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Detection-confirmation-standardization-quantification: a novel method for the quantitative analysis of active components in traditional Chinese medicines

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Background. Quantitative analysis of the active ingredients of Traditional Chinese Medicine is a research tendency. The objective of this study was to build a novel method, namely, Detection-confirmation-standardization-quantification (DCSQ) for the quantitative analysis of active components in traditional Chinese medicines, without individual reference standard.

Methods. Danshen (the dried root of Radix *Salvia miltiorrhiza*) was used as the matrix. The "extraction" function in high-performance liquid chromatography-mass (HPLC-MS) instrument was used to find the peaks corresponding to cryptotanshinone, tanshinone I, and tanshinone IIA in the total ion current (TIC) chromatogram of Danshen. The multicomponent reference standard (MCRS) containing the three tanshinones mainly was prepared by preparative HPLC. The contents of them in the resulting MCRS were determined by NMR; moreover, the constituents of the MCRS were confirmed. The MCRS containing known content of the three tanshinones was used as the reference standard for the quantitative analysis of cryptotanshinone, tanshinone I, and tanshinone IIA in Danshen Samples by analytical HPLC.

Results. Establishing the optimized HPLC conditions for the quantitative analysis of the active components in Danshen. And the assignments of the extracted peaks were confirmed by analyzing the characteristic fragments in their MS/MS product ion spectra and UV spectra. Then the MCRS containing the three tanshinones was prepared successfully. The results of determination about contents by NMR showed linearity fitted with high likelihood and calibration curves possessed high linearity. The results of determination on Danshen Samples obtained through DCSQ exhibited minimal deviations, in contrast to those obtained through individual reference standards.

Conclusion. The establishing DCSQ is independent and convenient for the quantitative analysis of the active components in TCMs by MCRS, without individual reference standard. This method is a great advance in quantitative analysis for complex composition, especially TCMs.

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2 Quantification: A novel method for the quantitative
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12 Abstract

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28 analyzing the characteristic fragments in their MS/MS product ion spectra and UV spectra. Then
29 the MCRS containing the three tanshinones was prepared successfully. The results of
30 determination about contents by NMR showed linearity fitted with high likelihood and
31 calibration curves possessed high linearity. The results of determination on Danshen Samples
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33 individual reference standards.
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37 Keywords. Quantitative analysis, Detection-confirmation-standardization-quantification (DCSQ),
38 Multicomponent reference standard (MCRS), Tanshinones, HPLC-MS/MS, NMR
39

Introduction

41 Traditional Chinese medicines (TCMs) have been widely used in the treatment of various
42 diseases because of their remarkable and reliable biological activities. Therefore, the
43 determination of the active components in TCMs by chromatography methods such as high-
44 performance liquid chromatography (HPLC), thin-layer chromatography (TLC), and high-
45 performance capillary electrophoresis (HPCE) are considered as the main strategy by which the
46 quality of TCMs may be controlled. Conventionally, the active components of TCMs are
47 quantified by the corresponding reference standards*(National Commission of Chinese*
48 *Pharmacopoeia, 2020)*. Popular compounds are purchased from authoritative organizations,
49 whereas rare compounds are purified inhouse. Another approach, namely, single standard to
50 determine multicomponents (SSDMC), had been developed to reduce the reliance of quantity
51 on the reference standards *(Fang et al., 2017; Liu et al.,2017)* . The reference standards are still
52 needed when assigning peaks and calculating conversion factors (the molar response ratio of
53 reference standard to analyte).
54 NMR spectroscopy is considered as a promising quantification method for the direct
55 determination of target compounds in mixtures without reference standards; the amount of
56 analytes can be calculated using the ratios of the signal intensities of the protons of different
57 compounds and internal reference standard*(Frezza et al., 2018; Luisa et al., 2017; Petrakis et al.,*
58 *2017; Chauthe et al., 2012; Staneva et al., 2011)*. However, a major challenge associated with
59 NMR is the difficulty involved in the quantification of complex mixtures. The NMR spectra of
60 complex mixtures often exhibit severe peak overlap, thereby affecting the accuracy of the
61 quantity of analyte. To prevent severe peak overlap, *Staneva et al.(2011)* fractionated the
62 extracts of TCMs before quantifying the target components by NMR*(Chauthe et al., 2012;*
63 *Staneva et al., 2011)*. This procedure often leads to the dissociation of target components into
64 separate parts or absorption by sorbents. Consequently, the results do not accurately reflect the
65 contents of target compounds in TCMs.
66 We proposed a novel method for the quantitative analysis of the active components in TCMs
67 without individual reference standards, namely, detection-confirmation-standardization-
68 quantification (DCSQ). Danshen (the dried root of *Salvia miltiorrhiza*), which was a popular
69 traditional Chinese medicinal herb best known for its putative cardioprotective and anti-
70 atherosclerotic effects, was used as the matrix*(Jia et al., 2019; Liu et al., 2015; Wang et al.,*
71 *2011)*. The main components responsible for its pharmacological properties were hydrophilic
72 depsides and lipophilic diterpenoid quinines*(Wang et al., 2013; Li et al., 2018)*. An application
73 of the DCSQ method for the quantitative analysis of three main diterpenoid quinones,
74 cryptotanshinone, tanshinone I, and tanshinone IIA **(Fig. 1, A-C)** was reported for the first time.

75   HPLC is a suitable technique for the quantitative analysis of the active components in TCMs
76 because they contain numerous compounds. The separation of target components from a
77 complex mixture in TCMs can be achieved by optimizing the HPLC conditions. Based on the
78 combination of HPLC, MS, and NMR techniques, the quantitative analysis of the target
79 components in TCMs without reference standard can be performed as follows: First, the
80 "extraction" function in HPLC-MS instrument was used to find the peaks corresponding to the
81 target components from the complex TIC chromatogram of TCMs, even without adequate
82 separation*(Wang et al.,2017)*. Next, the peaks were confirmed by the analysis of their MS/MS
83 spectra. Finally, the HPLC conditions were optimized for the quantitative analysis of target
84 components. The mixture mainly consisting of the target components, referred to as the
85 multicomponent reference standard (MCRS), was prepared by preparative HPLC. The contents
86 of the target components in the MCRS were determined directly by NMR, and the MCRS was
87 used as the reference standard instead of individual reference standards.
88

Materials & Methods
89

Materials and Chemicals
90
91 Danshen was purchased from Qiancao herbal wholesale company (Beijing). The standards of
92 cryptotanshinone (1), tanshinone I (2), and Tanshinone IIA (3) (**Fig. 1, A-C**) were obtained from
93 Traditional Chinese Medicine Solid Preparation National Engineering Research Center
94 (Nangchang, Jiangxi Province, China), with a purity of ≥98%. Dimethyl fumarate (4) (**Fig. 1, D**)
95 with a purity of 99% was obtained from Alfa Aesar (Ward Hill, Massachusetts, USA). $CDCl_3$
96 (99.8% pure) was obtained from Cambridge Isotope Laboratories Inc. (Andover, MA, USA).
97 Acetonitrile and methanol for HPLC were obtained from Fisher Scientific (Fair Lawn, New
98 Jersey, USA). Formic acid (>98% purity) for HPLC was obtained from Sinopharm Chemical
99 Reagent Co. Ltd. (Shanghai, China). All other chemicals were of analytical grade. Deionized
100 water was obtained from Wahaha Company (Hangzhou, Zhejiang, China).
101

Identification of cryptotanshinone, tanshinone I, and tanshinone IIA in Danshen by HPLC-
102
ESI-MS/MS
103
104 Sample preparation: Dried Danshen samples were powdered using a mill and sieved through a
105 No. 24 mesh. Approximately 0.3 g of the powder sample was weighed and extracted by refluxing
106 in 50 mL of methanol for 1 h. The weight loss was adjusted with methanol after the extraction.
107 One mL of the sample solution was filtered through a 0.45 μm nylon filter into a HPLC amber
108 sample vial for injection.
109   Data acquisition: An Agilent 1100 series HPLC system (Agilent Technologies, Santa Clara,
110 CA, USA) equipped with a quaternary solvent delivery system, an on-line degasser, an auto-
111 sample injector, a column temperature controller, and a variable wavelength detector (VWD)
112 was coupled to an Agilent 6460 triple quadruple mass spectrometer (QQQ-MS) equipped with a
113 dual electrospray ion source (ESI) (Agilent Technologies, Santa Clara, CA, USA) formed the
114 HPLC-ESI-MS/MS system.

115   The samples were separated using a Diamonsil C18 column (4.6 × 250 mm, 5 μm, Dikma
116   Technologies Inc.). The mobile phase was a mixture of acetonitrile (mobile phase A) and water
117   containing 0.1% formic acid (mobile phase B). The gradient elution started at 60% A, followed
118   by a linear increase to 80% A at 30 min, 90% A at 40 min, a linear decrease to 60% A at 41 min,
119   and held constant at 55 min before the next injection was performed. The flow rate was 1.0 mL
120   min^{-1}. The column temperature was maintained at 22 °C. The injection volume was 20 μL.

121   The mass spectrometric data were acquired using the positive electrospray mode. The full-
122   scan mass spectrum was recorded over the range of m/z 250-325. N_2 was used as the sheath and
123   auxiliary gas of the mass spectrometer. The capillary voltage for ESI spectra was 3.5 kV, and the
124   capillary temperature was set at 300 °C. Ultra-high pure helium was used as the collision gas in
125   the collision-induced dissociation (CID) experiments. The MS/MS product ion spectra were
126   acquired through the CID of the peaks with the protonated molecular ion [M + H] $^+$ of each
127   analyte. The collision energy for the target protonated molecular ions was set at 20 eV to obtain
128   the appropriate fragment information.

129

130   **Preparation of multicomponent reference standard**
131   Preparation of total tanshinones: The total tanshinones were prepared using a method of
132   preparing tanshinone extract as recorded in the ***Chinese Pharmacopoeia 2020 edition***. Fifty
133   gram of the Danshen powder was extracted by refluxing in 500 mL 95% ethanol for 2 h. The
134   Danshen extract was evaporated under reduced pressure at 40 °C. The resulting solid was
135   washed three times with hot water (80 °C) to remove the water-soluble components and obtain
136   the total tanshinones.

137   Preparation of multicomponent reference standard (MCRS): The MCRS containing the three
138   tanshinones was prepared using a CXTH LC-3000 semi-preparative HPLC series equipped with
139   a binary solvent delivery system, a Rheodyne 7725i manual injection valve (5 mL sample loop)
140   and a UV-visible detector (Chuangxintongheng Co. Ltd., Beijing, China). The total tanshinones
141   were dissolved in 30 mL of methanol and separated using a Thermo BDS Hypersil C18 prep-
142   column (22.2 × 150 mm^2, 5 μm, Thermo Scientific) eluting with methanol/water (77:23, v/v).
143   The flow rate was 9.0 mL min^{-1}, and the detection wavelength was set at 200 nm. The injection
144   volume was 1.0 mL. All the effluents containing cryptotanshinone, tanshinone I, and tanshinone
145   IIA were collected and evaporated to dryness. A total of 210 mg of the MCRS was obtained from
146   30 mL of the total tanshinone solution.

147

148   **Quantitative determination of each tanshinones in multicomponent reference standard by**
149   **QNMR**
150   Generation of NMR calibration series: A series of volumetric solutions containing 20.50, 10.25,
151   6.83, 3.42, and 1.71 mg/mL of the MCRS and 2.96, 1.48, 0.74, 0.37, and 0.19 mg/mL of
152   dimethyl fumarate in CDCl$_3$ were prepared and measured by NMR at 600 MHz.

153   NMR spectral acquisition and processing parameters: The spectra was acquired using a 14.1 T
154   Bruker Avance 600 MHz NMR spectrometer equipped with a 5 mm broad band (BB) inverse

155 detection probe tuned to detect ^1H resonances. The ^1H resonance frequency was 600.13 MHz.
156 All the spectra were acquired at 298 K. A total of 64 scans of 32 K data points were acquired
157 with a spectral width of 9615.4 Hz (16 ppm). A pre-acquisition delay of 6.5 μs, with an
158 acquisition time of 1.7 s, recycle delay of 24 s, and flip angle of 90° were used. The chemical
159 shift of all the peaks was referenced to the tetramethylsilane (TMS) resonance at 0 ppm. The
160 spectra were Fourier transformed to afford a digital resolution in the frequency domain of 0.293
161 Hz/Point. The phase and baseline corrections of the spectra were carried out manually.
162 Preliminary data processing was carried out using the Bruker software TOPSPIN 2.1.

163 The signals for the H-15 of cryptotanshinone (4.884 ppm, t, 2H), H-17 of tanshinone I (2.295
164 ppm, d, 3H), H-17 of tanshinone IIA (2.259 ppm, d, 3H), and olefinic H of dimethyl fumarate
165 (6.864 ppm, s, 2H) were used to determine the contents of cryptotanshinone, tanshinone I, and
166 tanshinone IIA in the MCRS.

167 The concentrations of the three tanshinones in the MCRS were calculated using the following
168 general equation:

169
$$C_x = \frac{A_x \times W_i \times N_i}{A_i \times M_i \times N_x \times V} \qquad \text{Eq. (1)}$$

170 where C_x (in mM) corresponds to the concentrations of the three individual tanshinones; A_x
171 and A_i correspond to the peak areas of tanshinones and the internal standard. W_i corresponds to
172 the mass of the internal standard (in mg); N_i and N_x correspond to the number of protons of the
173 respective signals of the internal standard and tanshinones used for the quantitative analysis; M_i
174 corresponds to the molecular weight (in Da) of the internal standard, and V (in L) corresponds to
175 the volume of $CDCl_3$.

176 The contents of the three tanshinones in the MCRS were calculated using the following
177 general equation:

178
$$P_x = \frac{C_x \times M_x \times V}{W_m} \times 100\% \qquad \text{Eq. (2)}$$

179 where P_x (%) corresponds to the percentage of the three individual tanshinones in the MCRS;
180 M_x corresponds to the molecular weight (in Da) of the three tanshinones, and W_m corresponds to
181 the mass of the MCRS (in mg).

182

183 **Quantitative analysis of the three tanshinones in the Danshen sample by HPLC using the**
184 **MCRS as the reference standard**
185 Sample preparation and HPLC conditions: The Danshen sample was prepared according to the
186 procedure described according to the above steps.

187 Analytical HPLC was carried out using a Shimadzu LC-20AT series equipped with a
188 quaternary solvent delivery system, an on-line degasser, and auto-sample injector, a column
189 temperature controller, and an SPD-M20A diode-array detector (DAD) (Shimadzu corporation,
190 Kyoto, Japan). Finally, the samples were separated using a Diamonsil C18 column (4.6 × 250
191 mm, 5 μm, Dikma Technologies Inc.). Notably, the HPLC conditions are compatible to the
192 HPLC-ESI-MS experiments.

193   Construction of calibration curves: Appropriate amounts of the MCRS were weighed and
194   dissolved into 100 mL of acetone. To construct the calibration curves, 2, 4, 8, 12, 16, and 20 μL
195   of the solution were injected in triplicates. The calibration curves were constructed by plotting
196   the peak areas versus the quantity of the three tanshinones in the MCRS.

197
198   Results

199   **Detection of the peaks corresponding to cryptotanshinone, tanshinone I, and tanshinone**
200   **IIA from the TIC and HPLC chromatograms of Danshen**

201   The initial investigation focused on determining the target components from the TIC and HPLC
202   chromatograms of Danshen and establishing the optimized HPLC conditions suitable for the
203   quantitative analysis of the active components in Danshen. A rough gradient elution was first
204   performed, after which the peaks corresponding to cryptotanshinone, tanshinone I, and
205   tanshinone IIA were searched with their protonated molecular ion $[M + H]^+$ from the total ion
206   chromatogram of Danshen*(Wang et al.,2017)*.The results (**Figs. 2, A and C**) indicated that the
207   peak with the retention times of 6.4, 6.2, and 8.5 min corresponded to the *m/z* of 297, 277, and
208   295, respectively. Under these conditions, cryptotanshinone and tanshinone I were not
209   adequately separated, even though the positions of cryptotanshinone and tanshinone I in the TIC
210   and HPLC spectra of Danshen were successfully assigned. The result obtained by the
211   optimization of gradient elution is shown in **Fig. 2 (B and D)**; the peaks with the retention times
212   of 17.2, 19.0, and 26.1 min corresponded to the *m/z* of 297, 277, and 295, respectively, in the
213   TIC of LC-MS **(Fig. 2, B)**. Moreover, the three tanshinones were adequately separated from
214   other compounds. This condition was adequate for the subsequent quantitative analysis of the
215   three tanshinones in Danshen.
216
217   **Confirmation of the peak assignments by ESI-MS/MS product ion and UV spectra**
218   The ESI-MS/MS product ion spectra were acquired through the CID of the peaks obtained with
219   the protonated molecular ion $[M + H]^+$ of the three tanshinones. The proposed MS fragmentation
220   pathway of the compounds with the protonated molecular ions of *m/z* 297, 277, and 295 were
221   summarized in **Fig. 3**, consistent with the MS fragmentation pathway of cryptotanshinone,
222   tanshinone I, and tanshinone IIA as studied by ***Wang et al., 2017***. The UV spectra shown in **Fig.**
223   **4** were obtained directly from the HPLC experiments. The UV spectra of the three peaks
224   matched with those of cryptotanshinone, tanshinone I, and tanshinone IIA, as reported in the
225   current literature*(Huang et al., 2018)*.
226

227   **Preparation of the MCRS containing mainly cryptotanshinone, tanshinone I, and**
228   **tanshinone IIA**

229 The MCRS containing mainly cryptotanshinone, tanshinone I, and tanshinone IIA was prepared
230 by the preparative HPLC of the total tanshinone to perform a quantitative analysis of the three
231 components, without individual reference standards. The chromatograms of the total tanshinone
232 and MCRS were shown in **Fig. 5 (picture A)**. Peaks 1, 2, and 3 were assigned to
233 cryptotanshinone, tanshinone I, and tanshinone IIA, respectively, by collecting the main peaks in
234 the preparative HPLC separately and then analyzing them by analytical HPLC.

235

236 **Determination of the contents of cryptotanshinone, tanshinone I, and tanshinone IIA in the**
237 **MCRS by NMR**
238 The contents of the three tanshinones in the resulting MCRS were determined directly by ^{1}H
239 NMR analyses. The ^{1}H NMR spectrum of the MCRS mixed with the internal standard, dimethyl
240 fumarate, was shown in **Fig. 6**. The main signals were assigned to the three tanshinones
241 according to the references, excluding the signals belonging to dimethyl fumarate*(Mei et al.,*
242 *2019; Zeng et al., 2017; Wu et al., 2015)*. The composition of the MCRS was confirmed again
243 by analyzing the signals observed in the ^{1}H NMR spectrum. To perform an accurate quantitative
244 analysis, the data processing technique was used which named the Lorentz deconvolution
245 function in the Bruker software TOPSPIN 2.1 . The linearity of signals were fitted by the Lorentz
246 method. Next, the deconvolution was performed, and the peak areas were automatically
247 generated. Regarding the quantitative analysis of the mixtures by NMR using the data
248 processing, the accuracy was determined by the likelihood of lineshape fitting. The results
249 showed that the contents of crytotanshinone, tanshinone I, and tanshinone IIA were at 1.635
250 g/100g, 0.718 g/100g, 1.953 g/100g in Danshen sample, respectively, by MCRS method.
251 Under the preparation of MCRS of Danshen, the selection of the signals in the NMR spectra
252 for the quantitative analysis of the three tanshinones and the validity of the NMR method were
253 performed. The analytical method of NMR was achieved a high separation efficiency which was
254 shown in **Fig. 7 (A-O)**. The NMR spectrum is very reproducible with good reproducibility and
255 target three compounds high separation efficiency.

256

257 **Determination of the contents of the three tanshinones in the Danshen sample by HPLC**
258 **using the MCRS as the reference standard and comparison of the results with those**
259 **obtained using individual reference standards.**
260 The calibration curve of the MCRS was constructed to investigate the validity of the NMR
261 method. The calibration curves were constructed by plotting the concentrations of the three
262 tanshinones versus that of dimethyl fumarate. Next, the linear regression lines were calculated.
263 The calibration graph demonstrating the linearity of the NMR response with increasing
264 concentrations of the three tanshinones was shown in **Fig. 8**.
265 Approximately 5 mg of the MCRS was dissolved into 100 mL of acetone to construct the
266 calibration curve of the three tanshinones, corresponding to ~1.5, 0.5, and 1.7 mg of individual
267 cryptotanshinone, tanshinone I, and tanshinone IIA, respectively. The contents of the three
268 tanshinones in the Danshen sample were determined according to the calibration curves based on

269 individual standards. The content of cryptotanshinone was determined at 1.635 g/100g and 1.627
270 g/100g, respectively, using MCRS and standard quantitation. Tanshinone I was detected at 0.718
271 g/100g and 0.727 g/100g, respectively, based on the two methods. Tanshinone IIA was measured
272 at 1.953 g/100g and 1.886 g/100g, respectively. The mean contents of the three compounds
273 obtained by the two methods were tested for no significance difference at the $P \leq 0.05$ level. The
274 results were compared with those obtained using individual reference standards. The deviations
275 of the three tanshinones determined by the two methods were minimal **(Fig. 9)**, and this could be
276 attributed to the maximum likelihood lineshape fitting of their signals in the NMR spectrum of
277 the MCRS.

278

279 Discussion

280 The multicomponent reference standard (MCRS) of Danshen was preparing successfully to use
281 for the samples' quantitative analysis. The MCRS containing cryptotanshinone, tanshinone I, and
282 tanshinone IIA was obtained by collecting peaks 1, 2, and 3 together **(Fig. 5, picture B)**. The
283 preparation of MCRS was found to be much easier than that of individual reference standards.
284 The presence of some other compounds whose NMR signals did not overlap severely with those
285 of the target components may be allowed. And the target components need not be separated from
286 each other in their preparative HPLC spectrum because they were finally collected for preparing
287 the MCRS.

288 Apart from structure elucidation, NMR had been progressed to become a useful technique for
289 the direct quantitative analysis of complex mixtures. However, it remained difficult to directly
290 quantitatively analyze the target components from the extracts of TCMs using the NMR
291 technique, owing to the existence of numerous components, which causing severe overlap of the
292 NMR signals. In this study, HPLC was selected for the quantification of the three tanshinones in
293 Danshen for its outstanding separation ability compared to NMR. By using the "extraction"
294 function in HPLC-MS instrument to find the target components and MCRS as the reference
295 standard diminished the dependency of individual reference standards in the quantitative analysis
296 of the target components in TCMs. Evidently, the increased amount of components in TCM
297 samples would not make the quantitative analysis of target components more difficult, which
298 could be attributed to the efficient separation function of HPLC. The components whose signals
299 overlap with each other could be divided into different MCRSs. The amount of MCRS prepared
300 from one sample could be adjusted according to the components requiring quantification and
301 considering the prevalence of NMR signal overlapping.
302 The results of MCRS shown in **Fig. 7** indicated that the extents of the likelihood of the
303 lineshape fitting of the signals for the three tanshinones performed by the Lorentz deconvolution
304 are different. Pictures **A-E, F-K, L-M, and N-O** showed the lineshape fitting results of the
305 signals for cryptotanshinone, tanshinone I, tanshinone IIA, and dimethyl fumarate, respectively.
306 The lineshapes of the following signals were fitted with relatively higher likelihood: the triplet in
307 **B** at 4.884 ppm belonged to cryptotanshinone, the doublets in **G** and **K (right side)** belonged to

308   tanshinone I, the doublet in **K (left side)** belongs to tanshinone IIA, and the singlet in **N**
309   belonged to dimethyl fumarate.

310   The calibration graph demonstrating the linearity of the NMR response with increasing
311   concentrations of the three tanshinones in **Fig. 8** indicated that the R^2 values calculated from the
312   signals in **C** and **K (left side) (Fig. 7)** belonging to cryptotanshinone and tanshinone IIA were
313   1.0000 and 0.9999, respectively. The signals in **G** and **K (right side) (Fig. 7)** belonging to
314   tanshinone I also exhibited a high likelihood of lineshape fitting, and the R^2 values calculated
315   from the two signals were 0.9995 and 0.9997, respectively. The result confirmed that the
316   concentrations of the three tanshinones in the MCRS could be accurately determined using [1]H
317   NMR after deconvolution. The average contents of three tanshinones was calculated with five
318   different concentrations from the NMR spectra of the MCRS. And the results were 28.01%,
319   9.43%, and 34.36%, respectively. The signals in **B, K (left side and right side) (Fig. 7)**, and **N**
320   **(Fig. 7)** were finally selected for the quantitative analysis of the three tanshinones, because their
321   linearity fitted with high likelihood and their calibration curves possessed high linearity.

322   The calibration curves of the three tanshinones were constructed using the same method as
323   that of individual reference standards. A significant advantage of using the MCRS as the
324   reference standard was that it reduced the dependency of quantitative analysis on the individual
325   reference standard and also reduced the consumption of reference standards.
326

Conclusions

328   In this study, the advantageous combination of HPLC, MS, and NMR techniques facilitated
329   the accurate quantification of the target components in TCMs, without individual reference
330   standards, which could be defined as Detection-confirmation-standardization-quantification
331   (DCSQ). By combination of multiple analytical techniques, the MCRS was detected, confirmed,
332   prepared and determinated successfully. In the study, NMR' direct quantitative analysis was
333   utilized to the MCRS even though the resolution of HPLC was not enough in the mixture. The
334   MCRS of danshen with three major constituents, cryptotanshinone, tanshinone I, and tanshinone
335   IIA, was prepared and used for samples. We provided an innovative method to get reference
336   standards. The MCRS could be a novel approach as standards for quantification about
337   corresponding samples, which was less dependent than individual reference standards. The
338   MCRS as the reference standard will be used to quantitative analysis and for the more industrial
339   applications. It will be more accurate and convenient for the target analyte. It was a great
340   advance in quantitative analysis for complex composition, especially TCMs.

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447   **Figure 1** Chemical structures of cryptotanshinone (A), tanshinone I (B), tanshinone IIA (C), and dimethyl
448   fumarate (D).

449

450   **Figure 2** Total ion current chromatograms of the Danshen extract and extracted ion chromatograms of
451   target protonated molecular ions (A-B) and UV spectrum of the Danshen extract (C-D). The HPLC
452   conditions were as follows: The water phase contained 0.1% formic water. The flow rate was 1.0 mL/min.
453   The detection wavelength was set at 270 nm. The eluting condition of (A-B) was as follows: 85%
454   acetonitrile was maintained for 15 min, and the column temperature was 35 °C. The eluting condition of
455   (C-D) was as follows: 60% acetonitrile was maintained for 0–30 min, and the concentration of acetonitrile
456   was increased from 60% to 80% and then to 90% of acetonitrile at 40 min. The column temperature was
457   22 °C.

458

459   **Figure 3** Positive ion ESI-MS/MS spectra and the proposed MS fragmentation pathways of peaks 1 (A),
460   2 (B), and 3 (C).

461

462   **Figure 4** UV spectra of the three peaks shown in Fig. 2 (picture D). (A) UV spectrum of the peak with the
463   retention time of 16.8 min; (B) UV spectrum of the peak with the retention time of 18.6 min; (C) UV
464   spectrum of the peak with the retention time of 25.7 min.

465

466   **Figure 5** Representative preparative HPLC chromatogram of total tanshinones (A) and analytical HPLC
467   chromatogram of the MCRS (B). The MCRS was analyzed under the same condition as HPLC–MS
468   experiment (Fig. 2, eluting gradient of (C-D)).

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470   **Figure 6** 600 MHz ^{1}H NMR spectrum of the MCRS mixed with dimethyl fumarate. The concentrations
471   of the MCRS and dimethyl fumarate in CDCl3 were 20.50 and 2.96 mg·mL^{-1} , respectively.

472

473   **Figure 7** Representative results of the lineshape fitting of the signals belonging to the three tanshinones
474   and dimethyl fumarate performed by the Lorentz deconvolution. Images A-E reflect the representative
475   lineshape fitting of signals belonging to cryptotanshinone. Images F-K (the right side) reflect the
476   representative lineshape fitting of signals belonging to tanshinone I. The doublet in the left side of K,
477   images L and M (the doublet in the right side) reflect the representative lineshape fitting of signals
478   belonging to tanshinone IIA. Images N and O reflect the lineshape fitting of signals belonging to dimethyl
479   fumarate.

480

481   **Figure 8** Concentrations of the three tanshinones determined by specific signals.

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483   **Figure 9** Deviations of the contents of the three tanshinones in the Danshen sample as determined by the
484   MCRS contrast to individual reference standards.

Figure 1

Figure 1 Chemical structures of cryptotanshinone (A), tanshinone I (B), tanshinone IIA (C), and dimethyl fumarate (D).

Cryptotanshinone (1)
Chemical Formula: $C_{19}H_{20}O_3$
Molecular Weight: 296.36

Tanshinone I (2)
Chemical Formula: $C_{18}H_{12}O_3$
Molecular Weight: 276.29

Tanshinone IIA (3)
Chemical Formula: $C_{19}H_{18}O_3$
Molecular Weight: 294.34

D

H_3COOC ⎯ $COOCH_3$

Dimethyl fumarate (4)
Chemical Formula: $C_6H_8O_4$
Molecular Weight: 144.13
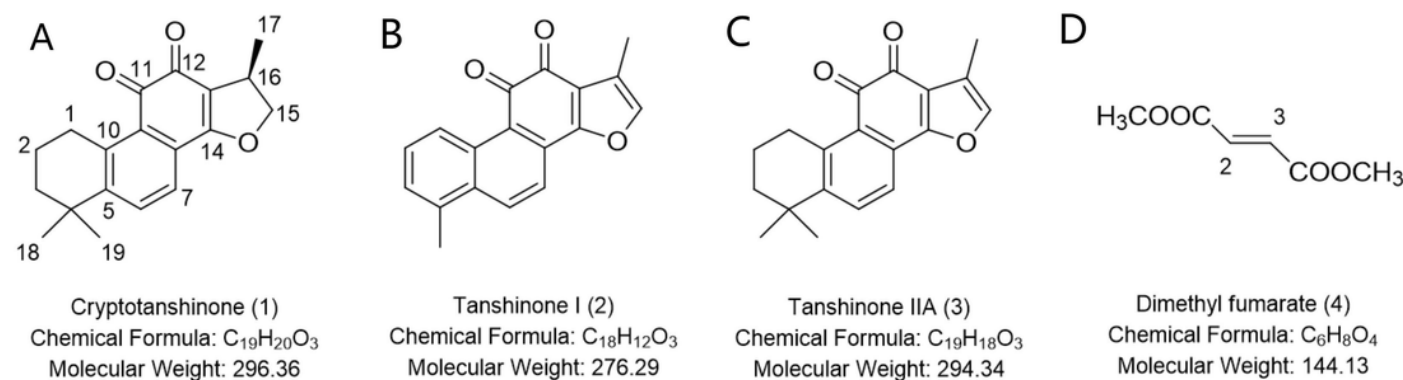
Figure 2

Figure 2 Total ion current chromatograms of the Danshen extract and extracted ion chromatograms of target protonated molecular ions (A-B) and UV spectrum of the Danshen extract (C-D).

The HPLC conditions were as follows: The water phase contained 0.1% formic water. The flow rate was 1.0 mL/min. The detection wavelength was set at 270 nm. The eluting condition of (A-B) was as follows: 85% acetonitrile was maintained for 15 min, and the column temperature was 35 °C. The eluting condition of (C-D) was as follows: 60% acetonitrile was maintained for 0–30 min, and the concentration of acetonitrile was increased from 60% to 80% and then to 90% of acetonitrile at 40 min. The column temperature was 22 °C.
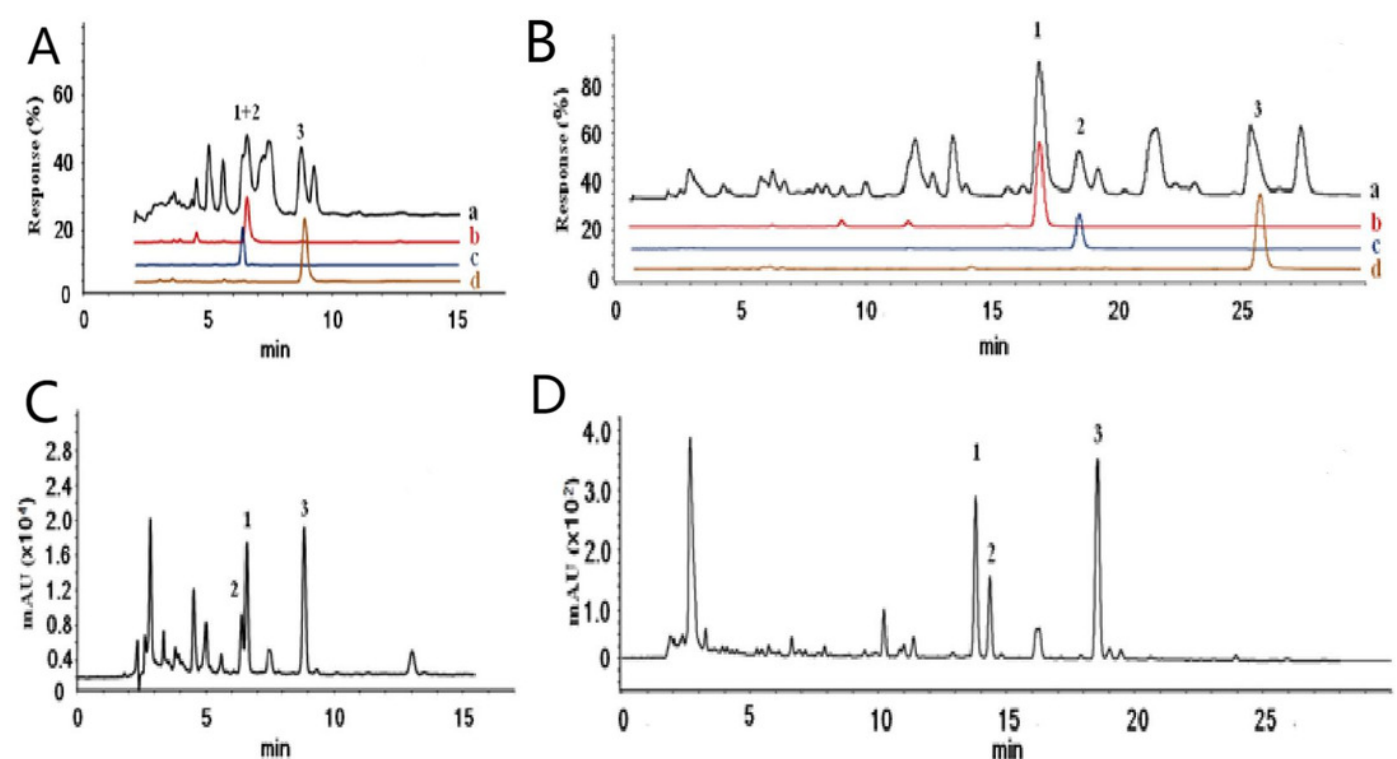
Figure 3

Figure 3 Positive ion ESI-MS/MS spectra and the proposed MS fragmentation pathways of peaks 1 (A), 2 (B), and 3 (C).
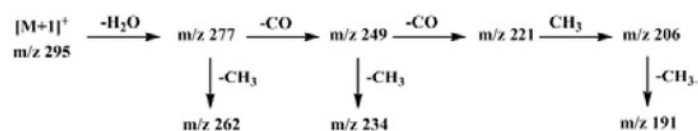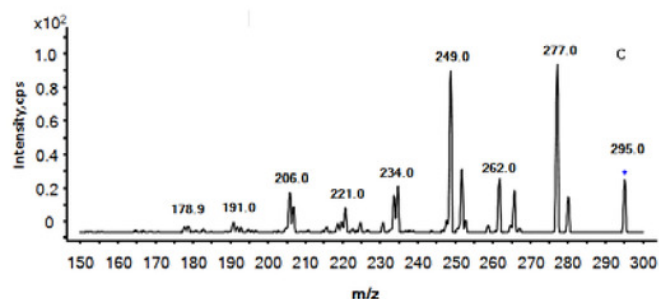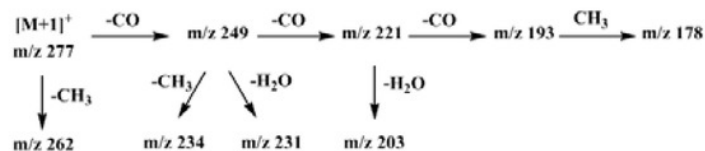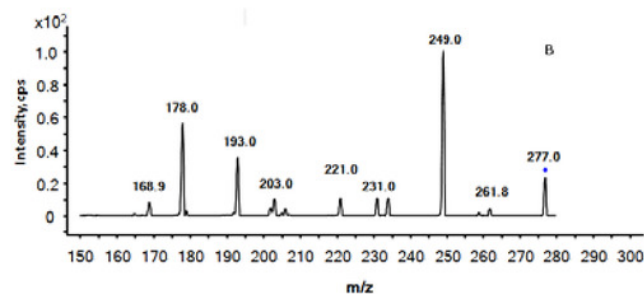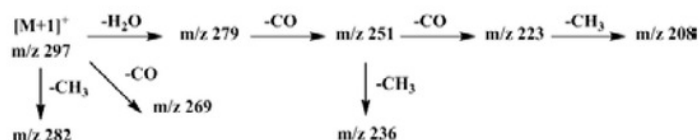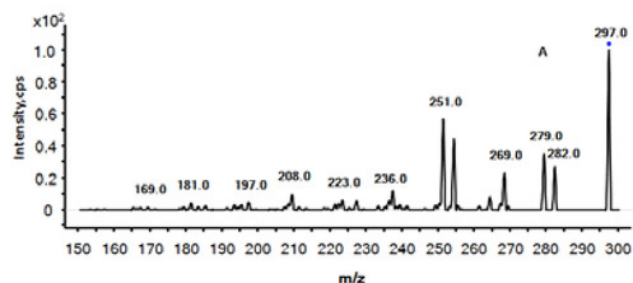
Figure 4

Figure 4 UV spectra of the three peaks

UV spectra of the three peaks shown in Fig. 2 (picture D) . (A) UV spectrum of the peak with the retention time of 16.8 min; (B) UV spectrum of the peak with the retention time of 18.6 min; (C) UV spectrum of the peak with the retention time of 25.7 min.
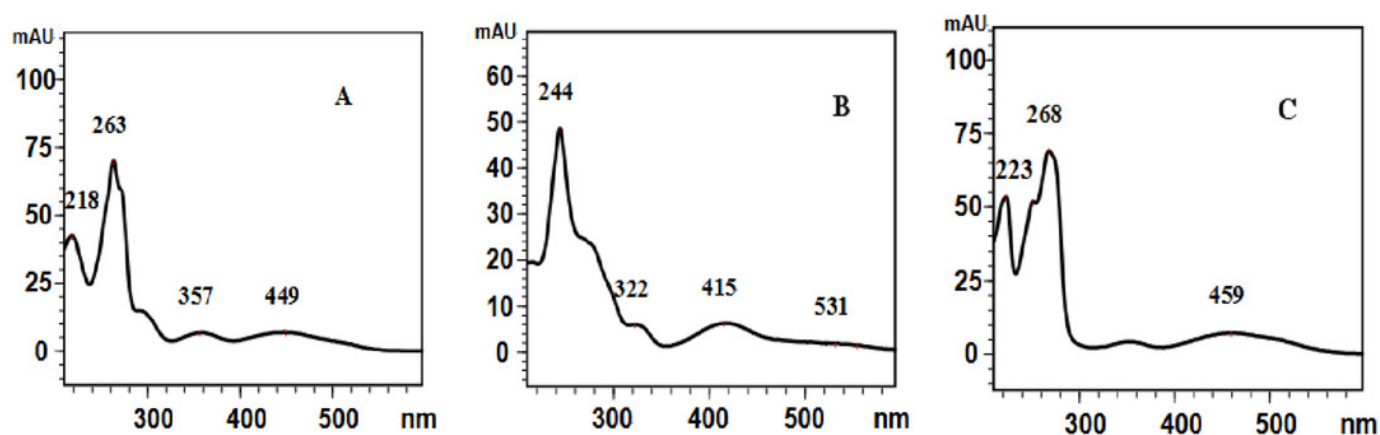
Figure 5

Figure 5 Representative preparative HPLC chromatogram of total tanshinones (A) and analytical HPLC chromatogram of the MCRS (B).

The MCRS was analyzed under the same condition as HPLC–MS experiment (Fig. 2, eluting gradient of (C-D)).
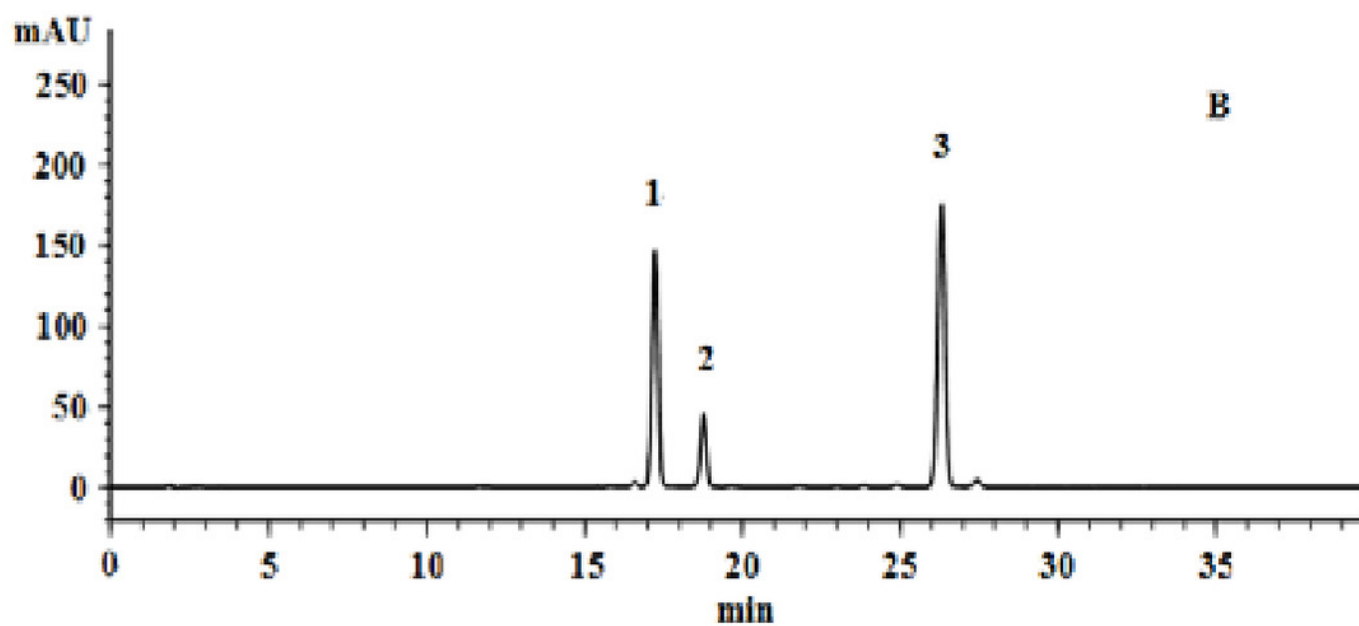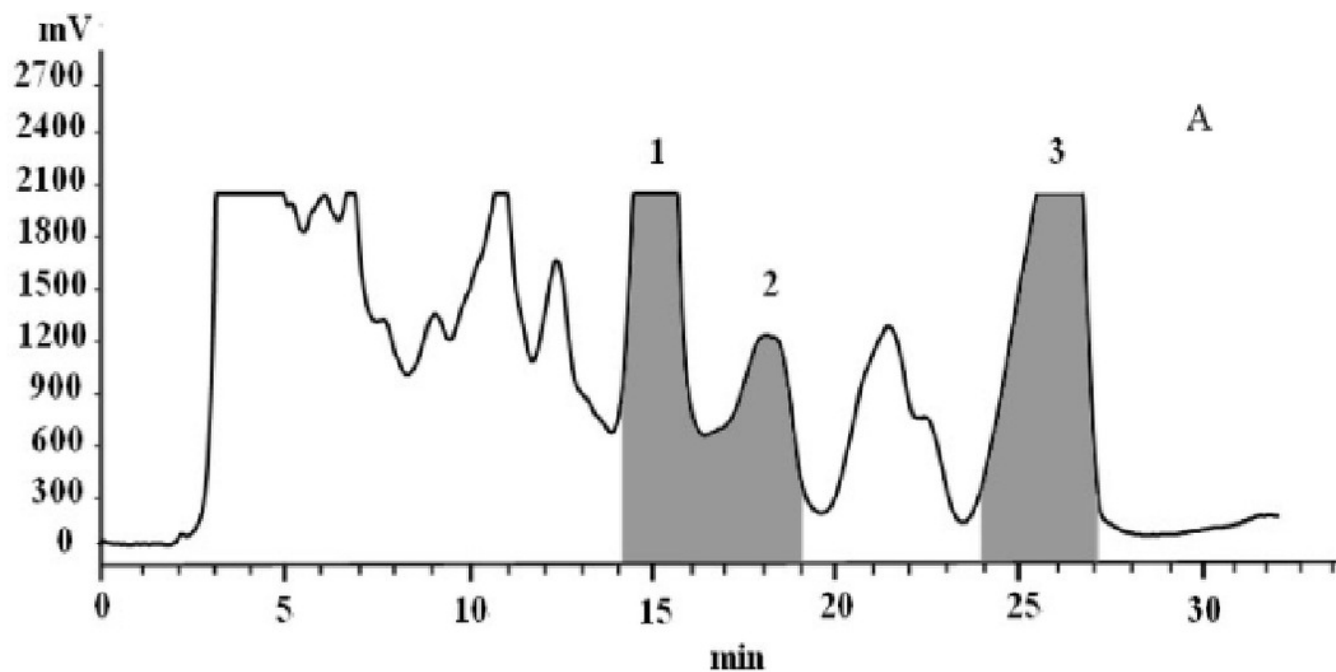
Figure 6

Figure 6 600 MHz ^{1}H NMR spectrum of the MCRS mixed with dimethyl fumarate.

The concentrations of the MCRS and dimethyl fumarate in CDCl3 were 20.50 and 2.96

mg·mL^{-1} , respectively.
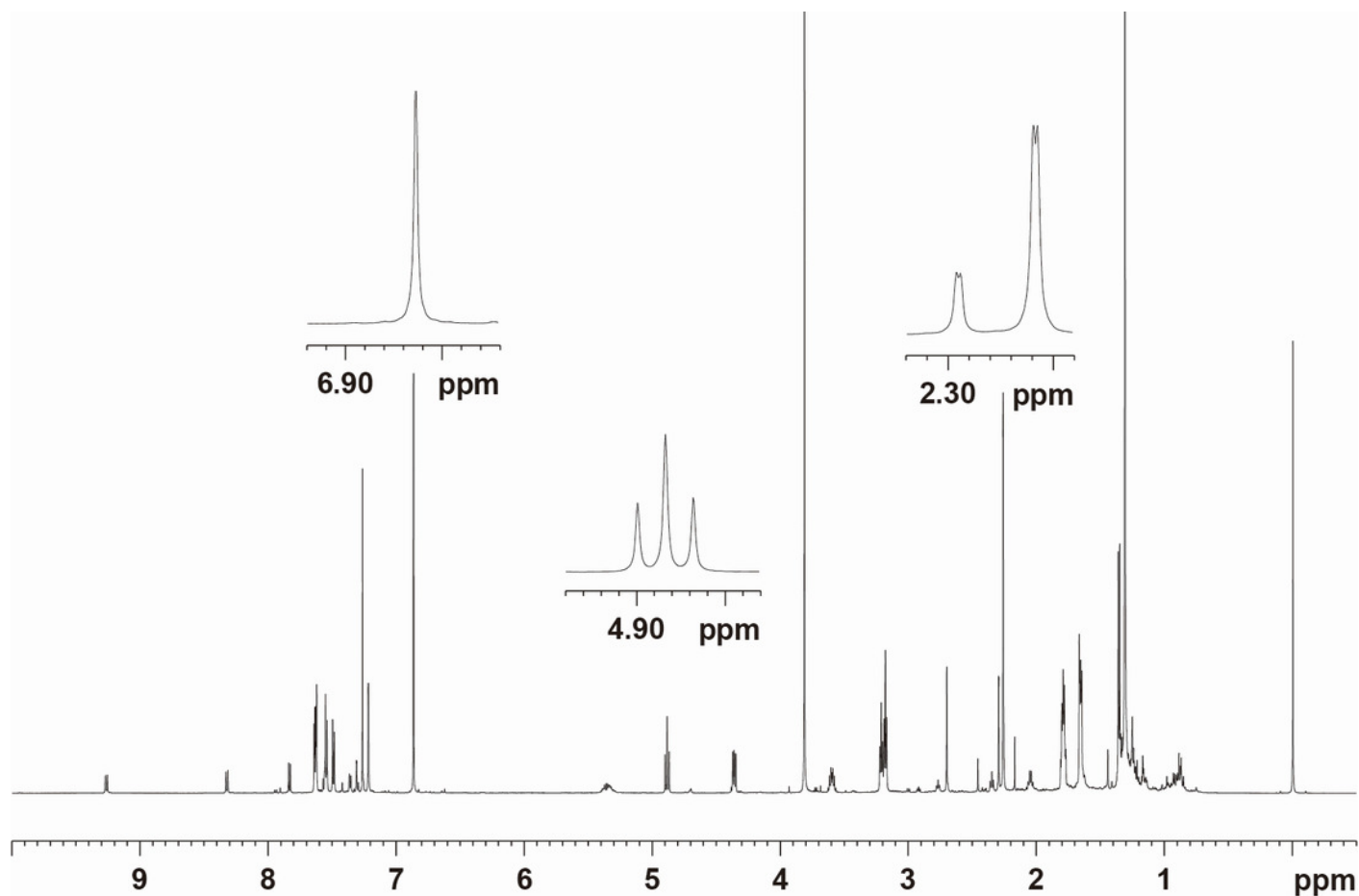
Figure 7

Figure 7 Representative results of the lineshape fitting of the signals belonging to the three tanshinones and dimethyl fumarate performed by the Lorentz deconvolution.

Images A-E reflect the representative lineshape fitting of signals belonging to cryptotanshinone. Images F-K (the right side) reflect the representative lineshape fitting of signals belonging to tanshinone I. The doublet in the left side of K, images L and M (the doublet in the right side) reflect the representative lineshape fitting of signals belonging to tanshinone IIA. Images N and O reflect the lineshape fitting of signals belonging to dimethyl fumarate.
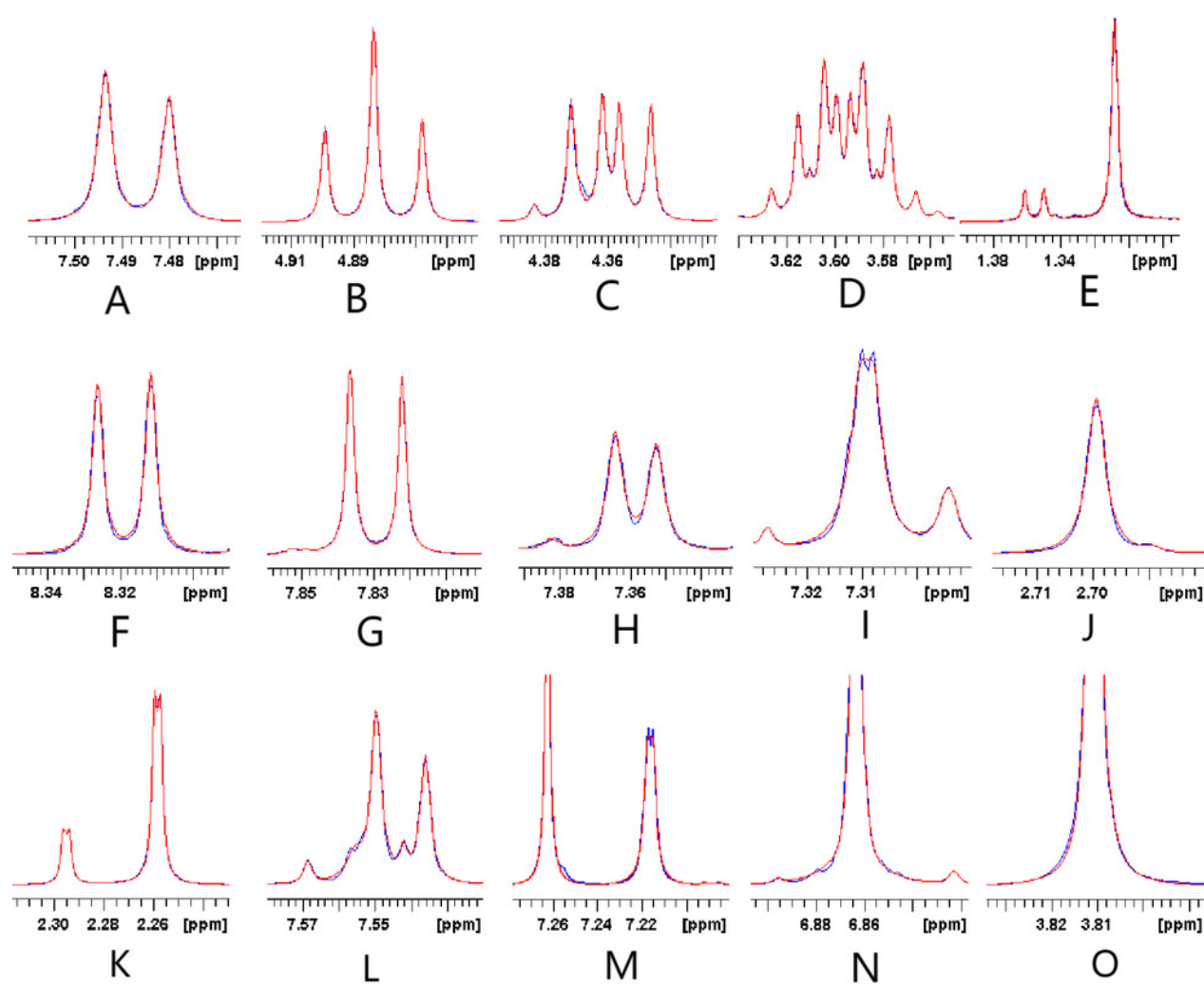
Figure 8

Figure 8 Concentrations of the three tanshinones determined by specific signals.
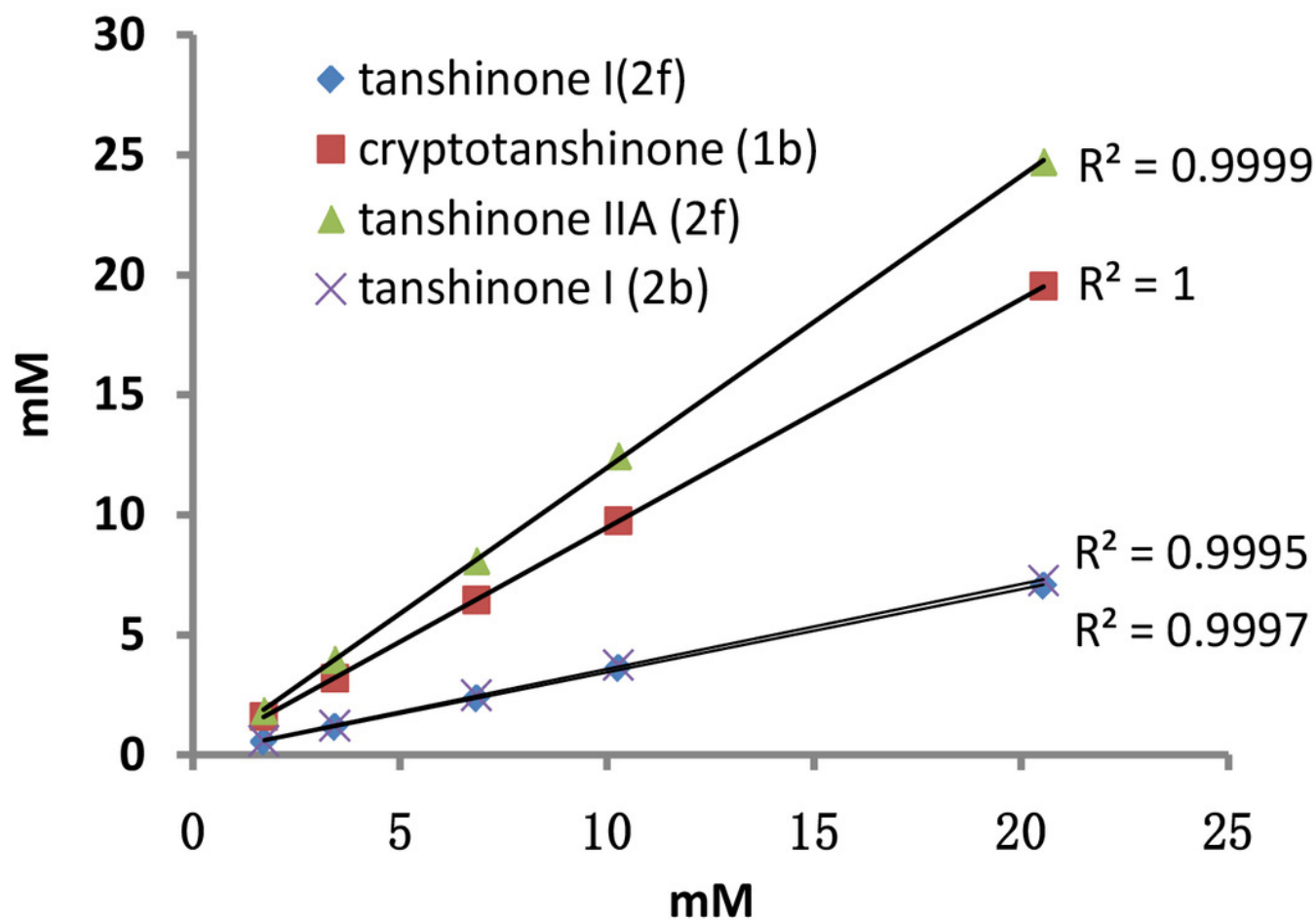
Figure 9

Deviations of the contents of the three tanshinones in the Danshen sample as determined by the MCRS contrast to individual reference standards.