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# Determination of five components in *Ixeris sonchifolia* by high performance liquid chromatography

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**Abstract**

A high performance liquid chromatography (HPLC) method was developed for the simultaneous quantification of five major active ingredients (markers) in *Ixeris sonchifolia* (Bge.) Hance, namely chlorogenic acid, caffeic acid, luteolin-7-*O*-β-D-glucuronide, luteolin-7-*O*-β-D-glucoside and luteolin. Samples were extracted with 70% methanol. The chromatographic separation was performed on a Hypersil $ODS_2$ column (250 mm × 4.6 mm i.d.; 5 μm) with a gradient of acetonitrile and 0.5% (v/v) aqueous acetic acid, at a flow rate of 1.0 ml/min, detected at 335 nm. Five regression equations showed good linear relationships ($r^2 > 0.999$) between the peak area of each marker and concentration. The assay was reproducible with overall intra- and inter-day variation of less than 3.2%. The recoveries, measured at three concentration levels, varied from 94.1% to 100.7%. This assay was successfully applied to the determination of the 5 bioactive compounds in 18 samples. The results indicated that the developed assay method was rapid, accurate, reliable and could be readily utilized as a quality control method for *I. sonchifolia* (Bge.) Hance.
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*Keywords:* HPLC; *Ixeris sonchifolia* (Bge.) Hance; Chlorogenic acid; Caffeic acid; Luteolin-7-*O*-β-D-glucuronide; Luteolin-7-*O*-β-D-glucoside; Luteolin

## 1. Introduction

*Ixeris sonchifolia* (Bge.) Hance which belongs to the plant family Compositae, mainly grows in northeastern areas of China [1]. It is a bitter, perennial herb and a well-known folk medicine in China. Dissipating blood stasis, invigorating the circulation of blood and relieving pain are the main actions of *I. sonchifolia*. Modern pharmacological studies focused on its analgesic and dissipating blood stasis effects. It was reported that *I. sonchifolia* could increase the coronary artery flow, decrease oxygen consumption of myocardium and improve the microcirculation [2–6]. Moreover, it also possesses anti-inflammatory, antioxidant, anti-tumor and hypocholesterolemic activities [16–23]. The chemical constituents in *I. sonchifolia* are quite complicate, the phytochemical studies on *I. sonchifolia* have revealed the presence of polyphenols, flavonoids, triterpenes, sesquiterpene lactone and amino acid, etc. [7–16]. Chlorogenic acid, caffeic acid and flavonoids including luteolin-7-*O*-β-D-glucuronide, luteolin-7-*O*-β-D-glucoside and luteolin are the important active components in *I. sonchifolia*. Their structures are shown in Fig. 1. They have proved to possess various activities, such as anti-bacterial [37,38], anti-inflammatory [23–26], antioxidant [27–29], anti-tumor [30–35] and hypocholesterolemic activity [20], etc. Hence, the five compounds were selected for analyzing and evaluating *I. sonchifolia*.

The development of quality control method is an essential issue for the effective clinical use of the medicinal herb. But up to now, there have been no specific quantification methods to control the quality of *I. sonchifolia* in the Chinese Pharmacopoeia. Although some studies on the quantitative determination of one or two constituents have been reported [36], to our knowledge, there has been no previous report on the simultaneous determination of multiple constituents in *I. sonchifolia*. The aim of this study was to develop a simple, rapid and accurate HPLC–UV–DAD method for the simultaneous determination of the five active compounds to evaluate the quality of this medicinal herb.

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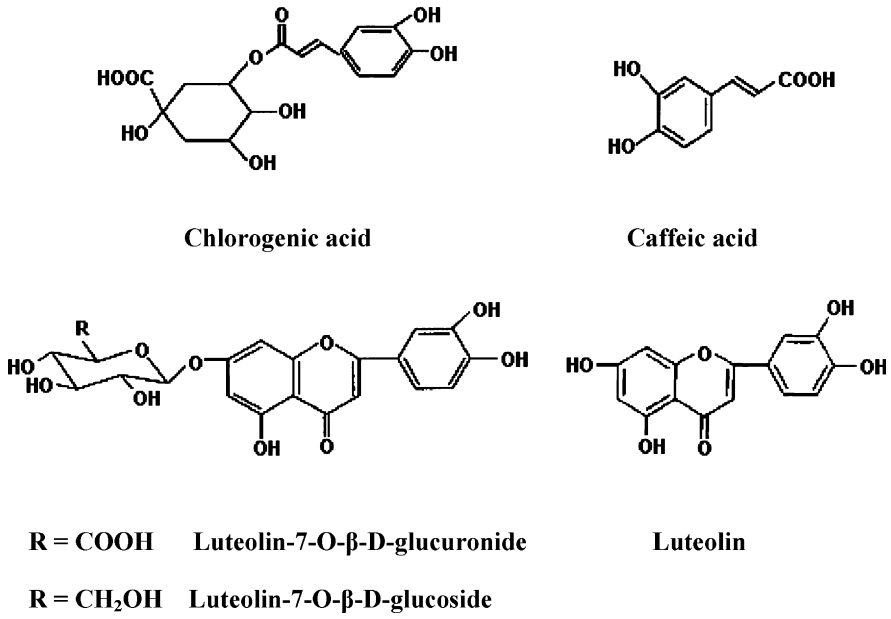


Fig. 1. Structures of the investigated compounds.

## 2. Experimental

### 2.1. Plant materials

The herb samples were collected from different provinces (Liaoning, Jilin, Neimeng, Hebei) of China in 2005, and authenticated as *I. sonchifolia* (Bge.) Hance by Prof. Qi-shi Sun (Department of Pharmacognosy, Shengyang Pharmaceutical University). A voucher specimen is deposited at the herbarium of Shenyang Pharmaceutical University (No. 5208), Liaoning, PR China.

### 2.2. Chemicals and standards

The standards of chlorogenic acid and caffeic acid were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The standards of luteolin-7-*O*-β-D-glucuronide, luteolin-7-*O*-β-D-glucoside and luteolin were isolated from the whole herb of *I. sonchifolia* by the author. Their structures were characterized by chemical and spectroscopic methods (UV, IR, NMR, MS) and then by comparing the data with those reported in literature [39–41]. Their purities were all above 98%. HPLC grade acetonitrile, methanol and glacial acetic acid were used for the HPLC analysis. Double distilled water was used throughout.

### 2.3. Apparatus and chromatographic conditions

The HPLC system consisted of a Shimadzu LC-10ATVP chromatograph, a SPD-M10AVP detector and column oven. The LC separation was carried out on a Hypersil $ODS_2$ column (250 mm × 4.6 mm i.d.; 5 μm) from Elite Analytical Instruments Co., Ltd. (Dalian, China) protected by a guard $C_{18}$ column (5 μm). The mobile phase consisted of: (A) acetonitrile and (B) 0.5% aqueous acetic acid (v/v). A gradient elution program of 7–11% A at 0–13 min, 11–18% A at 13–30 min, 18–28% A at 30–45 min, isocratic 28% A at 45–55 min was used to run the separation. Re-equilibration duration was 15 min between individual runs. Detection wavelength was set at 335 nm. The flow rate was 1.0 ml/min and aliquots of 20 μl were injected. The column temperature was maintained at 30 °C.

### 2.4. Preparation of standard solutions

The reference standards of the target compounds, i.e., chlorogenic acid (**1**), caffeic acid (**2**), luteolin-7-*O*-β-D-glucuronide (**3**), luteolin-7-*O*-β-D-glucoside (**4**) and luteolin (**5**) were accurately weighted and dissolved in methanol, then diluted to appropriate concentration ranges for the establishment of calibration curves. These solutions were stored at 4 °C.

### 2.5. Preparation of sample solutions

The powdered samples (about 0.5 g) were suspended in 70% methanol (15 ml) and extracted in an ultrasonic bath for 40 min. Then the extraction solutions were prepared by the method of weight relief, by which the weight lost in the extraction procedure was compensated. The obtained solution was centrifuged at 12,000 rpm for 3 min (Anke Centrifuge, Shanghai Anting Scientific instrument Co., Ltd., China), the supernatant was filtered through a 0.45 μm Millipore filter (Beijing Sunrise T&D Company, China) before injection. All samples were determined in triplicate.

## 3. Results and discussion

### 3.1. Optimization of extraction procedure

In order to obtain optimal extraction efficiency, variables involved in the procedure such as solvent, extraction method and

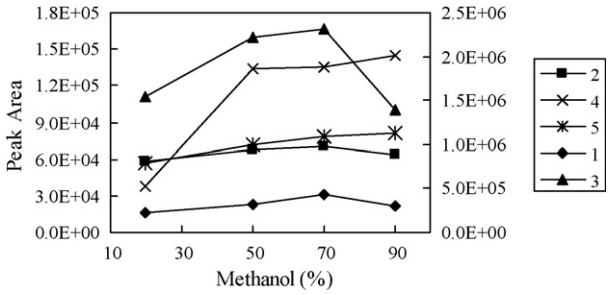


Fig. 2. Extraction efficiency of different solvents: chlorogenic acid (**1**), caffeic acid (**2**), luteolin-7-*O*-β-D-glucuronide (**3**), luteolin-7-*O*-β-D-glucoside (**4**) and luteolin (**5**).

extraction time were optimized. Pure water could not extract the luteolin efficiently and methanol is unfavourable for the extraction of chlorogenic acid and caffeic acid, therefore the effect of methanol concentration on extraction efficiency was investigated. Twenty, 50, 70 and 90% methanol were employed. The extraction efficiency was the highest when 70% methanol was used (see Fig. 2). Ultrasonic and reflux extraction were compared, and the ultrasonic method was found to be more suitable. Then the optimal extraction time was investigated. The peak areas of the marker constituents obtained by different extraction times (15, 30, 40 and 60 min) are shown in Fig. 3. It is seen that the five constituents were almost completely extracted within 40 min.

### 3.2. Chromatographic conditions

To obtain chromatograms with a good separation and resolution of adjacent peaks within a short analysis time, mobile phase, column temperature and flow rate were optimized. The mobile phase containing acid, which could suppress the ionization of phenolic hydroxyl and carboxyl groups was beneficial for good retention and separation of chlorogenic acid, caffeic acid and luteolin-7-*O*-β-D-glucuronide. Different mobile phase compositions (such as $CH_3OH$–$H_2O$–$H_3PO_4$, $CH_3OH$–$H_2O$–HOAc, $CH_3CN$–$H_2O$–$H_3PO_4$, and $CH_3CN$–$H_2O$–HOAc) were attempted to separate the investigated five components. Acetonitrile and water containing 0.5% acetic acid was chosen as the eluting solvent system because efficient separation and desired peak shape could be achieved. Under isocratic elution

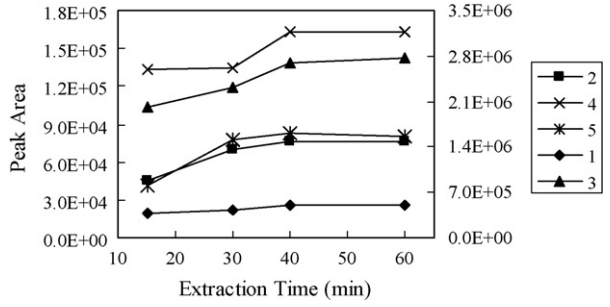


Fig. 3. Extraction efficiency at different extraction times: chlorogenic acid (**1**), caffeic acid (**2**), luteolin-7-*O*-β-D-glucuronide (**3**), luteolin-7-*O*-β-D-glucoside (**4**) and luteolin (**5**).

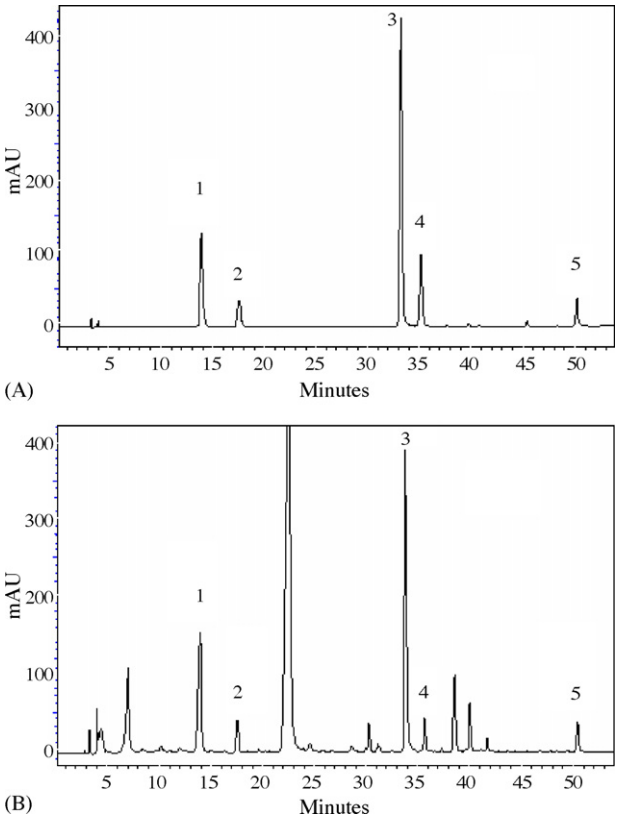


Fig. 4. HPLC chromatograms of standard mixture (A) and *Ixeris sonchifolia* sample (B). (**1**) Chlorogenic acid, (**2**) caffeic acid, (**3**) luteolin-7-*O*-β-D-glucuronide, (**4**) luteolin-7-*O*-β-D-glucoside and (**5**) luteolin.

modes, the five compounds could not be separated effectively. Gradient elution mode was therefore used, which can effectively separate the five ingredients. The effect of temperature on the separation was investigated in the range of 25–40 °C; 30 °C was found to be optimal. The most suitable flow rate was found to be 1.0 ml/min. Since their polarity, solubility and other characteristics differ greatly; at least 55 min of elution time was needed for the simultaneous quantification of the five target compounds.

On the basis of the UV spectra of the five components recorded by the DAD detector in the range from 200 to 390 nm, 335 nm was selected for monitoring. Chromatograms of standard mixture (A) and *I. sonchifolia* sample (B) are shown in Fig. 4.

### 3.3. Linearity, limits of detection and quantification

Calibration curves were obtained by plotting peak areas versus six different concentrations of standard solutions. The results are presented in Table 1. All compounds showed good linearity ($r^2 > 0.999$) in the given concentration range.

The limits of detection (LOD; S/N = 3) are summarized in Table 1. The lowest acceptable level of the calibration curve was regarded as the limit of quantification (LOQ). The LOQ of chlorogenic acid, caffeic acid, luteolin-7-*O*-β-D-glucuronide, luteolin-7-*O*-β-D-glucoside and luteolin were 1.69, 0.38, 3.86, 1.22 and 0.33 μg/ml, respectively.

Table 1
Calibration data for compounds 1–5 ($n = 6$)

| Compound | Regression equation ($y = ax + b$)[a] | $r^2$ | Linear range (μg/ml) | LOD (μg/ml) |
|---|---|---|---|---|
| Chlorogenic acid | $y = 5.3E+04x - 8247.6$ | 0.9999 | 1.694–84.70 | 0.11 |
| Caffeic acid | $y = 8.5E+04x - 5492.9$ | 0.9997 | 0.384–19.21 | 0.15 |
| Luteolin-7-*O*-β-D-glucuronide | $y = 7.7E+04x + 54558$ | 0.9999 | 3.860–193.0 | 0.17 |
| Luteolin-7-*O*-β-D-glucoside | $y = 5.0E+04x + 17724$ | 0.9998 | 1.224–61.20 | 0.15 |
| Luteolin | $y = 7.3E+04x - 625.65$ | 0.9999 | 0.333–16.65 | 0.11 |

[a] $y$ is the peak area, $x$ refers to the concentration of compound (μg/ml); $a$ and $b$ are the slope and the intercept of the regression line, respectively; $r^2$ is the correlation coefficient of the equation.

Table 2
Analytical results of intra- and inter-day variability for compounds 1–5 in *Ixeris sonchifolia*

| Compound | Intra-day | | Inter-day | |
|---|---|---|---|---|
| | Mean ± S.D.[a] | R.S.D (%) | Mean ± S.D.[a] | R.S.D (%) |
| Chlorogenic acid | 0.351 ± 0.006 | 1.75 | 0.351 ± 0.010 | 2.87 |
| Caffeic acid | 0.032 ± 0.001 | 2.93 | 0.032 ± 0.000 | 1.02 |
| Luteolin-7-*O*-β-D-glucuronide | 1.210 ± 0.015 | 1.27 | 1.210 ± 0.032 | 2.66 |
| Luteolin-7-*O*-β-D-glucoside | 0.127 ± 0.003 | 2.15 | 0.127 ± 0.003 | 2.74 |
| Luteolin | 0.040 ± 0.001 | 2.78 | 0.040 ± 0.001 | 3.12 |

[a] Data were mg compound per gram crude drug, and S.D. were calculated using SPSS (Statistical Package for the Social Science).

### 3.4. Repeatability, precision and stability

The repeatability of the method was examined by the intra- and inter-day variability. The intra-day variability was examined on six individual samples within 1 day, and inter-day variability was determined for three different days. The relative standard deviation (R.S.D.) was calculated as a measurement of method repeatability. The results shown in Table 2 indicated that the intra- and inter-day R.S.D. values of the five marker compounds were less than 3.2%, which showed good reproducibility.

The precision was evaluated using the results of five replicate injections of the standard solutions containing the five components. The results showed a good precision, and the relative standard deviations (R.S.D) were found to be 2.4% for chlorogenic acid, 1.9% for caffeic acid, 1.5% for luteolin-7-*O*-β-D-glucuronide, 1.8% for luteolin-7-*O*-β-D-glucoside and 1.9% for luteolin.

For the stability test, the same sample solution was analyzed at different times within 48 h at room temperature. The R.S.D. values of the peak areas were all lower than 2.0%, suggesting that it is safe to analyze the sample within 2 days.

### 3.5. Recovery test

Standard addition test was performed to determine the accuracy as well as the extraction recovery. In this test, the mixed

Table 3
Analytical results of recoveries ($n = 3$)

| Compound | Added (μg) | Measured (μg)[a] | Recovery[b] | R.S.D (%) |
|---|---|---|---|---|
| Chlorogenic acid | 42.65 | 41.86 | 98.1 | 1.63 |
| | 85.31 | 80.31 | 94.1 | 2.80 |
| | 127.96 | 122.89 | 96.0 | 2.89 |
| Caffeic acid | 4.32 | 4.27 | 98.7 | 1.67 |
| | 8.64 | 8.46 | 97.8 | 1.23 |
| | 12.97 | 12.49 | 96.4 | 2.15 |
| Luteolin-7-*O*-β-D-glucuronide | 168.75 | 167.13 | 99.0 | 1.12 |
| | 337.50 | 323.61 | 95.9 | 0.30 |
| | 506.25 | 502.12 | 99.2 | 2.61 |
| Luteolin-7-*O*-β-D-glucoside | 13.20 | 12.61 | 95.6 | 3.29 |
| | 26.39 | 25.59 | 97.0 | 2.21 |
| | 39.59 | 39.86 | 100.7 | 4.22 |
| Luteolin | 5.41 | 5.28 | 100.6 | 2.07 |
| | 10.82 | 10.08 | 96.1 | 1.70 |
| | 16.23 | 15.49 | 98.4 | 2.19 |

[a] Calculated by subtracting the total amount after spiking from the amount in the herb before spiking. Data were means of three experiments.
[b] Calculated as detected amount/added amount × 100%. Data were mean of three experiments.

Table 4
Contents of chlorogenic acid, caffeic acid, luteolin-7-*O*-β-D-glucuronide, luteolin-7-*O*-β-D-glucoside and luteolin (ingredients 1–5) in *I. sonchifolia* ($n = 3$)

| No. | Source | Concentration of ingredients 1–5 (mg/g)[a] | | | | |
|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 |
| 1 | Chifeng Neimeng | 1.996 ± 0.025 | 0.026 ± 0.000 | 2.955 ± 0.042 | 0.827 ± 0.014 | 0.042 ± 0.001 |
| 2 | Daxinganling Neimeng | 0.549 ± 0.012 | 0.046 ± 0.001 | 1.907 ± 0.031 | 0.206 ± 0.005 | 0.066 ± 0.001 |
| 3 | Neimeng | 1.762 ± 0.031 | 0.303 ± 0.004 | 2.053 ± 0.042 | 0.293 ± 0.005 | 0.212 ± 0.003 |
| 4 | Neimeng | 1.027 ± 0.020 | 0.185 ± 0.002 | 1.617 ± 0.023 | 0.183 ± 0.004 | 0.280 ± 0.005 |
| 5 | Fushun Liaoning[b] | 0.138 ± 0.002 | 0.029 ± 0.001 | 0.678 ± 0.008 | 0.055 ± 0.001 | 0.062 ± 0.001 |
| 6 | Fushun Liaoning[c] | 0.186 ± 0.004 | 0.030 ± 0.001 | 0.633 ± 0.009 | 0.049 ± 0.001 | 0.053 ± 0.001 |
| 7 | Yixian Liaoning[b] | 1.060 ± 0.021 | 0.176 ± 0.003 | 1.598 ± 0.032 | 0.219 ± 0.004 | 0.279 ± 0.004 |
| 8 | Yixian Liaoning[d] | 1.503 ± 0.041 | 0.015 ± 0.000 | 1.086 ± 0.030 | 0.326 ± 0.005 | 0.011 ± 0.000 |
| 9 | Beizhen Liaoning[b] | 1.221 ± 0.020 | 0.157 ± 0.003 | 1.934 ± 0.031 | 0.237 ± 0.003 | 0.189 ± 0.002 |
| 10 | Beizhen Liaoning[d] | 1.231 ± 0.020 | 0.015 ± 0.000 | 0.467 ± 0.010 | 0.138 ± 0.002 | 0.012 ± 0.000 |
| 11 | Linghai Liaoning[b] | 1.876 ± 0.030 | 0.244 ± 0.003 | 2.199 ± 0.031 | 0.377 ± 0.010 | 0.211 ± 0.004 |
| 12 | Linghai Liaoning[c] | 0.066 ± 0.001 | 0.015 ± 0.000 | 1.404 ± 0.021 | 0.132 ± 0.003 | 0.318 ± 0.005 |
| 13 | Beipiao Liaoning[b] | 1.940 ± 0.026 | 0.337 ± 0.004 | 2.282 ± 0.063 | 0.168 ± 0.002 | 0.234 ± 0.003 |
| 14 | Beipiao Liaoning[c] | 0.128 ± 0.003 | 0.074 ± 0.001 | 0.901 ± 0.023 | 0.063 ± 0.001 | 0.228 ± 0.003 |
| 15 | Jilin | 0.351 ± 0.006 | 0.032 ± 0.001 | 1.210 ± 0.015 | 0.127 ± 0.003 | 0.040 ± 0.001 |
| 16 | Tonghua Jilin | 0.225 ± 0.006 | 0.048 ± 0.001 | 0.464 ± 0.012 | 0.044 ± 0.001 | 0.216 ± 0.004 |
| 17 | Hebei | 0.752 ± 0.021 | 0.170 ± 0.004 | 0.592 ± 0.009 | 0.043 ± 0.001 | 0.114 ± 0.002 |
| 18 | Hebei | 1.019 ± 0.020 | 0.200 ± 0.003 | 1.211 ± 0.020 | 0.127 ± 0.002 | 0.053 ± 0.001 |

[a] Data were expressed as mean ± S.D. of three experiments.
[b] Samples collected in May.
[c] Samples collected in June.
[d] Samples collected in July.

standard solutions were prepared with three different concentration levels. The three standard solutions were added to the sample powder (0.25 g, Jilin Province). The resultant samples were extracted and analyzed with the proposed HPLC method. The experiments were repeated three times for each level. The ratio of measured and added amounts was used to calculate the recovery. As shown in Table 3, extraction recoveries were in the range of 94.1–100.7%, and their R.S.D. values were less than 5.0%. Considering the results of the recovery test, the method is accurate.

### 3.6. Application

The developed analytical method was successfully applied to the simultaneous determination of chlorogenic acid (**1**), caffeic acid (**2**), luteolin-7-*O*-β-D-glucuronide (**3**), luteolin-7-*O*-β-D-glucoside (**4**) and luteolin (**5**) in 18 samples of *I. sonchifolia*, which were obtained from various provinces and cities in China. Each sample was determined in triplicate. Peaks in the chromatograms were identified by comparing the retention times and on-line UV spectra with those of the standards. Retention time for compounds 1–5 were 13.68, 17.15, 33.50, 35.35 and 50.05 min, respectively (see Fig. 4).

From the results presented in Table 4, it was found that the contents of ingredients 1–5 varied greatly among the different samples. In the majority of cases luteolin-7-*O*-β-D-glucuronide (compound 3) was the main component, whose contents varied from 0.46 to 2.95 mg/g in 18 samples, with almost 6.4-fold variation. Similar variation could also be found for the other components. The reasons for the variation of the contents can be the difference of plant origin, the effect of environment and some other factors, such as season of collection, drying process and storage conditions, etc. Because variations of the marker compounds may influence the quality and potency of the medicinal herb, it was necessary to develop an effective qualitative and quantitative method to evaluate the quality of *I. sonchifolia*. The assay of one or two constituents only cannot give a complete assessment of the herb. Thus, in this paper, five major compounds in *I. sonchifolia* were analyzed simultaneously to evaluate its quality.

## 4. Conclusion

This is the first report on the simultaneous determination of chlorogenic acid, caffeic acid, luteolin-7-*O*-β-D-glucuronide, luteolin-7-*O*-β-D-glucoside and luteolin in *I. sonchifolia*. The developed method was found to be rapid, linear, reliable and accurate. The validation procedure and assay results suggested that this HPLC–UV method is promising for being used in quality control of *I. sonchifolia*.

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