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Ruggedness and robustness of conversion factors in method of simultaneous determination of multi-components with single reference standard

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ABSTRACT

Single standard to determine multi-components (SSDMC) is a novel and rational method for quality control of botanical products and traditional Chinese medicines (TCMs). However, it is restricted to wide application due to unknown fluctuation in conversion factors when it is performed in different laboratories. To evaluate the fluctuations of conversion factors, we selected *Salvia miltiorrhiza* as an example to determine three components of tanshinones by SSDMC method. Then ruggedness and robustness test were adopted to comprehensively investigate three kinds of factors that may influence stability of conversion factors, which were related with environmental parametric variables, operational parametric variables and peak measurement parametric variables. Nested-factorial-design was used to perform ruggedness tests. One-variable-at-a-time (OVAT) procedure and Plackett–Burman (PB) design were both used in robustness test. The results showed that stability of conversion factors was principally related with accuracy of wavelength of UV detector, peak measurement parameters and concentration of standard solution. The acceptable range of conversion factors was obtained from robustness test. Our results showed that conversion factors were inevitable to change, but when key parameters were well controlled, the range of its fluctuation was acceptable and the SSDMC method could be used widely in different laboratories.

1. Introduction

Traditional Chinese medicines (TCMs) have been widely used to treat various diseases for thousands of years in China. Recently, an increasing appreciation of the health-promoting benefits of herbal preparations has been observed in the United States [1]. To control the quality of the complex botanical products and traditional Chinese medicines (TCMs), determination of multi-components was considered to be one of the key methods by Chinese Pharmacopoeia and United States Pharmacopoeia. During the revision of Chinese Pharmacopeia 2005 edition (Ch.P. 2005), Chinese Pharmacopoeia Commission directed that analytical method and testing items of monograph being revised should embody the idea of comprehensive quality control of TCMs, which multi-components or fingerprint should be analyzed rather than single marker compound [2]. For the moment, there were two main methods for the quantitative determination of multi-components in herbal products. The first method is to use multiple reference standards for the analysis of multiple components. For instance, in monographs of Ch.P. 2010 edition, three reference standards (aconitine, hypaconitine and mesaconitine) were used to determine three alkaloids in *Aconitum carmichaelii* Debx (Chuanwu) [3], and four reference standards (polyphyllin I–IV) were used to determine four components in *Paris polyphylla* Smith var. *yunnanensis* (Franch.) Hand-Mazz. (Chonglou) [4]. In comparison, the second method only requires a single reference standard to simultaneously determine the contents of multi-components, which could be abbreviated as SSDMC (single standard to determine multi-components.) method. In the second method, the content of each component could be obtained directly or calculated by multiple conversion factors. Due to the difficulties and expenses to prepare bulk of all reference standards, the application of the first method was limited, and it would be especially difficult to determine more than five components. SSDMC method only needs the minimum number of reference standard with low cost. And it enables to determine more than ten components simultaneously. Thus, the use of SSDMC method was ideally explored.

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Conversion factors of SSDMC method could be divided into two types based on UV detection. The first type is that the values of conversion factors are considered as the value of 1, which could only be used when the molar absorptivity and molecular weights of all analytes possess high similarities. Such as in USP 33, the quality of Cat's Claw was controlled by this method and isopteropodine is used as single reference standard to directly determine the other five components [5]. The reason was that six components had the same structure belonging to pentacyclic indole alkaloids with the same molecular weight. The only difference was the configuration of those six components. Another example was that 15 anthocyanins in Bilberry Extract were determined because they had similar chromospheres structure [6]. However, the use of this method was limited since results obtained were just an approximation, which may deviate from the true value. The second type, which conversion factors of all analytes are different, could avoid this deviation. Molar absorptivity (ε) of analytes with different chromophores is generally different at the same wavelength. (Such as the log(ε) of tanshinone II_A, cryptotanshinone and tanshinone I are 4.47, 4.39 and 4.30 respectively at 270 nm.) Therefore the content of other components should be calibrated when they are determined by single reference standards. The conversion factors were just used to calibrate the contents, which were also called as relative response factors in some literatures. It could be defined as response ratio of reference standard and analyte at the same unit mass concentration. Such as in USP 33, the total phenols in *Echinacea pallida* (Nutt.) Nutt. were determined by conversion factors. The chlorogenic acid was used as one reference standard, and the contents of other three phenol acids (caftaric acid, chicoric acid and echinacoside) were calibrated by 0.881, 0.695 and 2.220, respectively [7]. Tu et al. analyzed seven anthraquinones in rhubarb rhizome by conversion factors. The emodin was selected as single reference standard, and maximum conversion factor was aloe-emodin that the value was 1:0.0759 [8]. The results clearly showed that it was absolutely necessary to calibrate the content by conversion factors.

As mentioned above, quality control of botanical products (herb drugs or TCMs) by multi-component quantification has reached a consensus. SSDMC with conversion factors has been applied in 20 of 108 monographs of herbal dietary supplements in USP 33 (such as red clover, *Echinacea angustifolia*, St. John's Wort., etc.). In European Pharmacopoeia 7.0 (EP7.0), 6 herbal drugs were assayed by this method (such as Ginkgo dry extract and Purple coneflower herb and its root) in 232 herbal drugs. While in the latest version of Ch.P. 2010 edition (volume I), only Coptides Rhizoma (Huanglian) was controlled by SSDMC method in all 593 Chinese crude drugs recorded, in which conversion factor was not used. The berberine hydrochloride was selected as single reference standard, and the contents of four alkaloids (epiberberine, coptisine, palmatine and berberine) were determined respectively without calibration by conversion factors [9]. The above facts showed that SSDMC method with conversion factors was not widely used in Ch.P. 2010 and EP7.0. One of the most important reasons was that the potential fluctuation in conversion factor in different laboratories was not fully taken into consideration [8]. Thus SSDMC method was heavily restricted by the ruggedness and robustness of conversion factors. And the factors which would influence the fluctuation of conversion factors have not been reported up to now. Therefore it should be investigated systematically.

In this study, to investigate the ruggedness and robustness of conversion factors, we selected Salvia Miltiorrhizae Radix et Rhizoma (Danshen) as an example. Known from our previous works in fingerprint of Danshen [10], the quality of lipophilic part in Danshen can be controlled by determining three main components – tanshinone I, cryptotanshinone and tanshinone II_A. A number of methods on the simultaneous determination of the three components had been reported [11–13]. There have no reports regarding the simultaneous determination of three tanshinones with SSDMC method as a compendia procedure. Therefore analytical procedure adopting a SSDMC method with conversion factors was validated initially. The three tanshinones (tanshinone II_A, tanshinone I and cryptotanshinone) of Danshen were simultaneously determined by tanshinone II_A as single reference standard, which is easy to obtain and abundant in the material, on high-performance liquid chromatography with diode-array detector (HPLC-DAD). And then all factors which might have influence on the conversion factors were studied comprehensively. (1) Whether it was related with environmental parameters, such as different days, analysts, instruments, and columns. If this was the case, what were the reasons? (2) Whether it was related with operational parameters, such as different acid concentration in mobile phase, ratio of components in mobile phase, wavelength of UV detector, column length, injection volume, column temperature and concentration of reference standard. (3) Whether it was related with peak measurement parameters which were rarely reported, such as different slit width, bandwidth and integration parameters. (4) What is the acceptable range of conversion factors? (5) Finally, the values of conversion factors and the analytical procedure were verified and confirmed by Shanghai Institute for Food and Drug Control (SIFDC) of China which has Laboratory Accreditation Certificate. These results provided a firm foundation for SSDMC method with conversion factors to use as compendia procedures.

2. Experimental

2.1. Chemicals and materials

Tanshinone II_A (A), cryptotanshinone (C) and tanshinone I (I) (Fig. 1) were obtained from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The purity of tanshinone I was determined to be 97% by peak area normalization method on HPLC, and purities of the other two compounds were more than 98%. Acetonitrile for HPLC was obtained from Honeywell (UV, NJ, USA). Methanol for analytical grade was obtained from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Phosphoric acid for HPLC was obtained from Tedia (USA). High purity deionised water was obtained from Millipore, Milli-Q (Bedford, MA, USA) purification system.

Salvia Miltiorrhizae Radix et Rhizoma (Danshen) were collected from Shandong Province of China, and identified by one of the coauthors (Dr. De-An Guo). Voucher specimens were deposited at Shanghai Institute of Materia Medica, Chinese Academy of Sciences.

2.2. Apparatus

Analyses were primarily performed by an Agilent 1100 HPLC System, comprised a quaternary solvent delivery system, an on-line degasser, an auto-sampler, a column temperature controller and a diode-array detector (DAD) coupled with an analytical workstation (Chemstation For LC 3D Systems A10.02) (Agilent Technologies, Palo Alto, CA, USA). Two additional different HPLC instruments were used. One is Agilent 1100 HPLC System comprised a variable wavelength detector (VWD) (Agilent Technologies, Palo Alto, CA, USA), another was a Waters 2996 HPLC System comprised a quaternary solvent delivery system, an on-line degasser, an auto-sampler, and photodiode array detector coupled with an analytical workstation (Empower 2 software) (Waters Corp, Milford, MA, USA). A BRANSON B3500S-DTH ultrasonic bath (140 W, 42 kHz) (BRANSON Ultrasonic, Shanghai, China) was used for sample preparation. Samples were primarily separated on a Zorbax Extend-C_{18} column (5 μm particles, 4.6 mm i.d. × 250 mm; Agilent) with a guard column (5 μm particles, 4.6 mm i.d. × 10 mm; Agilent).

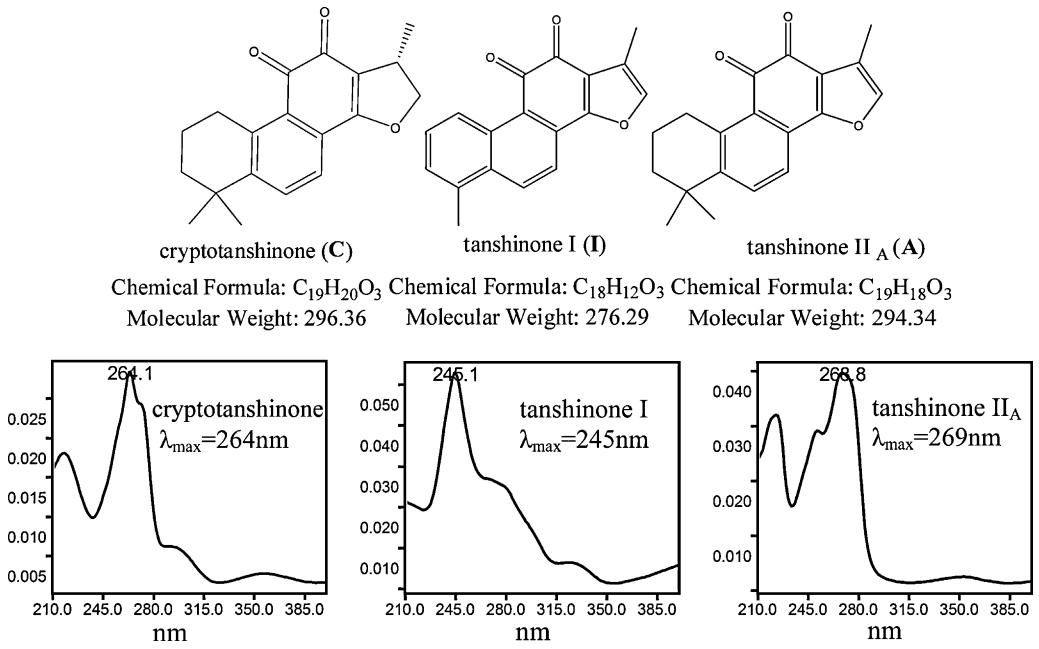

Fig. 1. Chemical structures and UV spectrums of tanshinone I, cryptotanshinone and tanshinone II_A.

2.3. Chromatographic conditions

The mobile phase was a mixture of acetonitrile (mobile phase A) and water containing 0.02% phosphoric acid (mobile phase B). The eluted gradient was as following: the initial composition was 61% of mobile phase A; after 6 min this was changed, with a linear gradient, over 18 min, to 90% of mobile phase A, then was changed to 61% in 0.5 min, which was maintained for 4.5 min. The mobile phase flow rate was 1.0 mL per minute. The DAD detector was operated at 270 nm with 4 nm bandwidth and no reference wave. The column temperature was at 20 °C.

2.4. Preparation of solutions

2.4.1. Preparation of standards solution

Tanshinone I stock solution was prepared by dissolving 12.98 mg in a 25-mL amber volumetric flask with 5 mL chloroform, then diluting to the volume with methanol. Then the mixed stock solution of tanshinone I, tanshinone II_A and cryptotanshinone were prepared by dissolving 13.40 mg tanshinone II_A and 11.36 mg cryptotanshinone in a 25-mL amber volumetric flask with 10.0 mL tanshinone I stock solution, then diluting to the volume with methanol. The mixed stock solutions were serially diluted (dilution factor = 10, 12.5, 16.67, 25, 50, 250, 500) to produce calibration standard solutions.

2.4.2. Preparation of sample solutions

Sample solutions were prepared by placing 300 mg powder of Danshen in a 100-mL glass-stopper conical flask, 40 mL methanol was added and sonicated for 30 min, and then the solution was filtered into a 50-mL amber volumetric flask, and 2 mL of methanol which was used to rinse the residual and the filter paper were combined into the volumetric flask, then diluted to volume with methanol.

2.5. Calculation of conversion factors

For traditional external standard method, all the reference standards solutions corresponding to analytes would be prepared first. Then the concentration of analyte (C_x) can be calculated by the ratio between responses of analytes in sample solution (r_x) and the response of its corresponding standard solution in a unit concentration (r_{sx}/C_{sx}).

$$C_x = \frac{r_x}{r_{sx}/C_{sx}} \tag{1}$$

However, when single reference was used to determine the multi-components in samples, the C_x values obtained by external standard method and SSDMC method may be somehow very different, because molar absorptivity (ε: means the absorbance of a 1 M solution of analyte in a 1 cm cell at a given wavelength) of different analytes were often different, Such as the conversion factor of emodin and aloe-emodin, their ratio of conversion factor was 1:0.0759 as mentioned before. If the content of aloe-emodin was calculated directly by the emodin without calibration, the content of aloe-emodin calculated would be only 7.59% of its true value. Therefore the concentration of analyte (C_x) should be calculated by the ratio between the responses of analytes in sample solution (r_x) and the responses of reference standard solution in a unit concentration (r_s/C_s), and then calibrated by conversion factor ($\mathbf{F}_x$).

$$C_x = \frac{r_x}{r_s/C_s} \times \mathbf{F}_x \tag{2}$$

To obtain the conversion factors, just as internal standard method, the conversion factor ($\mathbf{F}_x$) was the ratio of responses in a unit concentration between standard substance (r_s/C_s) and analyte (r_{sx}/C_{sx}):

$$\mathbf{F}_x = \frac{r_s/C_s}{r_{sx}/C_{sx}} \tag{3}$$

To calculate the conversion factors, (1) three independent calibration standard solutions were prepared as indicated in Section 2.4.1; (2) then the ratio at each concentration level of three independent standard solutions were calculated as Eq. (3); (3) the conversion factor of each analyte was obtained as the mean values calculated from the triplet of seven gradient concentrations.

Additionally, the influence of concentrations of standard solution on the conversion factors was studied. A series of standard solutions up to 600-fold concentration differences were used to observe its stability. It was prepared through diluting the mixed standard solution in order. The mixed stock solutions were serially

Table 1
Ruggedness tests of conversion factors (n = 2) (mean ± SD).

Instruments		Analyst 1		Analyst 2		Analyst 3	
		Column1 Ext_01	Column2 Ext_02	Column3 Kromasil_01	Column4 Kromasil_02	Column5 XDB	Column6 Capcell
Instrument1	F_C	1.18 ± 0.00	1.18 ± 0.00	1.20 ± 0.01	1.19 ± 0.01	1.19 ± 0.01	1.19 ± 0.01
(Agilent 1100 DAD)	F_I	1.33 ± 0.01	1.32 ± 0.01	1.30 ± 0.01	1.31 ± 0.02	1.32 ± 0.02	1.27 ± 0.00
Instrument2	F_C	1.18 ± 0.01	1.18 ± 0.01	1.20 ± 0.00	1.19 ± 0.00	1.18 ± 0.01	1.19 ± 0.01
(Agilent 1100 VWD)	F_I	1.33 ± 0.02	1.32 ± 0.02	1.36 ± 0.00	1.36 ± 0.00	1.29 ± 0.00	1.30 ± 0.00
Instrument3	F_C	1.21 ± 0.00	1.20 ± 0.00	1.22 ± 0.01	1.21 ± 0.00	1.23 ± 0.01	1.23 ± 0.01
(Waters 2996 PDA)	F_I	1.30 ± 0.01	1.29 ± 0.01	1.29 ± 0.02	1.31 ± 0.01	1.33 ± 0.02	1.27 ± 0.02

diluted (dilution factor = 1, 2.5, 6.25, 15.62, 39.06, 97.66, 244.14, 610.35) to produce eight gradient standard solutions in a broad range. The conversion factors were calculated as mentioned above.

2.6. Validation of analytical method

SSDMC method adopted to determine C, I and A in Danshen was validated based on the following parameters: selectivity, accuracy, linearity, precision (within- and between-day variability, different operators, different instruments and columns) and stability as guided in USP.

The results obtained by SSDMC method were compared to the results obtained by traditional external standard method (i.e. three reference standards were used for analysis).

2.7. Ruggedness and robustness of conversion factors

The ruggedness and robustness test was designed to determine all potential and changeable factors on an analytic procedure when it was performed in different laboratories, and to find out which factor has the most remarkable influence on the results. The definition and case studies had been reviewed [14]. As for our understanding, the factors in ruggedness test and robustness test were investigated from different angles. Environmental factors were mainly investigated in ruggedness test. These environmental factors include many known or unknown parameters. The operational parameters and peak measurement parameters were just the primary known parameters of environmental factors. And operational parameters were mainly investigated in robustness test. Now both ruggedness and robustness tests were used to evaluate stability of conversion factors in SSDMC method.

2.7.1. Ruggedness test

The ruggedness test (also called intermediate precision) was performed to examine the effects of environmental factors. These factors were often not described in assay procedure, such as different days, analysts, instruments and columns sources. The conversion factors might be changed with the above environmental factors when the analysis procedure was used in different laboratories.

A nested-factorial-design was adopted to evaluate the effect of the factors on the conversion factors [15,16]. Three analysts participated in the experiment design. Each analyst prepared mixed standard solutions (dilution factor = 25) in duplicate and used two columns which were different from others. Each column was used on all of three HPLC instruments. The design was showed in Table 1. The conversion factors on each column and instrument were calculated. The minitab V16 computer program was used for data manipulation and calculation based on literature [15]. Balanced ANOVA and the option of restricted form of the model were adopted.

2.7.2. Robustness test

Robustness test was performed to examine the effects of the operational factors. These factors were often embodied as analytical parameters in the context of analytical procedure, such as pH of mobile phase, ratio of components in mobile phase, time program of mobile phase, wavelength of UV detector, flow rate, injection volume and column temperature. Parameters of these factors might often be adjusted slightly to meet the system suitability requirements in different laboratories. For traditional external standard method, the results were generally not affected by adjustment in a narrow range. However, for SSDMC method, only single standard was used, the effects of above factors on the conversion factors were still unknown. The fluctuations of conversion factors were studied on these factors by changing these parameters in a narrow range based on USP (see Table 2). In order to know how those factors have influence on conversion factors, one-variable-at-a-time (OVAT) procedure was mainly applied.

2.7.3. Peak measurement parameter test

The peak measurement parameters can also be considered as part of robustness test which mainly include slit of detector, width of a wavelength and ways of integration. These parameters were called different names in different HPLC instruments. In this study, the Agilent HPLC with DAD detector was used as example. The three types of factors were investigated as follows: (1) slit of detector was often set 4 nm as default, and different slit values were compared on 2 nm and 8 nm. (2) Width of a wavelength was called bandwidth in Agilent HPLC, which means that signal was recorded in an average response of a length of wavelength (and the center of the length of wavelength was called wavelength of UV detector). This parameter was mainly used in DAD or PDA detectors. The bandwidth value was often set 4 nm as default, and different bandwidth were compared on 2 nm and 16 nm. (3) The ways of integration was a more subjective factor. In Agilent HPLC, the slope sensitivity and peak width were the most influent integration parameters on peak area. And different integration parameters were compared based on the stability of conversion factors (see Table 3). OVAT procedure was mainly used.

2.7.4. Retest the robustness test and peak measurement parameter test

The OVAT procedure can clearly showed the effect of every factors and easy to interpret. But neither statistical interpretation nor evaluation of the whole experimental domain can be obtained by OVAT. Hence a Plackett–Burman (PB) design was adopted to verify the results obtained in robustness test and peak measurement parameter test. Eleven factors of robustness test and peak measurement parameter test were retested simultaneously, in which 8 factors were selected from robustness test and 3 factors were selected from peak measurement parameter test except the factor of peak width. And the levels of 11 factors were same with levels listed in Tables 2 and 3, except for the factor of injection volume with its level (+1) changed to 15 μL and its level (−1) changed to

Table 2
Selection of factors and its levels in robustness test.

No.	Factors	Level (−1)	Level (+1)	Norminal
1	Concentration of acid (PH)	H_2O	0.1% H_3PO_4	0.02% H_3PO_4
2	Proportion of mobile phase (MP)[a]	59/59/88/59	63/63/92/63	61/61/90/61
3	Time programs of mobile phase (TP)	4 min	8 min	6 min
4	Wave length (WL)	267 nm	273 nm	270 nm
5	Column length (CL)	150 mm	250 mm	250 mm
6	Flow rate (FR)	0.8 mL/min	1.2 mL/min	1.0 mL/min
7	Injection volume (IV)	1 μL	5 μL	10 μL
8	Column temperature (CT)	15 °C	30 °C	20 °C

[a] The numbers means the percent of acetonitrile in mobile phase at different time programs.

5 μL. The PB test for 11 factors ($N = 12$) was designed as described in literature [17]. The conversion factors of 12 experiment runs were calculated. The half-normal plots and Pareto chart was drawn by excel software. And Dong's criterion was calculated according to the literature [17].

2.8. Verification by accredited laboratory

The analytical procedure of tanshinones was verified by Shanghai Institute for Food and Drug Control (SIFDC) of China which have Laboratory Accreditation Certificate. The conversion factors were re-calculated and the accuracy of the method was re-tested. Three batches of samples provided by our laboratory were determined and compared with our results.

3. Results and discussion

3.1. Calculation of conversion factors

In this study, the conversion factors of cryptotanshinone and tanshinone I were determined and calculated because tanshinones had different responses at the same concentration when they were simultaneously determined at same given wavelength. The tanshinone IIA was selected as the single reference standard.

Tanshinone I, cryptotanshinone and tanshinone IIA have different maximal absorbent wavelengths, although they all belong to diterpene quinone with similar structures (Fig. 1). The 270 nm was selected as determination wavelength. Because the maximal absorptive wavelength of cryptotanshinone and tanshinone IIA were obtained approximately at 270 nm, and the shoulder peak of tanshinone I can also be obtained at 270 nm.

The results from the triplet of seven gradient concentrations showed that the conversion factors of tanshinone I ($\mathbf{F_I}$) was 1.31 ± 0.02, and the cryptotanshinone ($\mathbf{F_C}$) was 1.18 ± 0.01. (Table S1-1 to S 1-4, the results are available in Supplementary data with the online version of the article.) The concentration of tanshinone I was calibrated as 97% due to its purity, while other two were not. The conversion factors were used to validate the analysis procedures.

Additionally, there were other two approaches used to calculate the conversion factors. The first one was calculated by the line regression equation, another was only by one single concentration solution. For the former, the conversion factor was obtained by calculating the ratio of equation slope of tanshinone IIA and tanshinone I (or cryptotanshinone). The results showed that conversion factor of $\mathbf{F_I}$ was 1.30, and the $\mathbf{F_C}$ was 1.18. Though the results were similar, but the line regression equation often had an interception which may not be neglected. For instance, the equation of aloe-emodin: $y = 2.03x - 3.01$, the interception was even larger than the slope [8]. Thus the conversion factors should not be calculated by line regression equation, especially when the interception was not neglected. For the latter, the reason was the same. The range of concentration used to calculate conversion factor should be same with linearity range at least. The ranges to calculate the conversion factors will be discussed in depth in Section 3.1.

Although all reference standards corresponding to all analytes should be prepared to develop a SSDMC method with conversion factors, just like the traditional external standard method, but only single reference standard was needed when the SSDMC method was applied.

Concentrations of standard solution were generally investigated in the linearity range test. But for SSDMC method, this factor should be further studied. When the conversion factors were calculated at first, its value showed a slight trend of change, especially for tanshinone I. Thus, it should be investigated in a more broad range of concentrations. The line regression equations were listed in Table S2. (Tables S1–S10 are available in Supplementary data with the online version of the article.)

The $\mathbf{F_I}$ and $\mathbf{F_C}$ were scattered in Fig. 3. Fig. 3A clearly showed that its value decreased with the increase of concentrations and change of $\mathbf{F_I}$ was more remarkable. The $\mathbf{F_C}$ was more stable when the concentrations were above the 10 μg/mL. Both $\mathbf{F_I}$ and $\mathbf{F_C}$ increased remarkably if the concentration was below the concentration of 10 μg/mL (Fig. 3B). Thus the conversion factors of tanshinone I and cryptotanshinone should be calculated above the concentration of 10 μg/mL. For the tanshinone I, the high concentration should also be limited. In this study, the highest concentration of tanshinone I was 20 μg/mL. The lowest concentration was 0.40 μg/mL because its content in the sample was very low, and a larger fluctuation was acceptable. Therefore, when the concentrations of analytes were lower than 10 μg/mL, the RSDs of results would increase.

Concentration of standard solution would affect the conversion factors and the influences were different for different analytes. The reasons could be: (1) when the concentration of standard solution was too low, the linearity may not establish. (2) Interception of regression equation was much different. It may relate with the UV spectrum, of which tanshinone I was much different from tanshinone IIA, while the UV Spectrum of cryptotanshinone was very similar as that of tanshinone IIA (Fig. 1). Therefore, when the conversion factors were calculated at high concentration of standard solutions, the interception of regression equation must not be neglected.

The above discussion showed that the conversion factors were not constant in a broad concentration range. It gave two hints, (1)

Table 3
Selection of factors and its levels in peak measurements parameters test.

No.	Factors	Level (−1)	Level (+1)	Norminal
1	Slit (SL)	2 nm	8 nm	4 nm
2	Band with (BW)	2 nm	16 nm	4 nm
3	Slope sensitive (SS)[a]	0.1	100	5
4	Peak width (PW)[a]	0.001	1	0.05

[a] Both the two parameters were integration events in Agilent 1100 HPLC.

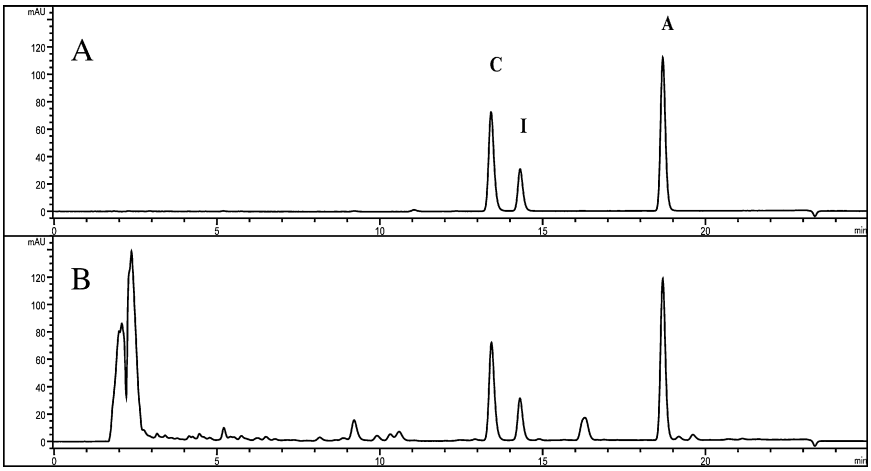

Fig. 2. Representative HPLC chromatograms of (A) mixed standards solution and (B) sample solution (Shandong).

conversion factors should be calculated not only in linearity ranges, but also in relative stable range. (2) When concentration of analyte in sample was too low or too high, the amount of sample should be adjusted.

3.2. Validation method

The method was validated based on the USP32 < 1225 VALIDATION OF COMPENDIAL PROCEDURES > by using the obtained conversion factors. The specificity was satisfactory and can easily separate from the unknown substances due to its high theoretical plates (Fig. 2). The accuracy of each analyte was 98.0–103.0% (relative standard deviations (RSDs) = 1.5–3.0%) at spiked 80%, 100% and 120% (Table S3). And the RSDs of repeatability was 0.14–1.30% at 50%, 100% and 150% of sample concentration (Table S4). The linearity could meet the requirement to analyze the samples in the range of 1.07–53.60 μg/mL, 0.91–45.44 μg/mL and 0.40–20.14 μg/mL for tanshinone IIA, cryptotanshinone and tanshinone I, respectively (Table S5). The RSDs of inter-day variability were in the range 1.3–1.7%, and the RSDs of analysts were in the range of 0.7–1.1%, which both showed no influence on the results (Tables S6 and S7). Five different columns were all used to assay on two HPLC instruments (Agilent 1100 with DAD detector and Waters 2695 with PDA detector). The RSDs of total content on different columns and instruments by SSDMC method was less than 1.0% (Table S8). The sample solution was stable within 24 h after preparation and RSDs of peak area was less than 0.9% (Table S9).

After validation, 10 batches of samples were analyzed by using two methods: three standard references (tanshinone I, cryptotanshinone and tanshinone IIA) were used to determine the three compounds of samples by Method I. And single reference standard by two conversion factors were used to determine the three compounds of same samples by Method II. The RSDs of sum contents of the three compound obtained by the two methods of each sample were less than 0.7%. And only for the contents of tanshinone I, the RSDs by the two methods of each sample was less than 3.2% (Table S10). The reason might relate with the low concentration of tanshinone I in samples as mentioned in Section 3.1. However, the difference can be neglected for quality control because the RSDs of sum contents meet the requirements. And the results showed that SSDMC method with conversion factors could assure to obtain consentaneous results with the external standard method. All these results obtained in single laboratory were acceptable, which were consistent with work of Tu et al. [8].

3.3. Ruggedness and robustness

The SSDMC method with conversion factor was often good enough in single laboratory as mentioned above. But it was often baffled when it was performed in different laboratories. Therefore the stability of conversion factor was investigated by ruggedness and robustness tests.

3.3.1. Ruggedness test

Ruggedness tests were often used to investigate environmental parameters. To conversion factors, different analysts, different instruments and columns were the main different, and may have interaction each other. In ruggedness test, one-variable-at-a-time (OVAT) procedure to evaluate the effects of the factors is not recommended, because the factor interactions should be taken into account. Hence a nested-factorial-design was adopted to evaluate the three main factors and their interactions. Three instruments and six columns were investigated. Two instruments were from Agilent, in which one detector was VWD (variable wavelength detector) and another was DAD. And the third HPLC with PAD (photodiode array detector) was from Waters. Six columns were all octadecyl silane chemically bonded to porous silica, and their inner diameter was 4.6 mm, length was 250 mm and particle diameter was 5 μm. The six columns came from three different manufacturers which were Agilent (Extent-C_{18} and Eclipse-XDB C_{18}, USA), SHISHEIDO (Capcell PAK C_{18}, Japan)

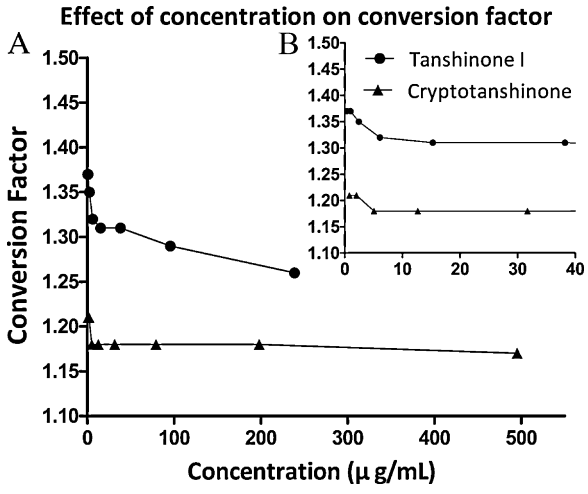

Fig. 3. Effect of different standard solution concentrations on conversion factors.

Table 4
ANOVA results of ruggedness test.

Source	$P(\mathbf{F_C})$	$P(\mathbf{F_I})$
Analyst	0.03	0.26
Column (analyst)	0.09	0.001
Instrument	<0.001	0.04
Analyst*Instrument	0.07	0.11
Instrument*Column (analyst)	0.16	0.01

and Elite (Kromasil C_{18}, China). All these columns meet with the requirements of system suitability parameters which the resolution between tanshinone I and cryptotanshinone was more than 1.5, and the number of plate of tanshinone II_A was more than 60,000.

The $\mathbf{F_C}$ and $\mathbf{F_I}$ on each column and instrument assayed by every analyst were listed in Table 1. The mean value of $\mathbf{F_C}$ and $\mathbf{F_I}$ on all columns and instruments were 1.20 and 1.31, respectively. And the RSDs of $\mathbf{F_C}$ and $\mathbf{F_I}$ were 1.67% and 2.29%, respectively. The values of RSDs seemed to be acceptable at normal time, but when the method was performed in another laboratory, it would be amplified. Through design of experiments (DOEs), factors which were most important to the value of conversion factors, were expected to be uncovered. The statistical results of the nested-factorial-design were listed in Table 4.

In Table 4, the source of "column (analyst)" means effect of column in each analyst because factor of columns was nested in the factor of analyst. The source of "Analyst*Instrument" means the interaction between factors of analyst and instrument. The source of "Instrument*Column (Analyst)" means the interaction between factors of instrument and column in each analyst. The results clearly showed that the factor of instrument was the most important one to $\mathbf{F_C}$ ($P < 0.001$), and the factor of column in each analyst was the most important one to $\mathbf{F_I}$ ($P = 0.001$). The factor of analyst only had influence on $\mathbf{F_C}$ ($P = 0.03$). And the factor of instrument also had influence on $\mathbf{F_I}$ ($P = 0.04$) and had interaction with column in each analyst ($P = 0.01$).

From results of the nested-factorial-design, it showed that different instruments and columns were the most important factors to conversion factors. And it was different for specific analytes. To apply the SSDMC method with conversion factors in different laboratories, the instruments and columns were inevitable to be changed. Therefore the potential mechanism should be clarified in order to better control those related parameters.

The potential mechanism was speculated that the factor of instrument was mainly related with its detector, and factor of column was mainly related with peak shape. (1) For different instruments, the detector system may be slightly different. While for cryptotanshinone, its UV spectrum showed a very sharp peak near the wavelength of 270 nm, and for tanshinone I, its UV spectrum was a relative gentle shoulder peak at the same wavelength. For this reason, the $\mathbf{F_C}$ would be more sensitive to the variability of detector, and so it is more sensitive to different instruments. (2) For different columns, the peak shape of different analytes, such as resolution and tailing factor, may be slightly different on each column. And the conversion factor with small peak area was more prone to be affected by different peak shape. While for tanshinone I, it was the lowest content in mixed standard solutions, and the content of cryptotanshinone was similar with that of tanshinone II_A. And it can also interpret the interaction between instruments and columns in each analyst, as that different void volume of instrument would have different influence on columns. Therefore, the $\mathbf{F_I}$ was more sensitive to different columns. Both above assumptions would be verified with following robustness test and peak measurement parameters test.

3.3.2. Robustness test

Different from the ruggedness test which the factors studied were inevitable changes in different laboratories, while for the robustness test, the factors studied were adjusted subjectively. And these factors are mainly related with detector, column or mobile phase, which were the key parameters of environmental factors. Therefore through robustness test, the hypothesis mentioned above might be verified.

Robustness test was mainly used to investigate operational parameters. Therefore eight related factors were studied, and each was selected at three levels. The range of variability was based on USP 33. The values of conversion factors at each level of eight factors were scattered in Fig. 4A and B. Fig. 4A clearly showed that all the factors had little influence on the value of $\mathbf{F_C}$ except for the factor of wavelength. And Fig. 4B showed that the factors of wavelength, injection volume and column temperature had some influence on the value of $\mathbf{F_I}$. The maximal difference of conversion factor to each factor was shown in Fig. 4C. The value of each factor was calculated by subtracting the conversion factor in level (+1) from that in level (−1). It showed the value and change tendency of effect of each factor on conversion factors. Fig. 4C clearly showed that the variability of wavelength was more remarkable to $\mathbf{F_C}$ than to $\mathbf{F_I}$, which verified our assumption in ruggedness test. It also showed that the factor of wavelength was the most remarkable factor among the eight factors.

For cryptotanshinone, all the differences of conversion factor were below 0.01 except for the wavelength. For tanshinone I, all the differences were in the range of ±0.01 except for the three factors mentioned above. It can be calculated that the RSDs of values of $\mathbf{F_C}$ and $\mathbf{F_I}$ were less than 2.0% at these variability of factors.

Factors regarding injection volume and column temperature have some influence on the $\mathbf{F_I}$. When the injection volume was decreased from 10 μL to 1 μL, the $\mathbf{F_I}$ was increased from 1.32 to 1.36. This change was proportionable. Potential mechanism was that the peak area was decreased too much when the injection volume was decreased. And the conversion factor was affected at low amount of analyte as mentioned in Section 3.1.

Regarding column temperature, when the temperature was increased from 20 °C to 30 °C, the $\mathbf{F_I}$ was decreased from 1.32 to 1.29. However, the $\mathbf{F_I}$ was stable when the temperature was decreased from 20 °C to 15 °C. The possible reason was that when the column temperature was high, the resolution of analytes would be decreased notably. It was verified that the resolution of tanshinone I and cryptotanshinone decreased from 3.77 and 3.02 to 1.76 when column temperatures were increased from 15 °C and 20 °C to 30 °C, respectively. The peak areas changed at same integration parameters when resolution was reduced. And the conversion factor with smaller peak area would much prone to be affected as mentioned above. The integration parameters may also play some function, which would be discussed in the following.

3.3.3. Peak measurement parameter test

The peak measurement parameters were rarely reported in literature and often neglected in external standard method [14]. But for SSDMC method, these factors should not be ignored. Four primary factors on peak measurement parameters were studied. The data was scattered in Fig. 5A and B, and analyzed as shown in Section 3.3.2. From Fig. 5C, it was found that the $\mathbf{F_C}$ was more sensitive to slit and bandwidth than other two integration parameters. The reason might be related with its UV spectrum as mentioned above. In contrary, the $\mathbf{F_I}$ was more sensitive to integration parameters, especially to the slope sensitive. This might be one of the reasons that the $\mathbf{F_I}$ was prone to be

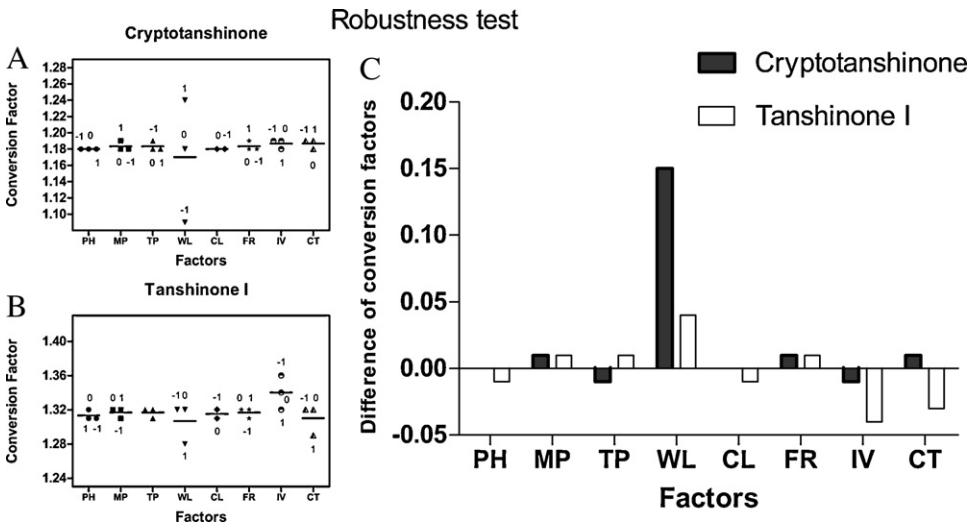

Fig. 4. Results of robustness test. (A) The conversion factors of cryptotanshinone at each level of eight factors, the line means the average value. (B) The conversion factors of tanshinone I at each level of eight factors, the line means the average value. (C) The max difference of conversion factor obtained by subtracting the conversion factor in +1 level from that of in −1 level at each factor. The abbreviations labels of *X*-axis (see Table 2).

affected by factors of column and column temperature as mentioned above. In this study, the range of integration parameters was set broad enough in order to observe the trend of effect of the factors. It will not be affected much in fact. But for different HPLC instruments, many other ways of integration can be chosen. Therefore, the rule of integration should be stated in detail.

3.3.4. Retest the robustness test and peak measurement parameter test

The results of PB design were clearly shown in Fig. 6. The half-normal probability plot was often used to interpret the PB design, and Pareto chart was more intuitive. Both the figures clearly showed that the factor of wavelength was the most important for $\mathbf{F_C}$ and factor of slope sensitive was the most important for $\mathbf{F_I}$. The results were same with the results obtained by OVAT procedure. But the PB design could provide more important information. Because there were no dummy effects available to estimate the standard error of effect in the PB design used, the algorithm of Dong was used to identify significant effects. The margin of error (ME) which is a critical effect, and the simultaneous margin of error (SME) were calculated according to literature [17]. An effect that exceeds the ME, but is below the SME, is called to be possibly significant and an effect that is above the SME, is considered to be significant [17]. Based on this rule, the effect of wavelength was significant, and the effect of slope sensitivity was possibly significant.

While for other non-significant factors, the order of importance was different from the results obtained in OVAT procedure. Such as the pH of mobile phase was the second important factor for $\mathbf{F_C}$, but in robustness test, it had no influence on the $\mathbf{F_C}$. The reason might be that the PB design is based on a hypothesis, which the interaction between factors is negligible, but the fact may not be the case, especially for the nor-significant factors. So it was difficult to

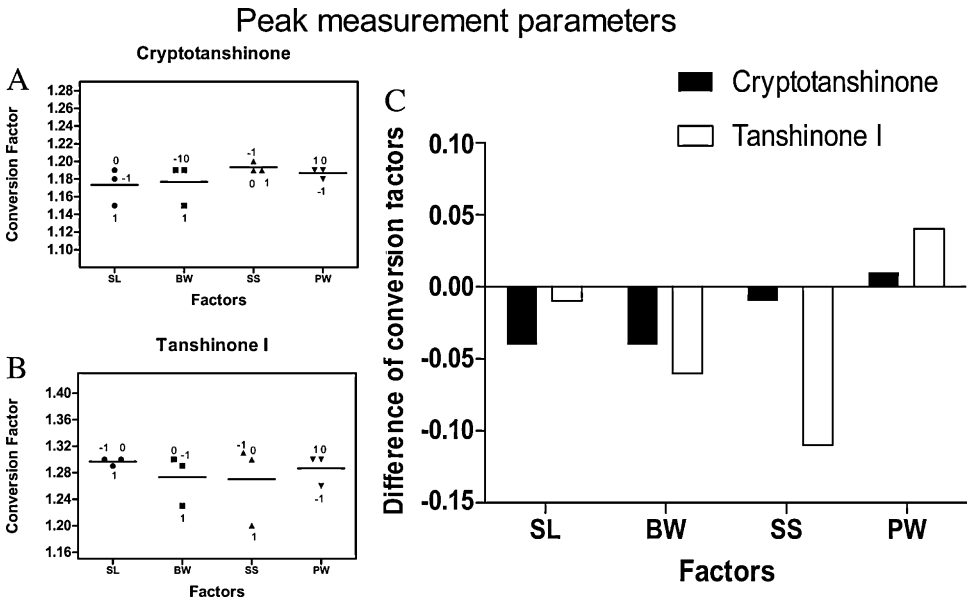

Fig. 5. Results of peak measurement parameters test. (A) The conversion factors of cryptotanshinone at each level of four factors, the line means the average value. (B) The conversion factors of tanshinone I at each level of four factors, the line means the average value. (C) The max difference of conversion factor obtained by subtracting the conversion factor in +1 level from that of in −1 level at each factor. The abbreviations labels of *X*-axis (see Table 3).

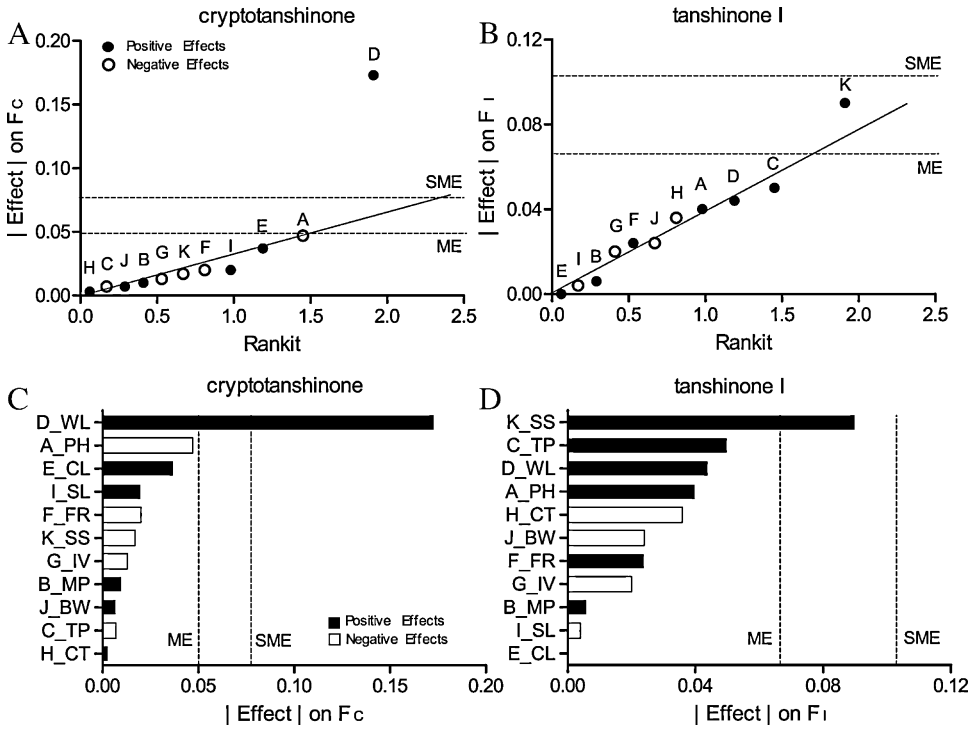

Fig. 6. Results of Plackett–Burman design. (A and B) Half-normal probability plot for the factor effects on the conversions factors with the identification of the critical effects margin of error (ME) and simultaneous margin of error (SME). (C and D) Pareto chart for the factor effects on the conversions factors with the identification of the critical effects ME and SME.

interpret each effect of factors. And the OVAT procedure was more suitable for this purpose.

3.4. Acceptable range of conversion factors

From the above discussion, we know that conversion factors were inevitable to change. Now the question will be what is the acceptable range of conversion factors? From our experiment, in the different instruments and columns tests, the range of $\mathbf{F_C}$ was 1.18 and 1.24, and the RSDs of cryptotanshinone content would be less than 3.5% theoretically calculated with the minimum and maximum, respectively. And the actual result also showed that RSDs of results of each analytes obtained by SSDMC method were less than 2.0% on different instruments and columns (Table S8). For the different samples, the RSDs of total content of tanshinones obtained by SSDMC method and external standard method were less than 1.0% (Table S10). For botanical products, the total contents were often adopted to evaluate its quality. Therefore, RSDs of total contents obtained by SSDMC were less than 2.0% compared with that obtained by external standard method, in which the fluctuation of conversion factors could be accepted. From the results of ruggedness and robustness tests, the range of $\mathbf{F_C}$ was (1.15–1.24), and $\mathbf{F_I}$ was (1.23–1.36) when the RSDs of total contents were below 2.0%.

3.5. Reproducibility

The developed analytical method had been verified by a certificated laboratory. The verified results were shown in Table 5. It showed that the deviation was less than 6.0% in the two laboratories which meet the requirements of Chinese Pharmacopoeia. The conversion factors of cryptotanshinone and tanshinone I were 1.15 and 1.23, respectively, which were both fall into the acceptable range as mentioned above.

3.6. Suggestions

From the above results, we found that the conversion factors were most sensitive to the UV detector, which the accuracy of UV

Table 5
Reproducibility of method.

Analytes	Samples	Total content of tanshinones Results I[a] (%)	Total content of tanshinones Results II[a] (%)	Deviation (%) |(A − B)|/(A + B)
Cryptotanshinone	01	0.20	0.19	2.56
	02	0.35	0.36	1.41
	03	0.32	0.32	0
Tanshinone I	01	0.10	0.09	5.26
	02	0.13	0.13	0
	03	0.15	0.15	0
Tanshinone II_A	01	0.24	0.25	2.04
	02	0.31	0.34	4.62
	03	0.40	0.42	2.44
Total	01	0.54	0.53	0.93
	02	0.79	0.83	2.47
	03	0.87	0.89	1.14

[a] Result I was obtained in our labs. And result II was obtained in SIFDC laboratory.

wavelength was the most remarkable factor. And the peak measurement parameters also play a crucial role in this process. In addition, the concentration of standard solution used to calculate the conversion should be in a suitable range.

Therefore, four suggestions would be useful for SSDMC method. (1) The robustness to determine wavelengths should be carefully chosen, which means that a common wavelength on the basis of the gentle range of absorptive peak on UV spectrum should be selected as the optimal wavelength of determination. (2) A second reference standard might be introduced, which means that the assay system should be adjusted based on a new system suitability parameter. The system suitability parameter is a range of conversion factor calculated with the two reference standards. The second standard may be one of the analytes or other compound which is not contained in the sample. (3) More detailed parameters on detector and peak measurement rules should be mentioned in the analytical procedure. (4) The concentrations of standard used to calculate the conversion factor should be in a suitable range.

4. Conclusion

The novel finding from current study is that the conversion factors were more prone to be affected in different laboratories. The primary and important factors were the detectors and its peak measurement parameters. But SSDMC method could be applied to control the quality of botanical products (herbal drugs and TCMs) with carefully controlling the parameter of detector. Four suggestions were concluded to improve the application of SSDMC method. To develop the SSDMC procedure for botanical products, the ruggedness and robustness test should be examined for the different instruments, different peak measurement parameters and columns because of the complexity of botanical products.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.06.058.

References

[1] United States Pharmacopeia Dietary Supplements Compendium, 2009, p. 1522.
[2] National Commission of Chinese Pharmacopoeia, Technological Requirements of Research and Drafting Monograph of Traditional Chinese Medicine in Ch.P. 2010. http://www.chp.org.cn/news/081119/349/fj.doc, 2008.
[3] National Commission of Chinese Pharmacopoeia, Pharmacopoeia of People's Republic of China, vol. I, China Medical Science Press, Beijing, 2010, p. 36.
[4] National Commission of Chinese Pharmacopoeia, Pharmacopoeia of People's Republic of China, vol. I, China Medical Science Press, Beijing, 2010, p. 243.
[5] United States Pharmacopeia 33-NF 28, vol. I, US Pharmacopoeial Convention, Rockville, MD, 2010, p. 1046.
[6] United States Pharmacopeia 33-NF 28, vol. I, US Pharmacopoeial Convention, Rockville, MD, 2010, p. 1037.
[7] United States Pharmacopeia 33-NF 28, vol. I, US Pharmacopoeial Convention, Rockville, MD, 2010, p. 1093.
[8] X.Y. Gao, Y. Jiang, J.Q. Lu, P.F. Tu, J. Chromatogr. A 1216 (2009) 2118.
[9] National Commission of Chinese Pharmacopoeia, Pharmacopoeia of People's Republic of China, vol. I, China Medical Science Press, Beijing, 2010, p. 285.
[10] A.H. Liu, Y.H. Lin, M. Yang, H. Guo, S.H. Guan, J.H. Sun, D.A. Guo, J. Chromatogr. B 846 (2007) 32.
[11] Z.H. Shi, J.T. He, T.T. Yao, W.B. Chang, M.P. Zhao, J. Pharm. Biomed. Anal. 37 (2005) 481.
[12] L.M. Zhou, M. Chow, Z. Zuo, J. Pharm. Biomed. Anal. 41 (2006) 744.
[13] Y. Liu, X.R. Li, Y.H. Li, L.J. Wang, M. Xue, J. Pharm. Biomed. Anal. 53 (2010) 698.
[14] B. Dejaegher, Y. Vander Heyden, J. Chromatogr. A 1158 (2007) 138.
[15] D.C. Motgomery, Design and Analysis of Experiments, 6th ed., Posts & Telecom Press, Beijing, China, 2009, p. 459, translated by J.S. Fu, J. Zhang, Z.Y. Wang, Y. Xie.
[16] C.A. Beasley, J. Shaw, Z. Zhao, R.A. Reed, J. Pharm. Biomed. Anal. 37 (2005) 559.
[17] Y. Vander Heyden, A. Nijhuis, J. Smeyers-Verbeke, B.G.M. Vandeginste, D.L. Massart, J. Pharm. Biomed. Anal. 24 (2001) 723.