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Quality Evaluation of a Herbal Prescription Through Quantification of 40 Components by HPLC–ESI–MS/MS

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ABSTRACT:

Introduction – The Kang-nao-shuai (KNS) capsule is a combined herbal prescription used in the treatment of insomnia, amnesia, neurasthenia, age-related dementia and brain injuries. Multiple constituents are considered to be responsible for the therapeutic effects of this herbal prescription. However, the quality control of the multicomponents is limited.

Objective – To establish a liquid chromatography–electrospray ionisation–mass spectrometry method for the analysis of 40 constituents in KNS capsules.

Methodology – The optimal chromatographic conditions were achieved on an Agilent C_{18}-column with a gradient elution that consisted of methanol and 0.1% formic acid in water. The precursor and product ions of analytes were monitored on a hybrid quadrupole linear ion trap mass spectrometer in positive and negative mode respectively using multiple-reaction monitoring.

Results – A total of 40 constituents including organic acid, flavonoid, quinone, terpene, alkaloid and saponin were quantified, most of the 40 components were determined for the first time in the KNS capsule. A quantitative HPLC–ESI–MS/MS method allowing the quantification of 40 marker compounds was optimised and validated for linearity, precision, accuracy, stability, specificity and limits of detection and quantification. The method was successfully applied to analyse 10 batches of KNS capsule.

Conclusion – The established method is simple and can be used as a tool for quality evaluation and control of this natural product. **Copyright © 2011 John Wiley & Sons, Ltd.**

Supporting information can be found in the online version of this article.

Keywords: HPLC–ESI–MS/MS; MRM; quality evaluation; quantification; Kang-nao-shuai

Introduction

The Kang-nao-shuai (KNS) capsule is an effective herbal prescription used in the treatment of insomnia, amnesia, neurasthenia, age-related dementia, brain injuries caused by stroke, cerebral trauma and encephalitis (Li *et al*., 1987; Tan *et al*., 1998; Cheng, 2002; Huang, 2003). Its ingtredients are prepared from 19 herbs in a specified ratio. Among them, the herbs Radix Paeoniae Alba, Radix Salviae Miltiorrhiae, Flos Chrysanthemi, Radix Puerariae, Radix Polygoni Multiflori, Radix Scutellariae, Radix Astragali, Fructus Lycii and Rhizoma Cyperi are considered as the main ingredients due to their higher ratios and significant pharmacological actions. Modern pharmacology studies have demonstrated that the KNS capsule possesses significant effects including sedative, hypolipidemic, anti-inflammatory, enhanced blood circulation, etc. (Tan *et al*., 1998; Huang, 2003).

To date, several high performance liquid chromatography coupled with ultraviolet detector (HPLC-UV) methods have been used for the quality control of KNS capsules. These studies mainly focused on the analysis of a few constituents with longer running times (Guo and Lu, 2008; Zhang *et al*., 2006; Xu *et al*., 2009). A fingerprint method also has been established to evaluate the quality of KNS capsules (Wang *et al*., 2007), but this displayed only the similarity of different samples calculated on the basis of the relative value of unidentified peaks. Owing to the extraordinary complexity of the KNS capsule, quantitation the active constituents as many as possible could better reflect its chemical properties. Therefore, a more efficient quality control approach is urgently needed.

In the present study, 40 constituents including organic acid, flavonoid, quinone, terpene, alkaloid and saponin (the chemical structures are shown in Fig. 1) were selected as the marker components in KNS capsules because they are the major effective constituents in the corresponding herb ingredients (Liu *et al*., 1989; Liu *et al*., 2001; Yu *et al*., 2002; Wen, 2006; Zhang *et al*., 2007; Zhang and Zhang 2007; Guan and Su, 2008; Liu *et al*., 2008; Liu *et al*., 2009). A selective, sensitive and rapid HPLC–MS/MS method was established for the identification and quantification of these 40 components for the first time. The verified method was successfully applied to the quantification of 40 major components in 10 batches of KNS capsules.

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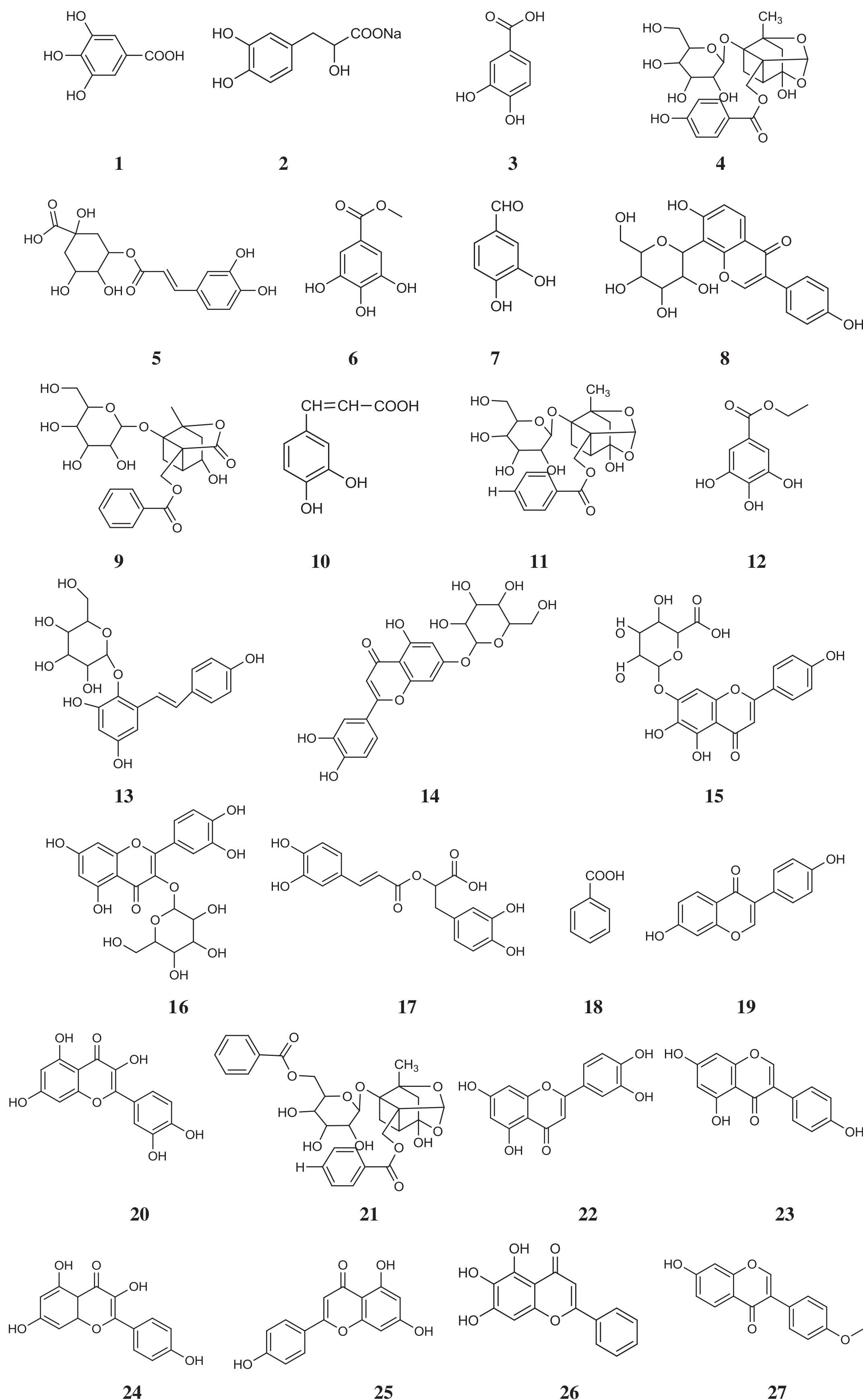

Figure 1. The structures of the 40 components in KNS capsules.

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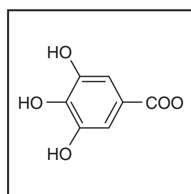

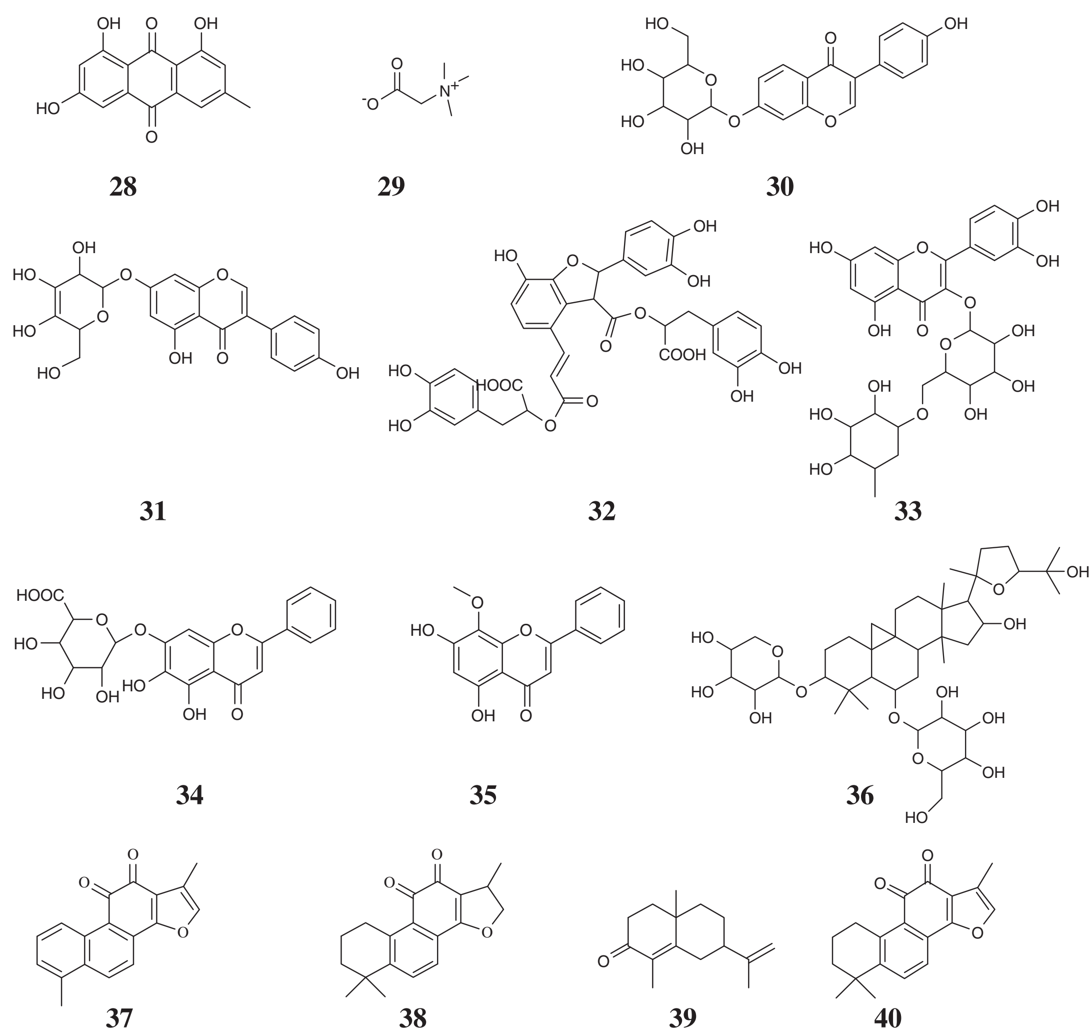

Figure 1. (Continued)

Experimental

Materials and reagents

Gallic acid (**1**), sodium danshensu (**2**), protocatechuic acid (**3**), oxypaeoniflorin (**4**), chlorogenic acid (**5**), methyl gallate (**6**), protocatechuic aldehyde (**7**), puerarin (**8**), albiflorin (**9**), caffeic acid (**10**), paeoniflorin (**11**), ethyl gallate (**12**), 2,3,5,4′-tetrahydroxystilbene-2-O-β-D-glucoside (**13**), luteoloside (**14**), scutellarin (**15**), hyperoside (**16**), rosmarinic acid (**17**), benzoic acid (**18**), daidzein (**19**), quercetin (**20**), benzoylpaeoniflorin (**21**), luteolin (**22**), genistein (**23**), kaempferol (**24**), baicalein (**26**), formononetin (**27**), emodin (**28**), betaine (**29**), daidzin (**30**), genistin (**31**), salvianolic acid B (**32**), rutin (**33**), baicalin (**34**), wogonin (**35**), astragaloside IV (**36**), tanshinone I (**37**), cryptotanshinone (**38**), α-cyperone (**39**) and tanshinone IIA (**40**) were all purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Apigenin (**25**) was obtained from Shanxi Huike Botanical Development Co., Ltd, China. All reference standards used in this study showed purities of > 98% by HPLC/UV analysis. The KNS samples of different production batches were provided by a pharmaceutical company in China.

HPLC grade methanol (Fisher, USA) was used for HPLC analysis. Deionised water was purified by Heal Force–PWVF Reagent Water System (Shanghai CanRex Analyses Instrument Corporation Limited, P. R. China). Formic acid was HPLC grade, purchased from Diamond Technology Incorporation. Analytical grade methanol (Tianjin Chemical Corporation, P. R. China) was used for the extraction of samples.

Instrumentation and conditions

Liquid chromatography. An Agilent 1200 liquid chromatography system (Agilent, USA) equipped with a quaternary solvent delivery system, an autosampler and a column compartment was used. The

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chromatographic separation was performed on an Agilent Zorbax SB–C_{18}-column (250 mm × 4.6 mm, 5 μm). The mobile phase consisted of A (methanol) and B (0.1% aq. formic acid, v/v). A gradient programme was used as follows: 30–90% A for 0–15 min and 90% A for 15–25 min. The flow rate was monitored at 0.8 mL/min. The injection volume was 10 μL and the column temperature was maintained at 25 °C.

Mass spectrometer. A 3200 QTRAP™ system from Applied Biosystems/MDS Sciex (Applied Biosystems, Foster City, CA, USA) was used for the detection, which is a hybrid triple quadrupole linear ion trap mass spectrometer equipped with Turbo V sources and a TurboIonspray interface. The analyses were performed using an electrospray ionisation (ESI) source in positive and negative modes. The operation conditions were as follows: ionspray voltage, 5500/-4500 V; curtain gas (CUR), 25 psi and interface heater was on; collision gas, medium; nebuliser gas (gas 1) and heater gas (gas 2), 60 and 65 psi; the turbo spray temperature, 600 ºC; entrance potential (EP), 10/-10 V; collision cell exit potential (CXP), 5/−5 V. Nitrogen was used in all cases. A multiple-reaction monitoring (MRM) mode was carried out for the detection. The results of the precursor ion, product ion, corresponding declustering potential (DP) and collision energy (CE) are shown in Table 1. The dwell time was set at 60 ms in negative mode and 120 ms in positive mode, respectively. Applied Biosystems/MDS Sciex Analyst software (version 1.4.2) was used for data acquisition and processing.

Table 1. The retention time (t_R), MS/MS fragment ions, declustering potential (DP), collision energy (CE), linear regression data, LOD and LOQ of the 40 constituents in KNS capsules

Compounds	t_R (min)	MS1 (m/z)	MS2 (m/z)	DP (V)	CE (eV)	Regression equation	r^2	Linear range (μg/mL)	LOD (ng/mL)	LOQ (ng/mL)
1	4.01	168.9	124.9	−37	−22	$y = 6.84e^{2}x + 1.04e^{5}$	0.9997	0.924–59.160	7.5	20.8
2	4.82	218.9	172.9	−78	−23	$y = 0.88e^{2}x + 1.68e^{4}$	0.9986	0.300–19.180	3.3	6.2
3	5.79	152.9	108.8	−33	−21	$y = 7.80e^{2}x - 5.84e^{3}$	0.9999	0.030–1.950	9.4	24.8
4	5.96	495.1	136.9	−32	−40	$y = 3.50e^{2}x - 1.30e^{4}$	0.9997	0.076–4.844	4.9	17.3
5	6.34	353.1	191.0	−20	−24	$y = 5.22e^{2}x - 1.20e^{4}$	0.9998	0.290–18.530	3.7	10.4
6	6.82	182.9	123.9	−54	−30	$y = 1.74e^{3}x + 8.43e^{3}$	0.9995	0.006–0.378	0.9	2.6
7	6.82	136.9	107.9	−53	−31	$y = 6.19e^{2}x + 7.14e^{3}$	0.9998	0.025–1.575	4.6	17.8
8	7.19	415.1	267.1	−50	−45	$y = 5.76e^{2}x + 1.00e^{6}$	0.9992	2.850–182.400	0.2	0.5
9	7.30	525.2	120.9	−5	−32	$y = 0.89e^{2}x + 1.33e^{5}$	0.9988	1.176–75.240	10.1	27.3
10	7.99	179.0	135.0	−31	−22	$y = 1.48e^{3}x + 2.31e^{4}$	0.9994	0.022–1.408	0.8	3.0
11	8.46	525.1	449.1	−12	−15	$y = 1.35e^{2}x + 1.56e^{5}$	0.9993	1.628–104.200	5.4	21.2
12	9.06	196.9	123.9	−50	−32	$y = 1.36e^{3}x + 5.29e^{5}$	0.9996	0.414–26.520	1.2	4.8
13	9.24	405.1	243.0	−50	−27	$y = 6.61e^{2}x + 2.41e^{5}$	0.9998	1.375–88.000	2.6	9.8
14	10.40	447.2	285.1	−65	−35	$y = 5.12e^{2}x + 1.60e^{5}$	0.9990	0.228–14.602	1.7	4.3
15	10.48	461.0	285.0	−30	−28	$y = 1.72e^{2}x + 5.01e^{3}$	0.9997	0.045–2.900	7.8	20.4
16	10.88	463.1	300.0	−66	−35	$y = 5.19e^{2}x + 3.25e^{3}$	0.9988	0.007–0.420	2.1	4.9
17	11.05	359.0	160.9	−29	−23	$y = 1.06e^{2}x + 2.18e^{4}$	0.9998	1.375–88.000	21.3	58.7
18	12.07	120.9	76.9	−24	−20	$y = 0.32e^{2}x + 6.17e^{2}$	0.9991	0.139–8.880	48.7	118.5
19	13.21	252.9	131.9	−52	−57	$y = 3.51e^{2}x + 9.62e^{4}$	0.9988	0.211–13.514	3.9	14.8
20	13.55	301.0	151.0	−50	−30	$y = 2.20e^{3}x + 4.09e^{3}$	0.9998	0.008–0.500	2.1	5.3
21	13.99	583.1	120.8	−25	−37	$y = 0.55e^{2}x + 3.86e^{3}$	0.9998	0.189–12.120	16.9	50.5
22	14.28	285.0	133.0	−70	−47	$y = 1.69e^{3}x + 2.14e^{4}$	0.9998	0.079–5.058	1.3	3.3
23	14.49	268.9	132.9	−54	−42	$y = 6.56e^{2}x + 6.83e^{3}$	0.9996	0.015–0.990	1.2	4.1
24	15.05	285.0	93.0	−67	−53	$y = 0.50e^{2}x + 2.98e^{3}$	0.9992	0.089–5.720	20.4	71.5
25	15.53	269.0	116.9	−67	−52	$y = 2.18e^{3}x + 6.63e^{4}$	0.9996	0.055–3.520	0.3	1.3
26	15.91	268.9	194.9	−50	−37	$y = 0.97e^{2}x + 9.33e^{4}$	0.9997	1.179–75.430	15.2	46.5
27	16.27	267.0	251.9	−42	−28	$y = 8.36e^{4}x - 1.32e^{5}$	0.9987	0.005–0.305	0.1	0.5
28	21.09	268.9	224.9	−55	−36	$y = 4.34e^{3}x + 4.08e^{5}$	0.9992	0.142–9.100	1.6	5.9
29	2.78	118.1	58.1	43	40	$y = 1.25e^{3}x + 1.32e^{5}$	0.9994	0.368–23.578	2.1	8.4
30	8.29	417.2	255.2	24	25	$y = 5.15e^{2}x + 4.65e^{5}$	0.9991	1.012–64.800	1.7	4.3
31	9.74	433.2	271.1	40	26	$y = 9.36e^{2}x + 1.97e^{4}$	0.9996	0.062–3.960	3.2	8.5
32	10.72	741.2	561.2	55	38	$y = 0.21e^{2}x + 2.21e^{4}$	0.9997	2.781–178.000	75.9	287.0
33	10.75	611.4	303.3	30	33	$y = 0.15e^{2}x + 1.20e^{3}$	0.9994	0.112–7.200	16.4	50.0
34	12.65	447.2	271.1	31	30	$y = 2.96e^{2}x + 2.00e^{6}$	0.9993	5.771–369.360	2.5	7.1
35	17.45	285.1	270.1	48	33	$y = 7.26e^{2}x + 6.47e^{5}$	0.9993	0.983–62.900	2.3	5.7
36	19.89	785.6	143.1	35	25	$y = 0.25e^{2}x + 1.04e^{3}$	0.9997	0.050–3.200	21.7	44.0
37	20.85	277.1	249.1	60	28	$y = 1.38e^{3}x + 3.71e^{4}$	0.9994	0.052–3.302	0.7	2.5
38	21.26	297.2	251.2	55	31	$y = 1.30e^{3}x + 8.06e^{4}$	0.9998	0.129–8.280	0.8	2.1
39	21.63	219.2	111.1	35	31	$y = 8.40e^{2}x + 8.13e^{3}$	0.9997	0.023–1.500	1.4	3.8
40	23.27	295.2	277.2	60	26	$y = 1.58e^{3}x + 5.68e^{4}$	0.9996	0.064–4.120	1.0	2.5

In the regression equation $y = ax + b$, x refers to the concentrations, y is the peak area, and r^2 is the correlation coefficient of the equation. LOD, limit of detection; LOQ, limit of quantification.

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Standard solution preparation

Each reference compound, **1–40**, was accurately weighed and dissolved in 50% aqueous methanol to make 40 different of stock solutions. Then, each stock solution was diluted and mixed with 50% aqueous methanol to prepare a final mixed standard solution. A series of seven calibration solutions was prepared by appropriate dilution of the final mixed standard solutions with 50% aqueous methanol for construction of the regression equations. All solutions were stored at 4 °C. The concentration ranges are given in Table 1.

Sample solution preparation

The constituents of twenty KNS capsules were ground and well mixed. The powder of the KNS capsules (0.3 g) was accurately weighed and extracted with 25.0 mL of 50% aqueous methanol for 30 min in an ultrasonic bath. The extracted solution was adjusted to the original weight by adding 50% aqueous methanol. After the centrifugation at 12000 rpm for 10 min, the supernatant was injected into the HPLC system after filtering through a 0.45 μm microporous membrane.

Results and Discussion

Selection of LC columns

Four different types of LC columns with different lengths and particle sizes, i.e. (C1) Agilent Zorbax SB C_{18}-column (250 mm × 4.6 mm, 5 μm), (C2) Thermo C_{18}-column (250 mm × 4.6 mm, 5 μm), (C3) Agilent Zorbax SB C_{18}-column (150 mm × 4.6 mm, 5 μm) and (C4) Sepax GP C_{18}-column PN (100 mm × 2.1 mm, 3 μm), were tested for the peak shape, signal intensity and separation efficiency of 40 constituents. The same sample solution was used for this optimisation test. According to the property of the four columns, both the flow rate and the proportion of mobile phase were optimised, then the chromatograms acquired on different columns under the optimised conditions were compared. It was found that the separation efficiency of C1 was obviously superior to that of the others, especially when analysing quercetin and baicalein. C2 presented worse peak shape and resolution than C1, even though they have identical lengths and particle sizes. C1 and C3 showed resemblance in the resolution of components, but the former was better probably due to its longer column length. C4 is a shorter column packed with smaller particle size material. Yet it was not preferable to the others, no improvement in eliminating peak tailing and shortening analysis time was observed. So C1 was accordingly selected as the separation column. Figure 2 shows the extract ions chromatograms (XIC) of quercetin and baicalein, which were obtained by performing the same sample solution with different columns under their optimal conditions.

Selection of mobile phase and buffers

The mobile phase composition was studied in view of achieving higher signal intensity, better resolution and shorter analysis time of target compounds. Various mixtures of water and acetonitrile were used as the mobile phase, but the signal intensity of several compounds was not satisfactory. However, when acetonitrile was replaced by methanol, the situation was greatly improved and satisfactory signal intensity was obtained.

The effect of different buffers was also examined, including formic acid (0.05%, 0.1%, 0.2%), acetic acid (0.05%, 0.1%, 0.2%) and ammonium acetate (0.2, 1, 2 mmol/L). Compared with acetic acid and ammonium acetate, addition of formic acid in the mobile phase was found to enhance the resolution and eliminate the peak tailing of the target compounds. However, it could also restrain ionisation efficiency of the major compounds detected under the negative ESI mode. Considering the combined factors between separation and ionisation efficiency, addition of 0.1% (v/v) of formic acid was a compromise and reasonable choice.

Optimisation of MS parameters

The precursor and product ions of 40 reference compounds were selected by infusing individual standard solutions into the mass

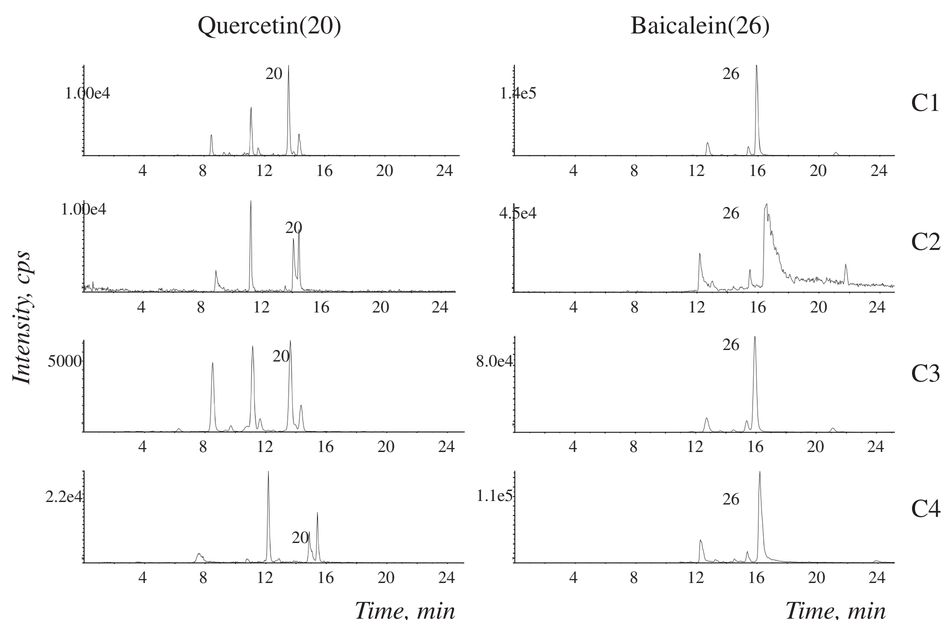

Figure 2. The extract ion chromatograms (XIC) of multiple-reaction monitoring (MRM) chromatograms of quercetin and baicalein obtained by using the same sample solution with four different columns under their optimal HPLC conditions.

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spectrometer using a syringe pump. In the MS1 scan mass spectra, most analytes formed predominant deprotonated molecular ions $[M-H]^-$ and protonated molecular ions $[M+H]^+$, except that the most abundant ions of albiflorin, paeoniflorin and salvianolic acid B were $[M+HCOO]^-$ (*m/z* 525.2), $[M+HCOO]^-$ (*m/z* 525.1) and $[M+Na]^+$ (*m/z* 740.2), respectively. So these predominant precursor ions of each analyte were chosen for MS/MS analysis. Several fragment ions of the analytes were observed in the product ion spectra and the predominant fragment ions were selected in MRM acquisition for quantification. Then DP and CE were optimised in order to obtain the maximum sensitivity of analysis. Both negative and positive ion modes were conducted, which showed that the negative ion mode was more sensitive for compounds **1–28**, whereas the positive mode was more sensitive for compounds **29–40**.

Initially, in order to shorten analysis time, the determination was set in positive and negative mode simultaneously, but this advantage can be diluted due to increasing the background effects, in particular for the low abundance compounds in KNS capsules. Therefore, individual positive and negative mode runs were carried out to analyse the KNS capsules.

Validation of the assay

Linearity, limit of detection and limit of quantification. The linear calibration curves containing seven different concentrations of each reference compound were performed by the external standard method. The peak area values were the average values of three replicate injections. All calibration curves were constructed from peak areas of the reference compounds versus their

Table 2. Precision of the 40 ingredients for quantitative analysis

Compounds	Instrument precision($n=6$)		Intra-day precision($n=6$)		Inter-day precision($n=9$)	
	Mean ± SD (μg/mL)	RSD (%)	Mean ± SD (μg/g)	RSD (%)	Mean ± SD (μg/g)	RSD (%)
1	14.79 ± 0.08	0.54	500.17 ± 5.55	1.11	498.48 ± 10.23	2.05
2	9.59 ± 0.06	0.62	177.39 ± 1.24	0.70	176.68 ± 2.05	1.16
3	0.97 ± 0.01	1.03	16.67 ± 0.08	0.48	16.87 ± 0.09	0.53
4	1.21 ± 0.01	0.83	30.66 ± 0.08	0.26	31.97 ± 0.28	0.87
5	9.26 ± 0.04	0.43	160.27 ± 2.00	1.25	163.48 ± 2.63	1.61
6	0.19 ± 0.001	0.53	2.65 ± 0.04	1.51	2.55 ± 0.05	1.96
7	0.79 ± 0.01	1.26	17.99 ± 0.22	1.22	17.88 ± 0.39	2.18
8	45.60 ± 0.31	0.68	1633.46 ± 9.65	0.59	1630.39 ± 15.24	0.93
9	37.62 ± 0.23	0.61	811.75 ± 5.41	0.67	810.21 ± 4.86	0.60
10	0.35 ± 0.002	0.57	8.42 ± 0.09	1.07	8.37 ± 0.08	0.96
11	52.10 ± 0.67	1.29	1934.03 ± 16.85	0.87	1931.14 ± 20.38	1.06
12	13.26 ± 0.09	0.68	442.34 ± 3.54	0.80	441.50 ± 3.12	0.71
13	44.00 ± 0.22	0.50	1068.24 ± 7.31	0.68	1065.51 ± 9.10	0.85
14	7.30 ± 0.10	1.37	207.37 ± 3.03	1.46	207.44 ± 2.15	1.04
15	1.45 ± 0.01	0.69	32.43 ± 0.15	0.46	30.77 ± 0.13	0.42
16	0.21 ± 0.001	0.48	4.11 ± 0.03	0.73	4.01 ± 0.03	0.75
17	22.00 ± 0.14	0.64	996.11 ± 5.86	0.59	993.12 ± 8.97	0.90
18	4.44 ± 0.03	0.67	171.56 ± 1.23	0.72	173.53 ± 2.50	1.44
19	3.38 ± 0.03	0.89	107.96 ± 1.95	1.81	106.50 ± 2.44	2.29
20	0.12 ± 0.001	0.83	3.13 ± 0.05	1.60	3.09 ± 0.06	1.94
21	6.06 ± 0.07	1.16	145.06 ± 1.10	0.76	144.36 ± 3.99	2.76
22	2.53 ± 0.02	0.79	121.17 ± 0.90	0.74	121.34 ± 0.57	0.47
23	0.49 ± 0.004	0.82	11.88 ± 0.15	1.26	11.03 ± 0.14	1.27
24	2.86 ± 0.02	0.70	57.10 ± 0.41	0.72	56.64 ± 0.36	0.64
25	1.76 ± 0.01	0.57	45.83 ± 0.49	1.07	45.59 ± 0.52	1.14
26	37.71 ± 0.18	0.48	1193.64 ± 15.36	1.29	1197.32 ± 18.53	1.55
27	0.08 ± 0.001	1.25	2.68 ± 0.01	0.37	2.47 ± 0.03	1.21
28	4.55 ± 0.04	0.88	133.13 ± 0.65	0.49	134.01 ± 0.87	0.65
29	11.79 ± 0.03	0.25	197.83 ± 2.54	1.28	199.01 ± 3.32	1.67
30	32.40 ± 0.24	0.74	865.47 ± 6.28	0.72	856.54 ± 6.09	0.71
31	1.98 ± 0.01	0.50	40.13 ± 0.20	0.50	40.05 ± 0.45	1.12
32	1.60 ± 0.01	0.62	2546.83 ± 24.78	0.97	2558.69 ± 22.41	0.87
33	3.60 ± 0.02	0.56	96.84 ± 1.25	1.29	96.34 ± 2.04	2.11
34	184.68 ± 1.15	0.62	4155.12 ± 33.26	0.80	4150.20 ± 45.38	1.09
35	31.45 ± 0.18	0.57	603.37 ± 5.35	0.89	601.17 ± 8.54	1.42
36	89.00 ± 1.02	1.15	30.28 ± 0.27	0.89	29.11 ± 0.16	0.55
37	1.65 ± 0.01	0.61	34.21 ± 0.62	1.81	33.67 ± 0.55	1.63
38	4.14 ± 0.03	0.72	62.20 ± 0.73	1.17	62.53 ± 0.95	1.52
39	0.75 ± 0.01	1.33	13.67 ± 0.22	1.61	13.82 ± 0.17	1.23
40	2.06 ± 0.01	0.48	42.31 ± 0.71	1.68	42.40 ± 0.90	2.12

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concentrations. All the analytes showed satisfactory linearity within their test ranges. Limit of detection (LOD) and limit of quantification (LOQ) were determined by serial dilution of standard solution until the S/N ratio for each compound reached 3 and 10, respectively. The results are given in Table 1.

Precision. Instrument precision was evaluated by analysing the mixture standard solution in six replicate injections. Intra- and inter-day variability was utilised to determine the precision of the method. The intra-day precision was performed using six replications prepared from the KNS sample (20080721) within one day, while inter-day precision was performed over consecutive three days. The relative standard deviation (RSD) was taken as a measure of precision. As shown in Table 2, the instrument, intra-day and inter-day precisions (RSD) of the components investigated were less than 1.37, 1.81 and 2.76%, respectively.

Accuracy. To further evaluate the accuracy of the method, a recovery test was carried out by spiking three concentration levels (low, middle and high) of the mixture standard solution to known amounts of KNS samples (20080721). Triplicate samples at each level were extracted. The recovery was calculated with the detected amounts versus the added amounts. Recovery was in the range of 96.5–103.8% with RSD less than 3.28%.

Stability. For the stability test, the same KNS sample solution was stored at room temperature and analysed every 12 h within 2 days. The solution was found to be rather stable (RSD values of the peak area were lower than 2.40%).

Specificity. Specificity was ascertained with the IDA employed to trigger the EPI scans by analysing MRM signals. All the peaks of target compounds in KNS capsules were unambiguously identified by comparison of retention time, parent and product ions with standards in MRM–IDA–EPI spectra. (Typical XIC of MRM chromatograms of standards and sample (20080721) are shown in Fig. S1 of the supplementary material.)

Quality evaluation of KNS capsules

The method developed was applied to determine the content of 40 constituents in 10 batches of KNS capsules. Each batch was determined in triplicate and RSD values of each compound were also calculated from 10 batches. (The data acquired are given in Table S1 of the supplementary material.)

Compounds **8**, **11**, **26**, **32** and **34** were the main components ($>$ 1 mg/g) in KNS capsules, but the contents of compounds **26**, **32** and **34** had a fluctuation with the RSD values higher than 10%. Furthermore, the RSD values of compounds **6**, **36** and **40** were 33.98%, 30.64% and 35.61%, which would significantly influence the quality stability of this preparation. In order to give a clear overview of the 40 constituents in KNS capsules from different production batches, the numbers of compounds and their related RSD percentages are reported in Fig. 3. About 12.5% of the compounds (5 cases) exhibited good RSD of 0% ~ 10%, 47.5% (19 cases) presented 10% ~ 20%, 32.5% (13 cases) showed 20% ~ 30%, while the remaining 7.5% was 30% ~ 40% (3 cases).

These results demonstrated that the contents of these analytes differed significantly between batches, which might lead to variances in the pharmacologic actions, even their therapeutic effects. Thus determination of multiple components is essential for the quality evaluation of KNS capsules. In addition, the observed variations in target compound concentrations might relate to factors such as disparities in the quality of the herbs or variances in the manufacturing process. Of these, the quality of the herbs might be the most important factor for KNS. Growing place, harvesting season, storage circumstance and the processing procedure would affect the quality of herbs. This the collecting procedure of each herb involved should in the future be standardised to assure the quality stability of the herbs prior to preparation.

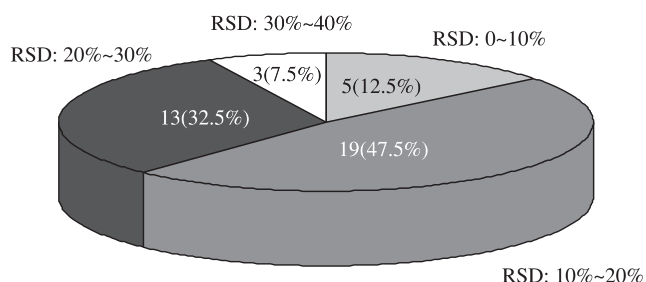

Figure 3. Relative distribution of RSD values of 40 constituents in KNS capsules from different production batches.

Summary

A selective and rapid HPLC–ESI–MS/MS method was established for the simultaneous quantification of 40 constituents in KNS capsules, and the quality of 10 batches of KNS capsule was evaluated. This study also provided a model for the quantitative analysis of complex herbal prescriptions.

Supporting information

Supporting information can be found in the online version of this article.

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