Research Article

Quality assessment of a formulated Chinese herbal decoction, Kaixinsan, by using rapid resolution liquid chromatography coupled with mass spectrometry: A chemical evaluation of different historical formulae

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Kaixinsan is an ancient Chinese herbal decoction mainly prescribed for patients suffering from mental depression. This decoction was created by Sun Si-miao of Tang Dynasty (A.D. 600) in ancient China, and was composed of four herbs: Radix and Rhizome Ginseng, Radix Polygalae, Rhizoma Acori Tatarinowii and Poria. Historically, this decoction has three different formulations, each recorded at a different point in time. In this study, the chemical compositions of all three Kaixinsan formulae were analyzed. By using rapid resolution LC coupled with a diode-array detector and an ESI triple quadrupole tandem MS (QQQ-MS/MS), the Radix and Rhizome Ginseng-derived ginsenosides including Rb_1, Rd, Re, Rg_1, the Radix Polygalae-derived 3,6′-disinapoyl sucrose, the Rhizoma Acori Tatarinowii-derived α- and β-asarone and the Poria-derived pachymic acid were compared among the three different formulations. The results showed variations in the solubility of different chemicals between one formula and the others. This systematic method developed could be used for the quality assessment of this herbal decoction.

Keywords: Chemical assessment / Depression / Herbal mixture / LC-MS / TCM
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1 Introduction

Traditional Chinese Medicine (TCM) has been used as medicines and daily dietary supplements in Asia for thousands of years. Usually, TCM is prepared as a formula by a unique method with a specific combination of different herbs [1, 2]. According to the needs of patients, the formulae vary in combination, or in dosage, to acquire the best therapeutic effects while minimizing any side effects there might be [1–4]. With the increase in popularity of herbal medicine worldwide and the rapid expansion of its global market, the safety and efficacy of TCM herbs and their products have become a major concern for health authorities, pharmaceutical industries and the public. However, the limited ability to recognize bioactive chemicals and the uncertainty of quality of herbal products severely hinders the development of TCM. The accurate identification of target chemicals from a single herb, or from a formula having a combination of different herbs, is a real challenge for us today.

Nowadays, HPLC equipped with diode-array detector (DAD) is frequently applied in determining the chemical composition and has become a routine method for the quality control of TCM products. Owing to the extreme complexity and diversity of the chemical composition in TCM formulae, the establishment of an ideal HPLC separation system to acquire the best resolution may consume a long time. At the same time, the commonly used DAD detector has no way of analyzing the chemical without instauration radicals or poor ultraviolet absorbance, which limits the detection accuracy. In recent years, MS has been used in TCM analysis, especially in qualitative analysis. In particular, triple quadrupole tandem mass spectrometer (QQQ-MS/MS) can achieve high sensitivity and selectivity without the need to establish the baseline chromatographic separation of target analytes from each other by using the

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Abbreviations: DAD, diode-array detector; **KXS,** kaixinsan; **MRM,** multiple reaction monitoring; **PO,** poria; **RAT,** Rhizoma Acori Tatarinowii; **RG,** Radix and Rhizome Ginseng; **RP,** Radix Polygalae; **RRLC,** rapid resolution LC; **TCM,** traditional Chinese medicine multiple reaction monitoring (MRM) scan mode. This method greatly facilitates the quantification of chemical markers in the complex matrixes with only a small amount of sample. Meanwhile, the development of particle columns with small diameters (e.g. 1.8 μm) has turned rapid separation into a reality. Although the LC–MS method has largely been utilized in qualitative identification of multiple chemicals in herbal products, rarely has it been used for the quantification of marker chemicals in TCM decoction.

Kaixinsan (KXS) is a famous TCM formula for the treatment of mental disorders, especially morbid forgetfulness and depression [5]. Indeed, KXS has been reported to enhance the memory [6] and to relieve the symptoms of depressive disorder, as revealed in animal studies [7]. The first description of KXS is in *Beiji Qianjin Yaofang <Thousand Formulae for Emergency>* written by Sun Si-miao of Tang Dynasty in 652 A.D. This herbal decoction is composed of four herbs: Radix and Rhizome Ginseng (root and rhizome of *Panax ginseng* C. A. Mey., Renshen, RG), Radix Polygalae (root of *Polygala tenuifolia* Wild., Yuanzhi, RP), Rhizoma Acori Tatarinowii (rhizome of *Acorus tatarinowii* Schott, Shichangpu, RAT), and Poria (sclerotium of *Poria cocos* (Schw.) Wolf, Fuling, PO). Owing to a lack of (or insufficient) printing technologies in Tang dynasty, the original edition of *Beiji Qianjin Yaofang* (652 A.D.) has never been seen. At least three different KXS formulations have been recorded in historical books, and which vary greatly in the herb ratio. The earliest edition of *Beiji Qianjin Yaofang* that can be found today was edited by Lin Yi and Gao Baoheng in the Song Dynasty. It described one of the KXS formulae, the KXS-652, which has a ratio of 1:1:25:50 (RG/RP/RAT/PO). In addition, the formula of KXS-984 was recorded in *Yi Xin Fang* written by Nima Yasunori from Japan in 984 A.D. (Song Dynasty). The author recorded an herb ratio of 1:1:1:2 for KXS-984 and cited *Beiji Qianjin Yaofang*. The KXS-1130 was recorded in *Sunzhenren Qianjin Fang* that was published in 1130 A.D. (Song Dynasty), and the herb ratio was 1:1:200:300 (Table 1). The disappearance of the original *Beiji Qianjin Yaofang* could be a major reason for the emergence of different KXS formulae. The original formula might have been rearranged by other practitioners, who revised the KXS composition according to individual experience. Among them, the ratio of RG/RP/RAT/PO, as written in 652 A.D, is 1:1:25:50, and which is the most widely used today. However, the efficacy and chemical composition of these different formulae of KXS had not been determined, hindering the clinical application of KXS, until now.

As a first step to reveal the efficacy of KXS, we developed a rapid method to simultaneously identify different chemicals from the four members of KXS as a means of quality assessment, which might be not only used for the quality control, but also useful for the compatible principle elucidation. However, in the different KXS formulae, the amount of each herb varies greatly. The chemical amounts are on the different levels, which make it impossible to detect them simultaneously by the conventional HPLC-DAD method. Here, we used LC coupled with a DAD and a QQQ-MS/MS to reveal the amount of different chemicals within the three listed KXS formulae: these chemicals included RG-derived ginsenosides such as Rb_1, Rd, Re, Rg_1 [8], RP-derived 3,6′-disinapoyl sucrose [9], RAT-derived α- and β-asarone [10] and PO-derived pachymic acid [11] (Fig. 1). The results established the chemical criteria for the quality assurance of KXS and supplied a tool for analyzing different amount level chemicals in the sophisticated TCM formulae simultaneously.

Table 1. Historical variation of different KXS formulae

Notation[a]	Record	Ratio			
		RG	RP	RAT	PO
KXS-652	*Beiji Qianjin Yaofang*[b]	1	1	25	50
KXS-984	*Yixin Fang*[c]	1	1	1	2
KXS-1130	*Sunzhenren Qianjin Fang*[d]	1	1	100	200

a) The notation of KXS was corresponding to the years of recording.

b) *Beiji Qianjin Yaofang* was written by Sun Si-miao in 652 A.D., which was re-edited in 1066 A.D. (Song Dynasty). The composition of KXS-652 was 0.26 g RG, 0.26 g RP, 6.50 g RAT and 12.98 g PO.

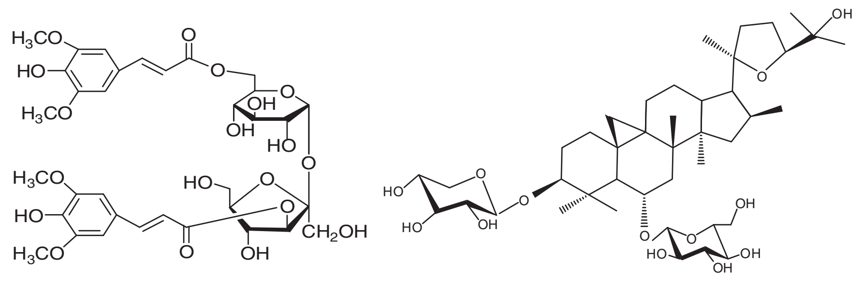
c) *Yixin Fang* was written by Nima Yasunori in 984 A.D. The composition of KXS-984 was 4 g RG, 4 g RP, 4 g RAT and 8 g PO.

d) *Sunzhenren Qianjin Fang* was published in about 1130 A.D., and the author was listed as Sun Si-miao. The composition of KXS-1130 was 0.07 g RG, 0.07 g RP, 6.62 g RAT and 13.24 g PO.

2 Materials and methods

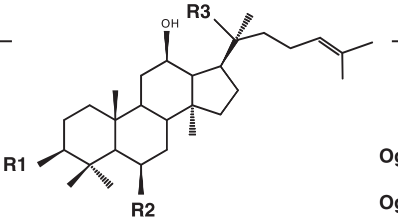
2.1 Chemicals, reagents and herbal materials

HPLC-grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). Formic acid and ammonium formate were purchased from Riedel-de Haën International (Hanover, Germany). Ultra pure water was prepared from a Milli-Q purification system (Millipore, Molsheim, France). The chemical standards of ginsenoside Rb_1, ginsenoside Rd, ginsenoside Re, ginsenoside Rg_1 and astragaloside IV were obtained from the Shanghai R&D Center for Standardization of TCM (Shanghai, China). 3,6′-Disinapoyl sucrose was extracted and purified in house. α-asarone and β-asarone were obtained from the Guangzhou University of TCM. Pachymic acid was purchased from the Hong Kong Jockey Club Institute of Chinese Medicine. The chemical standards of all materials used have a purity of at least 98% based on the HPLC profile, UV, MS, ^1H NMR and ^{13}C NMR data. Their chemical structures are shown in Fig. 1. The plant materials were purchased from Qingping Market of TCM in Guangzhou China, and were authenticated by one of the authors, Dr. Tina T. X. Dong, according to their morphological characteristics. The

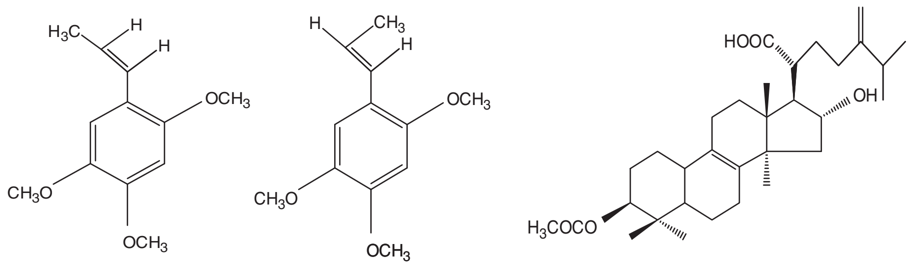


[1] 3,6'-Disinapoyl sucrose

[5] Astragaloside IV (Internal standard)



No.	Ginsenoside	R1	R2	R3
[2]	Rg_1	OH	Oglu	Oglu
[3]	Re	OH	Oglu (2-1) rha	Oglu
[4]	Rb_1	Oglu (2-1) glu	H	Oglu (6-1) glu
[6]	Rd	Oglu (2-1) glu	H	Oglu



[7] β-Asarone **[8] α-Asarone** **[9] Pachymic acid**

Figure 1. Chemical structures of marker chemicals analyzed in KXS. The denotations from 1 to 9 correspond to the peak numbers in the chromatogram as listed in Fig. 2, including 3,6′-disinapoyl sucrose (1), ginsenoside Rg_1 (2), ginsenoside Re (3), ginsenoside Rb_1 (4), ginsenoside Rd (6), β-asarone (7), α-asarone (8), pachymic acid (9) and astragaloside IV (5; an internal standard). The inserted table shows the functional groups of ginsenoside at R1, R2 and R3.

voucher specimens were deposited in the Centre for Chinese Medicine R&D at the Hong Kong University of Science & Technology. All other reagents used in this study were of analytical grade.

2.2 Sample preparation

According to different formulations of KXS including KXS-652, KXS-984 and KXS-1130 (see Table 1; notated according to the year of recording), the appropriate amounts of RG, RP, RAT and PO were weighed separately to form a combined weight of 20 g. The herbal extraction was performed in 160 mL of boiling water for 2 h, and the herbs were extracted twice. For the second extraction of KXS, the residue from the first extraction was filtered: the same extraction condition was applied on the filtered residue. Then the extracts were combined, dried under vacuum and stored at −80°C. This extraction condition was also applied to each individual herb as well as a combination of herbs under the same extraction method as described above. For the chemical analysis, an appropriate amount of each freeze-drying powder was weighed into a 15 mL centrifugal tube, and 5 mL of 50% methanol was added for sonication for 30 min. The supernatant was centrifuged at 4000 rpm at 4°C for 5 min before the rapid resolution LC (RRLC) analysis.

2.3 RRLC-DAD-QQQ-MS/MS analysis

Rapid analysis was performed on an Agilent RRLC 1200 series system (Agilent, Waldbronn, Germany), which was equipped with a degasser, a binary pump, an auto-sampler, a DAD and a thermostated column compartment. The herbal extract was separated on an Agilent ZORBAX Eclipse XDB-C18 column (1.8 μm id, 50 mm × 4.6 mm). The mobile phase was composed of 0.1% formic acid in acetonitrile (A) and 0.1% formic acid in water (B) using the following gradient program: 0–14 min, linear gradient 20.0–42.0% (A); 14–17 min, linear gradient 42.0–75.0% (A); 17–18 min, isocratic gradient 75.0–75.0% (A); 18–25 min, linear gradient 75.0–85.0% (A). A pre-equilibration period of 4 min was used between each run. The flow rate was 0.4 mL/min. The column temperature was 25°C. The injection volume was 5 μL. To establish the fingerprint of KXS, the UV detector wavelength was set to 330 nm with full spectral scanning from 190 to 400 nm. For the determination of 3,6′-disinapoyl sucrose, the wavelength was set to 330 nm. For the determination of α-asarone and β-asarone, the wavelength was set to 258 nm. Then the effluent was directed into the MS for the further analysis.

For the MS/MS analysis, an Agilent QQQ-MS/MS (6410A) equipped with an ESI ion source was operated in negative ion mode. The drying gas temperature was 325°C; drying gas flow: 10 L/min; nebulizer pressure: 35 psig; capillary voltage: 4000 V; delta electro multiplier voltage: 400 V. Two suitable transition pairs were chosen for acquisition in MRM mode for ginsenosides Rb_1, Rd, Re, Rg_1, pachymic acid and an internal standard astragaloside IV. The fragmentor voltage and collision energy values were optimized to obtain the highest abundance. Agilent Mass Hunter workstation software version B.01.00 was used for data acquisition and processing.

2.4 Method validation

To validate the analytic method, the linearity, sensitivity, precision, repeatability and accuracy of the analytes were determined. For the linearity, the calibration curve of each chemical was constructed using a range of concentrations of working standards, and each line was based on six different concentrations. The LOD and LOQ were used to evaluate the sensitivity. The LOD was estimated with a signal 3 times higher than that of the baseline noise while the LOQ was 10 times higher. The assay precision was determined by intra-day and inter-day variations, which were performed by analyzing standard solutions during a single day ($n = 6$) and on 3 executive days ($n = 6$), respectively. For repeatability test, five independent sample solutions were prepared in the procedures noted in Section 2.2. The accuracy was evaluated as the percentage recovery of analytes in the spiked samples. The recoveries were calculated by the following formula: recovery (%) = 100 × (amount found-original amount)/amount spiked. RSD was used to describe precision, repeatability and recovery.

3 Results and discussion

3.1 Optimization of extraction conditions

KXS was originally recorded in *Beiji Qianjin Yaofang*. According to different historical recording, three formulae were listed, namely KXS-652, KXS-984 and KXS-1130 (Table 1). The preparation of KXS required simple grinding and mixing. A herbal decoction of KXS, as compared with a powder of it, is the most common way of taking TCM [1–2]. The herbal preparation in the form of powder is used only when the patient has to take the drug for a prolonged period of time. In the extraction of KXS, water was selected as the solvent for two reasons: (i) water is the common solvent in TCM preparation and frequently used for the KXS preparation, and (ii) the water extract of KXS was reported to exert anti-depressant-like effects on the chronic mild stress animal model [7]. The extracts were dried and stored at −20°C. For the LC-DAD-MS/MS analysis, the methanol and acetonitrile were tested as the extraction solvent: the best solvent was found to be 50% methanol, which allowed a complete extraction of the tested chemicals. In addition, the extractions under sonication for 30 min, refluxing for 2 h, and Soxhlet extraction for 5 h were compared. Then 50% methanol with 30 min sonication was used for the analysis.

3.2 Method development and optimization of LC-MS conditions

The known chemical markers within KXS were determined, which included RG-derived ginsenosides such as Rb_1, Rd, Re, Rg_1, RP-derived 3,6′ -disinapoyl sucrose, RAT-derived α- and β-asarone and PO-derived pachymic acid (Fig. 1). In the three KXS formulae, the amounts of 3,6′-disinapoyl sucrose, α- and β-asarone were high enough for DAD detection while that of ginsenosides and pachymic acid were not. Therefore, we employed another MS/MS detection system for such purpose. In this case, we could simultaneously quantify the change of all the markers in the herbal decoction by a single injection, which the measurement error could be minimized. Thus, the 3,6′-disinapoyl sucrose, α- and β-asarone analyzed by the DAD at each highest absorbance wavelength, which was 330 and 258 nm, respectively. Under the optimal condition, these chemicals were well separated within 30 min (Fig. 2A). During the MS/MS analysis, the ginsenosides and pachymic acid were analyzed by the MRM scan mode. Although the ginsenosides Rg_1 and Re had not been baseline separated in the total ion chromatogram, they could also be separated and integrated in an MS/MS detector due to their difference in m/z (Fig. 2B). The amount of β-asarone in KXS was rather high, which could cause contamination when it flowed into the MS detector. Thus, the segment was set from 15 to 20 min on the MS/MS acquisition software, and the elution from the DAD detector was directed to the waste channel to avoid interference.

The MS conditions were also optimized. The roles of detection mode, buffer, capillary voltage, electron multiplier voltage and gas temperature were examined. The experimental parameters were set as follows: detection ion mode (positive, negative), buffer (5 mM ammonium formate, 10 mM ammonium formate, 0.1% formic acid), capillary voltage (3500, 4000, 4500 V), gas temperature (300, 325, 350°C), nebulizer pressure (30, 35, 40 psig) and the markers' abundance and shape were taken as the criteria for optimization. From the previous literatures [12–14], the positive mode and negative mode all had been used for the ginsenosides detection. But we found that the negative mode was more suitable for the detection, especially using the MRM mode. The optimum conditions were decided as follows (data not shown): negative ion mode, 0.1% formic acid, capillary voltage 4000 V, gas temperature 325°C, nebulizer pressure 35 psig, and electron multiplier voltage 400 V. Otherwise, the fragmentor, collision energy and the

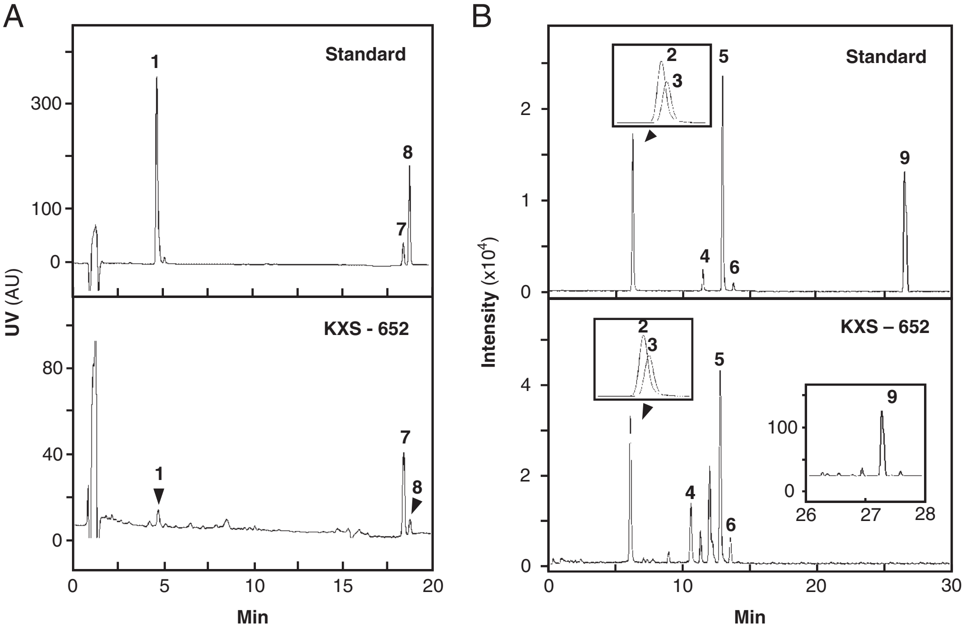


Figure 2. Typical RRLC-DAD-QQQ MS/MS chromatograms of marker chemicals in KXS-652. (A) The chromatographic method is described in Section 2. The identification of 3,6′-disinapoyl sucrose (1), α-asarone (8) and β-asarone (7) was made by a DAD detector (330 or 258 nm) in both standard markers and KXS-652. (B) The identification of ginsenoside Rg_1 (2), Re (3), Rb_1 (4), Rd (6), astragaloside IV (5) and pachymic acid (9) was made by an MS detector. Representative chromatograms are shown, $n = 3$.

ion pairs were optimized to achieve the highest abundance of the detected chemicals when the MRM detection mode was used. The characteristics of those chemicals in the MS/MS analysis are shown in Table 2 and Supporting Information Figure.

In MS/MS quantization, an internal standard should always be included: this compensates for sample-to-sample difference not only in extraction efficiency, but also in ionization efficiency during the transfer of analytes from liquid phase to gas phase. Here, astragaloside IV was used as an internal standard (Figs. 1 and 2; Table 2). The structure of astragaloside IV is similar to ginsenosides and pachymic acid, but more importantly, astragaloside IV is absent from KXS. Moreover, astragaloside IV had no signal in the DAD detector and did not interfere with the detection of 3,6′-disinapoyl sucrose, α- and β-asarone.

3.3 Validation of RRLC-DAD-QQQ-MS/MS methods

Each calibration curve was obtained from (drawn using) six different concentrations of chemical markers. The squared values of all the correlation coefficients (r^2) of these calibration curves were higher than 0.999 in DAD analysis and 0.990 in MS/MS analysis (Table 3). The LOD and LOQ were determined at a S/N of 3 and 10, respectively. For 3,6′-disinapoyl sucrose, α- and β-asarone by DAD analysis, the LOD and LOQ were in the range of 1–3 and 2–5 ng. For gisenosides and pachymic acid by MS/MS analysis, the LOD and LOQ were in the range of 0.004–0.021 and 0.025–0.220 ng. The intra- and inter-day precision were determined by analyzing the known concentrations of eight analytes in six replicates on a single day and duplicated on 3 successive days, respectively. In order to confirm the repeatability, five different working solutions prepared from the same KXS sample were analyzed. The RSD was taken as a measure of precision and repeatability: the precision and repeatability of the eight analytes were determined (Table 4). The RSDs of all intra- and inter-day variations were less than 5.0% for the eight analytes. Besides, the validation studies proved that the current assay had good reproducibility with RSD less than 5.0% ($n = 5$) for the eight analytes (Table 4). Recovery test was used to evaluate the accuracy of this method. Accurate amounts of the eight analytes were added to KXS extracts, which were then analyzed as described before. The total extracts containing known amounts of marker chemicals were subjected to the extraction and filtration steps as described in Section 2.2, and were then analyzed as described before. The developed analytical method showed an excellent accuracy with overall recovery of between 95.53 and 98.96% ($n = 3$) for the analytes (Table 4). Therefore, the RRLC-DAD-QQQ-MS/MS methods were precise, accurate and sensitive enough for simultaneous, quantitative evaluation of marker chemicals in KXS formulae.

3.4 Quantification of marker chemicals in KXS formulae

The developed method was applied to quantify eight chemical markers in three different historical formulae of KXS. The results of the quantitative analysis are summarized in Table 5. It was found that the eight chemical markers were detected in all three KXS formulae. Among the three formulae, KXS-984 showed the highest concentration of ginsenosides and 3,6′-disinapoyl sucrose, which indeed was not surprising because the KXS-984 formula recruited higher amounts of RG and RP herbs. In contrast, the amounts of RAT-derived asarone (both α and β) were the

Table 2. Mass spectra properties of marker chemicals in KXS

Chemical	Formula	Calculated mass [M]	Precursor ion [M−H][a]	Fragmentor energy[b]	Collison energy[c]	Production[d]	Retention time (min)[e]
Ginsenoside Rb_1	$C_{54}H_{92}O_{23}$	1108.6	1107.6	250	41	945.5	11.854
					49	783.5	
Ginsenoside Rd	$C_{48}H_{82}O_{18}$	946.5	945.5	250	33	783.5	14.058
					45	621.6	
Ginsenoside Re	$C_{48}H_{82}O_{18}$	946.5	945.5	250	41	637.5	6.772
					53	475.5	
Ginsenoside Rg_1	$C_{42}H_{72}O_{24}$	800.5	799.5	250	21	637.3	6.745
					37	475.4	
Pachymic acid	$C_{33}H_{52}O_5$	528.3	527.3	250	37	467.2	27.297
					41	465.3	
Astragaloside IV	$C_{41}H_{68}O_{14}$	784.9	829.5[f]	190	5	829.5	13.329
					25	783.2	

a) The detected chemicals had the greatest responses under the negative mode: the $[M-H]^-$ was used as the precursor ion.
b) The fragmentor energy was optimized to have the greatest ionize efficiency.
c) The collision energy was optimized to have the greatest product ion intensity, which was the key factor in the MRM mode.
d) Two product ions were used for the MRM analysis. The upper one was used for quantitative analysis and the lower one was for qualitative analysis, which could guarantee the precision of analytes.
e) The retention time was determined by 3 different individual analyses ($n = 3$).
f) The precursor ion of astragaloside IV was $[M+HCOOH-H]^-$ under the negative mode.

Table 3. Calibration curves, LOD and LOQ for eight marker chemicals in KXS

Chemical	Calibration curve[a]	Correlation coefficient (r^2)	Linear range (ng)	LOD[b] (ng)	LOQ[c] (ng)
Ginsenoside Rb_1	$y = 0.0090x - 0.00006$	0.9955	0.125–12.5	0.021	0.086
Ginsenoside Rd	$y = 0.0034x + 0.00008$	0.9914	0.5–50	0.049	0.220
Ginsenoside Re	$y = 0.0415x - 0.00005$	0.9955	0.05–5.00	0.004	0.025
Ginsenoside Rg_1	$y = 0.0370x - 0.00006$	0.9958	0.05–5.00	0.006	0.025
Pachymic acid	$y = 1.2056x - 0.0666$	0.9910	0.025–2.50	0.005	0.025
3, 6′-disinapoyl sucrose	$y = 54.196x - 3.4607$	0.9999	10–500	3	5
α-Asarone	$y = 84.349x - 4.8318$	0.9999	5–500	1	2
β-Asarone	$y = 65.104x - 0.5823$	0.9999	5–500	1	2

a) These calibration curves were constructed by plotting the peak area versus the concentration of each analyte. Each calibration curve was derived from six data points, $n = 3$.
b) LOD refers to the limits of detection.
c) LOQ refers to the limits of quantification.

lowest in the KXS-984 formula. The variation in the concentration of these chemicals within different KXS decoctions could be a result of two reasons: (i) the variation in the herb amount and (ii) the variation of chemical solubility in the presence of other herbs.

To explore these variations, especially the quantitative change of the selected markers, we compared the solubility of marker chemicals in a single herb with that in a mixture of herbs. The total amount of ginsenoside Rb_1, Rd, Re and Rg_1 extracted from 1 g of RG was about 4 mg. This amount was markedly increased when RG was extracted together with RP or PO: about 50% more than when RG was extracted alone (Fig. 3A). In contrast, the extraction together with RAT decreased the amount of ginsenosides by over 50%. This suppression could not be recovered in the presence of RP but could be recovered by extraction together with PO. The amounts of ginsenosides soluble in KXS were similar in all three historical decoctions (Fig. 3A). In RP, the amount of 3,6′-disinapoyl sucrose soluble in water was about 1.8 mg/g of herb, which could however be increased in the presence of RG and RAT (Fig. 3B). The suppression effect was revealed in the presence of PO. KXS-1130 was the best formula to have 3,6′-disinapoyl sucrose extracted from RP. The amount of RAT-derived asarone (both α and β) was similar in a single herb extraction or in a mixture (Fig. 3C). In parallel, the amounts of extractable asarones in the three KXS formulae were similar. The amount of pachymic acid from PO was about 40 ng/g of herb: this amount was markedly increased by about 4-fold when the extraction was conducted in the presence of RP (Fig. 3D). The solubility of PO-derived pachymic acid was the highest in KXS-984.

Table 4. Precision, repeatability and recovery of 8 marker chemicals in KXS

Chemical	Precision				Repeatability ($n = 5$)		Recovery[a] ($n = 3$)	
	Intra-day ($n = 6$)[b]		Inter-day ($n = 6$)[c]					
	Mean (mg/L)	RSD (%)	Mean (mg/g)	RSD (%)	Mean (mg/L)	RSD (%)	Mean (%)	RSD (%)
Ginsenoside Rb_1	0.86	3.21	0.80	3.51	0.204	3.65	95.53	3.25
Ginsenoside Rd	0.38	4.01	0.36	3.12	0.482	3.52	96.35	3.78
Ginsenoside Re	0.82	3.65	0.79	3.98	0.190	3.02	95.65	3.21
Ginsenoside Rg_1	1.06	3.32	1.02	3.51	0.246	2.98	98.12	4.02
Pachymic acid	0.01	2.21	0.009	2.29	0.408 e^{-3}	1.16	96.74	2.65
3,6′-Disinapoyl sucrose	1.62	1.05	1.61	2.01	0.588	1.31	98.96	1.78
α-Asarone	2.47	0.45	2.45	1.89	0.749	1.49	97.54	1.36
β-Asarone	53.51	0.16	53.49	2.31	16.86	1.05	96.21	1.85

a) Recovery (%) = 100 × (amount found − original amount)/amount spiked. The data were presented as average of three independent determinations, and the SD was <5% of the mean, which was not shown for clarity.

b) The intra-day analysis refers to the sample examined for six replicates within one day.

c) The inter-day analysis refers to the sample examined in duplicates over three consecutive days.

Table 5. Quantitative assessment of eight marker chemicals in three KXS formulae.

Marker chemical[a]	KXS-652	KXS-984	KXS-1130
Ginsenoside Rb_1	0.204 ± 0.017[b]	1.841 ± 0.080	0.044 ± 0.001
Ginsenoside Rd	0.080 ± 0.006	0.667 ± 0.045	0.023 ± 0.001
Ginsenoside Re	0.190 ± 0.013	1.655 ± 0.068	0.037 ± 0.001
Ginsenoside Rg_1	0.246 ± 0.022	1.966 ± 0.070	0.052 ± 0.001
Pachymic acid	0.211 e^{-3} ± 0.004 e^{-3}	0.177 e^{-3} ± 0.007 e^{-3}	0.138 e^{-3} ± 0.001 e^{-3}
3,6′-Disinapoyl sucrose	0.336 ± 0.003	2.838 ± 0.009	0.115 ± 0.011
α-Asarone	0.514 ± 0.002	0.304 ± 0.059	0.307 ± 0.003
β-Asarone	11.124 ± 0.019	4.895 ± 0.073	7.028 ± 0.025

a) The analyses of ginsenosides and pachymic acid were carried out by a QQQ-MS/MS detector, while the other three chemicals were revealed by a DAD detector.

b) Values are expressed in mg/g of dried powder of KXS, mean ± SD, $n = 3$.

Our results showed that a TCM formula is not simply a mixture of different herbs; there are sophisticated solubility changes during boiling and compatibility issues in the mixtures [2, 3], which may affect the extraction efficiency of chemicals strongly brought by the combination and the dosage of different herbs [3]. According to the TCM theory, the four herbs of KXS can be separated into two herb-pairs: one is RAT and PO and the other one is RG and RP. The RAT and PO pair is considered as *Jun* (king), while the RG and RP pair is considered as *Chen* (minister). It is well known that saponin, the main active chemical in RG and RP, is able to enhance the solubility of other chemicals in the decoction [3, 15]. Thus, the enhanced extraction efficiency of asarone and pachymic acid could be a result of the existence of saponin. On the other hand, polysaccharide, like chitosan, has been reported to enhance the chemical solubility of various chemicals, and is indeed the major component of PO [16]. By increasing the amount of PO, the RG-derived ginsenosides could be extracted more extensively, resulting in an increased extraction of RAT-derived asarone and PO-derived pachymic acid. In contrast, the 3,6′-disinapoyl sucrose extraction was inhibited when RP was boiled with PO.

Although the three KXS formulae vary greatly in the herb ratio, they are all designed to treat morbid forgetfulness [17]. Indeed, the previous studies have focused on the effects of KXS on memory enhancement and the potential to treat neurodegenerative diseases. KXS-652 and KXS-984 are the two most commonly used formulae, which have been demonstrated to enhance the hippocampus long term potentiation [18], to elevate the SOD activity, and to reduce the MDA content in brains and livers in animal studies [19, 20]. Meanwhile, with the higher risk of depression these days and the proven action of RG and RP on depression disorder, the potential of using KXS to treat depression disorders has become an important research focus. Since the disruption of neurotrophin is one of the hypotheses on the pathogenesis of mental depression [21], the three KXS formulae were also tested for activity that induces neurite outgrowth of cultured neurons. Based on our current study,

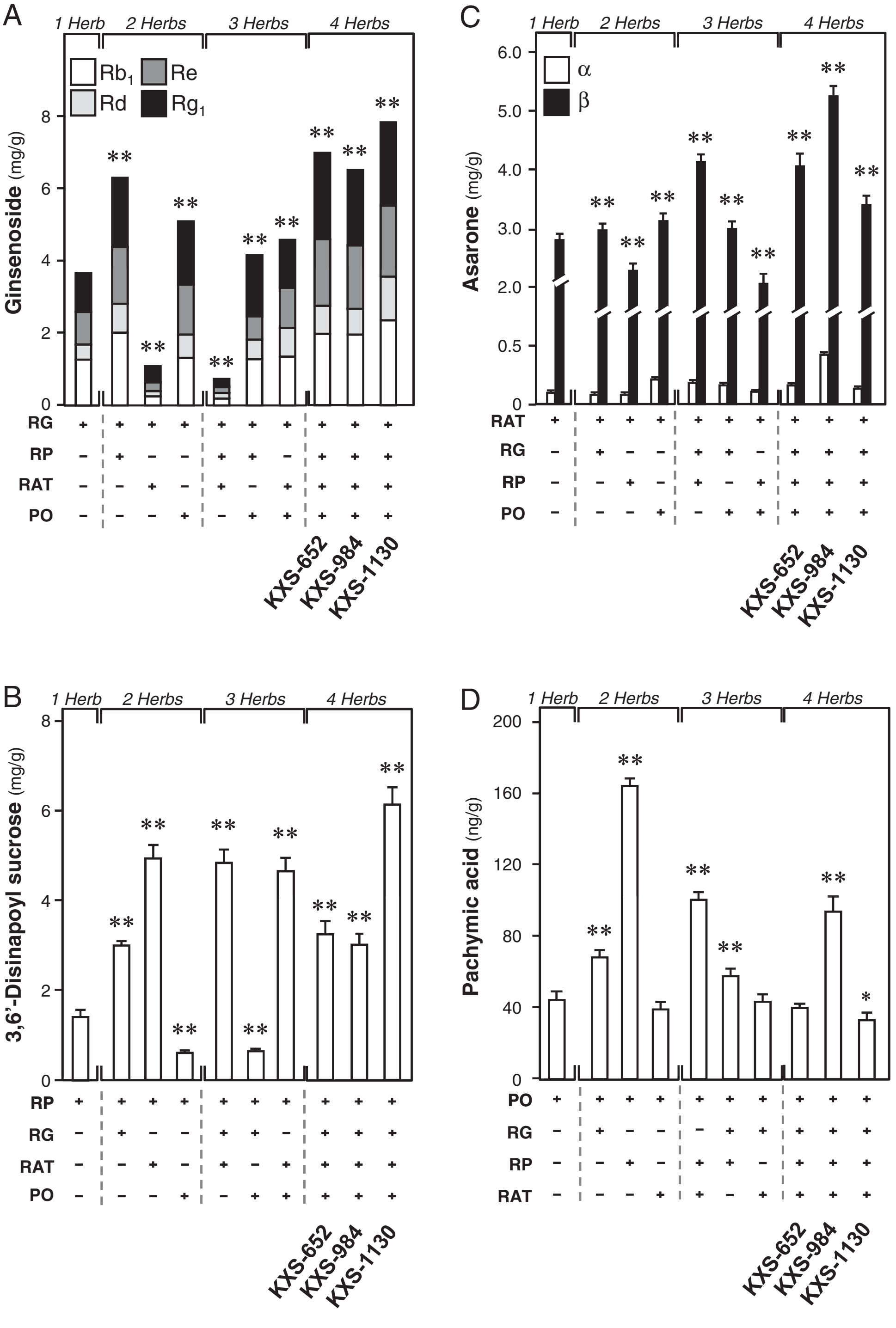


Figure 3. The solubilities of marker chemicals in single herb compared with that in KXS. The marker chemicals including RG-derived ginsenosides (A), RP-derived 3,6′-disinapoyl sucrose (B), RAT-derived α- asarone and β-asarone (C), and PO-derived pachymic acid (D) were determined in single herb as well as in a combination of other herbs of KXS. The amounts of these marker chemicals were also determined in the three formulae of KXS. Values are expressed in mg/g or ng/g of dried single herb, and they are in mean ± SD, where $n = 3$. Statistical comparison was made with the single herb; $^{*}p < 0.05$; $^{**}p < 0.01$.

KXS-652 was the strongest in this respect (Zhu et al., unpublished result).

The four individual herbs of KXS are known to affect our nervous system. The ginsenosides derived from RG are the main chemicals with strong neurotrophic and neuroprotective effects [22, 23]. Oligosaccharide ester derived from RP has also been reported to induce neurite outgrowth and have the potential to enhance memory [24], thus have been used to treat mood disorders [25]. In parallel, the RAT-derived asarones have been shown to protect neurons from β-amyloid-induced cell death [26], and have anti-depressant-like activity in mice [27]. The water extract of PO protected cells by suppressing the oxidative stress, which also prevented the apoptosis induced by β-amyloid [28]. In addition, the triterpenoids of PO (*e.g.* pachymic acid) regulated the expressed 5-HT3A receptors in *Xenopus* oocytes

[29]. The amount of PO used is the highest in KXS. Hence, the action of PO on depression is well worth studying in the future.

4 Concluding remarks

In this study, we have established a systematic approach for the interpretation of chemical composition and quality assessment of KXS formulae. By using RRLC coupled with a DAD and an ESI triple quadrupole tandem spectrometer, a rapid, simple and reliable method to simultaneously determine eight marker chemicals (ginsenoside Rb_1, Rd, Re, Rg_1, 3,6′-disinapoyl sucrose, α-asarone, β-asarone and pachymic acid) in KXS was first developed and validated. This method provides an excellent quantitative tool for quality assessments of TCM formulae due to its high capacity, high sensitivity, high selectivity and short analysis time. From the analysis of marker chemicals solubility with this method, our results show that the combination and the dosage of different herbs strongly affect the extraction efficiency of chemicals. There are sophisticated solubility changes of chemicals during boiling and compatibility issues in the mixtures, which shows that the TCM formula is not simply a mixture of different herbs.

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