Cinnamomum* sp., and *Morus alba* Linn.

Sample	Recovery data of apigenin			Recovery data of quercetin		
	Spiked concentration (ng/ml)	Mean recovery (%)	RSD (%)	Spiked concentration (ng/ml)	Mean recovery (%)	RSD (%)
SVF	400	93.92	5.42	600	109.17	6.80
	500	88.33	1.25	800	99.80	2.52
	600	98.26	0.84	900	101.54	1.50
Andrographis paniculata (Burm. F.) Wall. ex Nees	600	100.21	4.95			
	700	100.92	0.25	-	-	-
	900	99.15	0.71			
Cinnamomum sp.	1000	95.10	4.36	2000	99.27	12.69
	1200	103.23	8.01	2500	90.71	7.62
	1400	111.52	4.46	3000	100.54	10.38
Morus alba Linn.				700	97.52	5.63
	-	-	-	800	98.40	4.89
				1000	105.73	2.27

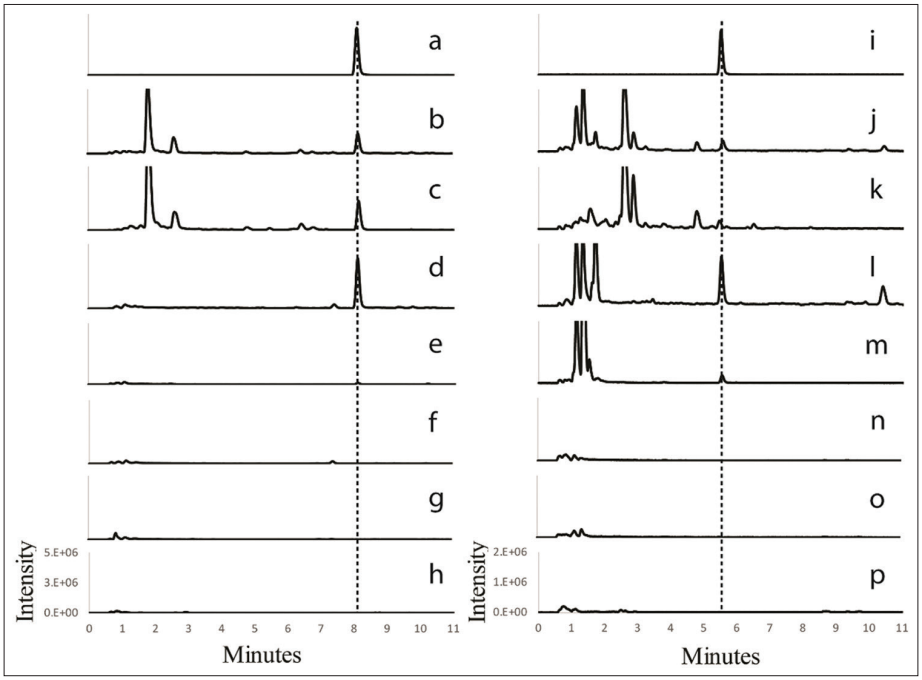

Figure 1: UPLC-MS analysis selected ion monitoring (SIM detection mode) chromatograms of m/z 271 from standard Apigenin (a), SVF (b), *AP* (c), *CN* (d), *MA* (e), *CA* (f), *MO* (g), *AC* (h), and UPLC-MS analysis selected ion monitoring (SIM detection mode) chromatograms of m/z 303 from standard Quercetin (i), SVF (j), *AP* (k), *CN* (l), *MA* (m), *CA* (n), *MO* (o), *AC* (p)

or flavonoids, an acid modifier was added to avoid peak tailing. In this study, various ratios of mobile phase combinations containing acetonitrile and 0.1% formic acid as modifier were compared. Finally, the most appropriated gradient ratio was selected for the proper separation.

The calibration curve of apigenin and quercetin was investigated in the range of 100–1400 ng/mL (100, 500, 800, 1000, 1200, and 1400 ng/mL) and 300–3000 ng/mL (300, 500, 800, 1000, 2000, and 3000 ng/mL), respectively. The linear regression equation of apigenin and quercetin was y = 20,318.64822x+73,857.06904, R^2 = 0.999063, and y = 4541.12742x–599,255.95261, R^2 = 0.999436, respectively. Weight of non-linear fit curve, LOD, and LOQ are presented in Table 2.

The results of precision and recovery rates were in acceptable range, as shown in Tables 3 and 4. The RSDs of intraday precision were 0.62–1.59 for apigenin and 0.65–1.39 for quercetin. RSDs of interday, on the other hand, were higher with 1.96–6.02 for apigenin and 1.25–4.01 for quercetin [Table 3]. The recovery of apigenin and quercetin was in the range of 88.33–111.52% and 90.71–109.17%, respectively [Table 4].

SVF [Figure 1b and j], AP [Figure 1c and k], CN [Figure 1d and l], and MA [Figure 1m] presented apigenin and quercetin peaks in the UPLC-MS analysis (SIM detection mode) when compared the RT with standard compounds [Figure 1a and i], although the amount of apigenin in MA [Figure 1e] and quercetin in AC [Figure 1p] was less than LOQ [Table 5]. Whereas, (SIM detection mode) chromatogram peaks were absent for CA [Figure 1f and n], MO [Figure 1g and o], and AC [Figure 1h].

In MS/MS mode of UPLC-MS-Q-TOF, the possible fragmentation pathways of apigenin and quercetin that were used in the confirmation of the precursor ions are presented in Figure 2. In particular, the apigenin fragment products by the C-ring fission resulted in $^{0,2}A^+$, $^{1,3}B^+$, and $^{1,3}A^+$ fragments. The m/z of these ions was 121.0282, 153.0204, and 119.0484, respectively. The C-ring fission products of quercetin resulted in $^{0,3}B^+$ and $^{1,3}B^+$ fragments, with m/z of 137.0230 and 153.0204, respectively. The loss of two carbon and two oxygen from A-ring and one oxygen from C-ring of quercetin generated $[M+H-H_2O-2CO]^+$ with m/z of 229.0527. Mass error was 14.76 ppm and 8.25 ppm for apigenin and quercetin, respectively.

MS/MS mode of UPLC-MS-Q-TOF was used to confirm the result obtained from UPLC-MS analysis (SIM detection mode) in two aspects. The first one was comparison of the MS/MS spectra. Since, it could provide an additional information of both precursor and product ions. In this study, the MS/MS spectra of SVF, AP, CN, MA, and AC showed complete matches with standard apigenin [Figure 3] and quercetin [Figure 4] Whereas, quercetin peak in AP did not show the same pattern of MS/MS spectra as standard quercetin [Figure 4a, 4c]. This led to the conclusion that the m/z 303 found in MS (SIM

Table 5: Amount of apigenin and quercetin in the extract of SVF and its components at concentration 10 mg/ml (n=6)

Sample	Amount of							
	Apigenin				Quercetin			
	Mean (ng/mL)	SD	% RSD	% w/w	Mean (ng/mL)	SD	% RSD	% w/w
SVF	502.04	21.52	4.29	0.00046	745.56	34.58	4.64	0.00068
AP	728.71	10.88	1.49	0.00094	ND	-	-	-
CN	1215.41	20.98	1.73	0.00112	2538.11	65.09	2.56	0.00234
MA	<LOQ	-	-	-	835.20	37.73	4.52	0.00082
CA	ND	-	-	-	ND	-	-	-
MO	ND	-	-	-	ND	-	-	-
AC	ND	-	-	-	<LOQ	-	-	-

<LOQ = less than limit of quantification

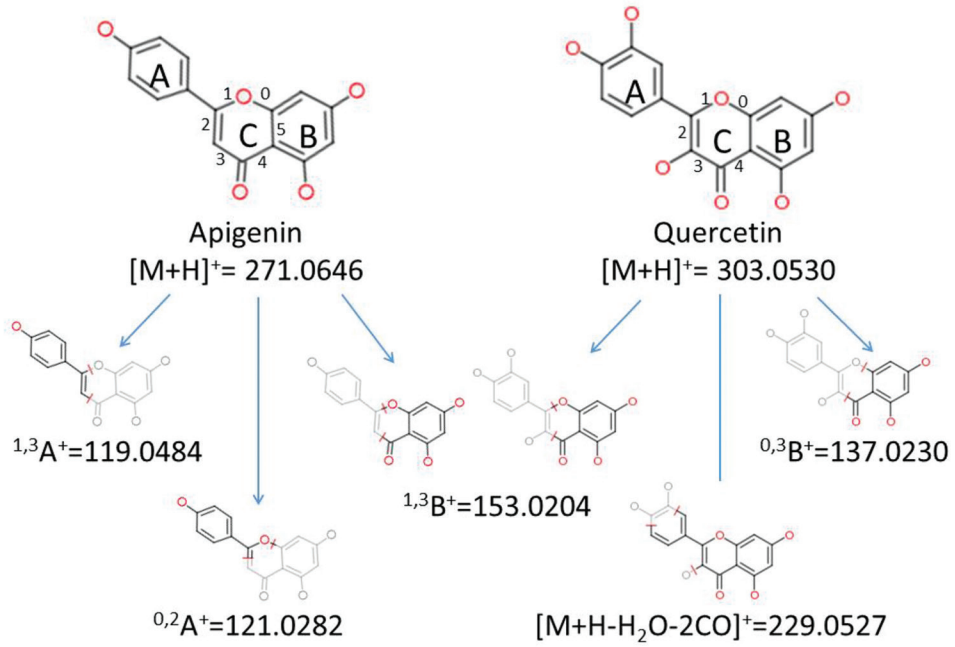

Figure 2: The structures of apigenin and quercetin standard with three fragments by Mass FragmentTM software (Waters Corp., MA, USA)

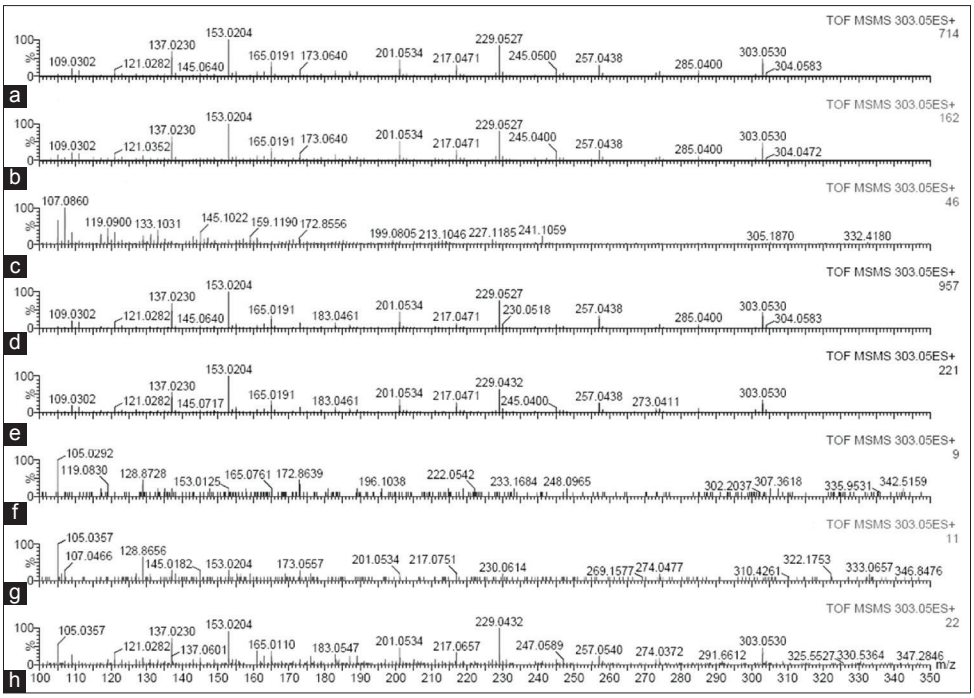

Figure 3: The representative MS/MS spectra of m/z 271.0646 from standard Apigenin (a), SVF (b), *AP* (c), *CN* (d), *MA* (e), *CA* (f), *MO* (g), *AC* (h)

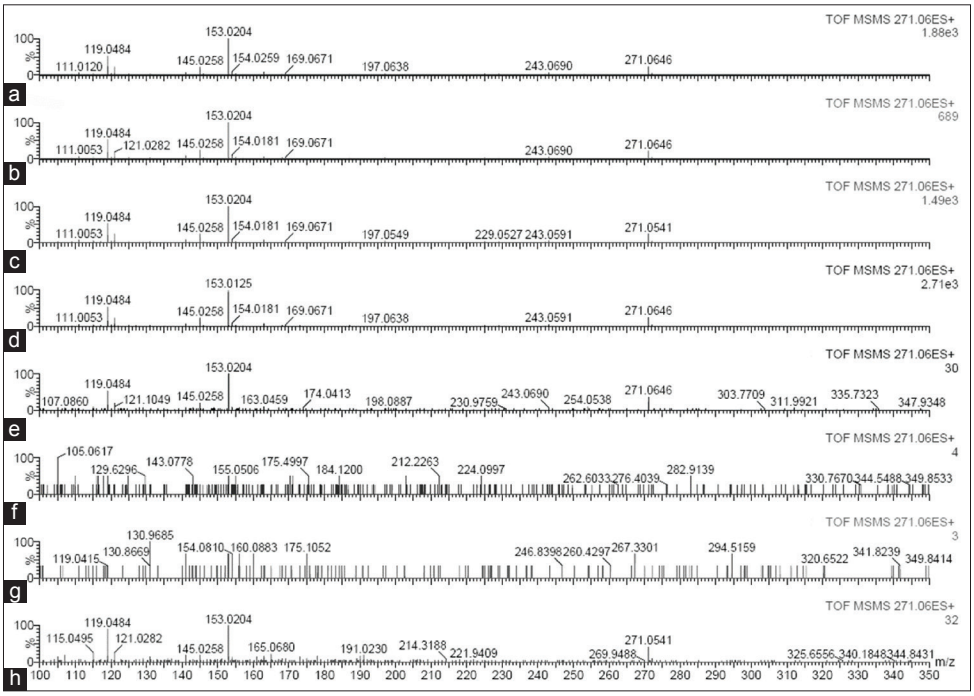

Figure 4: The representative MS/MS spectra of m/z 303.0530 from standard Quercetin (a), SVF (b), *AP* (c), *CN* (d), *MA* (e), *CA* (f), *MO* (g), *AC* (h)

detection mode) chromatogram of AP was not quercetin. It is hypothesized that this was a parent compound with identical mass per charge of 303 m/z to quercetin and should have very similar polarity due to their comparable RT. The secondary aspect of UPLC-MS-Q-TOF was to confirm the presence or absence of the peaks in high resolution mass.

In conclusion, apigenin was calculated quantify in SVF, AP, CN and quercetin was was calculated quantify in SVF, CN and MA. The highest levels of both apigenin and quercetin were found in CN. No signal of apigenin and quercetin were found in both UPLC-MS analysis (SIM detection mode) and scan mode of UPLC-MS-Q-TOF in CA, MO and AC.

DISCUSSION

The use of single or multiple chemical makers is important to quality control[28] of herbal medicine. Due to its complex

formula, identification of reliable chemical markers for SVF is the key in initiating the quality control process. We have established UPLC-MS as the method for identification and quantification in this study. However, the variety and complexity of SVF and its components present significant challenges for identifying of apigenin and quercetin using conventional methods. In our study, the MSMS spectra from a peak of quercetin in AP [Figure 1k] show difference pattern compared with the spectra of standard quercetin even at the same RT. This clearly demonstrates that data from LC–MS may yield the improper results and reiterate the importance of using MS/MS as a double check. In MS/MS mode of UPLC-MS-Q-TOF, apigenin and quercetin in SVF and its components were identified or presumed based on comparing RT, MS/MS spectrum, accurate mass, and fragment patterns with the standard.

However, once the identification process has been clearly developed, UPLC-MS is a suitable equipment for quantification of the compounds. This study provides the reliable optimization and robust UPLC-MS method for simultaneous quantification of apigenin and quercetin in SVF and its components. The well-validated method presented here demonstrates acceptable precision and accuracy, as well as adequate sensitivity. This finding contributes to the development of future methods for analyzing other Thai herbal formula which, in turn, serves to enhance the progress in the quality control of herbal medicine and products.

CONCLUSION

Apigenin was found in SVF, AP, and CN and quercetin was found in SVF, CN, and MA. The highest levels of both apigenin and quercetin were found in CN. No signal of apigenin and quercetin was found in both UPLC-MS analysis (SIM detection mode) and scan mode of UPLC-MS-Q-TOF in CA, MO, and AC.

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