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Identification and determination of the major constituents in Traditional Chinese Medicinal formula Danggui-Shaoyao-San by HPLC–DAD–ESI-MS/MS

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ABSTRACT

Danggui-Shaoyao-San (DSS), a famous traditional Chinese medicine formula consisting of six herbal medicines (*Paeonia lactiflora*, *Angelica sinensis*, *Ligusticum chuanxiong*, *Poria cocos*, *Atractylodis macrocephalae* and Rhizoma *Alismatis*), has been used as a classical gynecological remedy in China for centuries. However, its active substances have remained unknown. In this paper, an HPLC/DAD/ESI-MS/MS method was developed for the qualitative and quantitative analysis of the major constituents in DSS. The ESI-MS/MS fragmentation behavior of the reference compounds was proposed for aiding the structural identification of components in DSS extract. Forty-one compounds including monoterpene glycosides, phenolic acids, phathalides, sesquiterpenoids and triterpenes were identified or tentatively characterized by comparing their retention times, UV and MS spectra with those of authentic compounds or literature data, and 14 of them (gallic acid, albiflorin, paeoniflorin, ferulic acid, benzoic acid, senkyunolide I, coniferyl ferulate, senkyunolide A, 3-butylphthalide, Z-ligustilide, Z-butylidenephthalide, atractylcnolide II, atractylcnolide I and levistolide A) were determined by HPLC–DAD using a C_{18} column and gradient elution of acetonitrile/water–formic acid (100:0.1, v/v). The linearity, precision, accuracy, LOD and LOQ were validated for the quantification method, which proved sensitive, accurate and reproducible. The study might provide a basis for the quality control of DSS extracts and preparations.

1. Introduction

Traditional Chinese medicine (TCM) has been widely used in China due to its special efficacy in some instances in which the conventional Western therapies failed or proved to be insufficient to provide a palliative cure [1]. Commercially available TCM preparations are usually composed of several herbs with numerous constituents. Thus the analysis of such a complex mixture brings a great challenge to pharmaceutical analysts. Liquid chromatography coupled with DAD and electrospray ionization tandem mass spectrometry (LC/DAD/ESI/MS/MS) is a powerful analytical tool for the analysis of the known compounds and elucidation of unknown compounds in complex matrix, showing suitability for the analysis of TCM products [2,3].

Danggui-Shaoyao-San (DSS), comprising Radix *Paeoniae Alba*, Radix *Angelica sinensis*, Rhizoma *Chuanxiong*, *Poria cocos*, Rhizoma *Atractylodis macrocephalae* and Rhizoma *Alismatis*, is a widely used formula of TCM derived from "Jingui Yaolue", a medical classic written by Zhongjing Zhang in the Eastern Han Dynasty. This medicine has been used in China as a blood-activating and stasis-eliminating drug to treat gynecological disorders such as dysmenorrhea, amenorrhea and infertility for thousands of years. It has also been widely prescribed for the clinical practice in China and Japan [4,5]. Recent studies show that it also possesses the capability of treating neural dysfunctions such as senile dementia, memory loss, and other cognitive disorders, thus the formula is used as a remedy for Alzheimer's disease in Japan [6,7]. Although so many beneficial effects have been shown, the actual bioactive components of DSS are still unclear. Recently, some active ingredients related to pharmacological functions are gradually being revealed [8]. Among them, monoterpene glycosides, phenolic compounds and phthalides are the most representative components of DSS as far as both the contents and their biological activities are concerned. Monoterpene glycosides are responsible for the efficacy of R. *Paeoniae Alba*. A case in point is albiflorin and paeoniflorin, which exhibits analgesia, spasmolysis, anti-inflammation and anticoagulation activities [9–12]. Phenolic acids and phthalides in R. *Angelica sinensis* and R. *Chuanxiong* also have vasodilatative, antithrombotic, antioxidative, anti-inflammatory and muscle relaxant effects [13–18]. In addition, atractylenolides from R. *Atractylodis* showed gastrointestinal inhibitory, anti-inflammatory and antioxidative activity [23]. Meanwhile, cytotoxic, anti-inflammatory and antioxidant activity of triterpenes in R. *Alismatis* and *Poria cocos* have also been documented [24–26]. Current experimental evidences suggest a close relationship between these components and bioactive mechanism of DSS [19–22]. Although a number of studies on the

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quality evaluation of individual herb in DSS have been carried out using TLC [27], CE [28], HPLC–DAD [13,23,29,30], HPLC–ELSD [25], GC–MS [31] and LC–MS [30–33], the available methods of quality control for DSS products were still mainly based on the contents of one or two indicative compounds [34], while the simultaneous determination of multiple constituents in DSS has not been reported so far. For a complex formula, the comprehensive quality evaluation method should be based on the identification of its chemical components in a full spectrum. However, current studies on the constituents of the formula are inadequate to objectively assess the bioactive components of DSS. Therefore, it is necessary to develop a rapid and sensitive method to identify and quantify the compounds in DSS, which is beneficial to searching the bioactive substances and controlling the quality of the medicine. In our study, an LC/DAD/ESI/MS/MS method was developed to identify and quantify the major constituents of this formula for the first time. A total of 41 compounds in the formula were identified or tentatively characterized. In addition, quantification of 14 bioactive components was performed with HPLC–DAD and 9 commercial samples were analyzed, which is expected to provide comprehensive information for quality control of DSS preparations.

2. Experiment

2.1. Reagents and materials

HPLC grade acetonitrile was from Tedia (Fairfield, OH, USA); water for HPLC analysis was purified by a Milli-Q academic water purification system (Milford, MA, USA). Methanol and formic acid were of analytical grade (Jiangsu Hanbon Sci.&Tech. Co. Ltd., Jiangsu, China).

Crude drugs were purchased from Fengyuan Tongling crude drug company (Anhui, China) and were identified by Professor Boyang Yu. DSS products were purchased from six different brand manufacturers in China and Japan.

Gallic acid, protocatechuic acid, catechin, phthalic acid, vanilic acid, paeoniflorin, ferulic acid, benzoic acid and atractylenolide I, II, III were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Ligustilide was obtained from Tianjing Zhongxin Pharmaceutical Co. Ltd. (Tianjing, China). Albiflorin, lactiflorin and benzoylpaeoniflorin were isolated from *Paeonia lactiflora*. Senkyunolide I, coniferyl ferulate, senkyunolide A, 3-butylphthalide, Z-butylidenephthalide and levistolide A were isolated from essential oil of *Angelica sinensis*. Alisol C 23-acetate, alisol F, alisol B and alisol B 23-acetate were isolated from Rhizoma *Alismatis*, and pachymic acid was isolated from *Poria cocos* in the authors' laboratory. Their structures were elucidated by their spectral data (MS, ^{1}H NMR and ^{13}C NMR). The purity of each compound was determined to be higher than 98% by HPLC. Each reference compound was accurately weighed and dissolved in methanol as stock solutions.

2.2. Preparation of standard solutions

Stock solutions of the 14 standard substances for the determination were prepared in methanol at the concentration (mg/ml) of: gallic acid (0.40), albiflorin (1.60), paeoniflorin (2.30), ferulic acid (0.216), benzoic acid (0.104), senkyunolide I (0.32), coniferyl ferulate (1.12), senkyunolide A (1.85), butylphthalide (0.30), ligustilide (2.16), butylidenephthalide (0.15), atractylenolide II (0.021), atractylenolide I (0.036) and levistolide A (0.218). A series of working standard solutions with gradient concentration were obtained by diluting the mixed standard stock solution. All the solutions were stored in the refrigerator at 4 °C and brought to room temperature before use.

2.3. Preparation of sample solutions

One gram powder of the crude drugs compounded according to the formula in "Jin Gui Yao Lue" was extracted in 20 ml methanol–water (75:25, v/v) for 30 min in an ultrasonic water bath. The extraction was repeated twice. The extracted solutions were combined and concentrated nearly to dryness at 50 °C in vacuo. The evaporated residue was dissolved with methanol–water (75:25, v/v) into a 25 ml volumetric flask. The commercial preparations of DSS were uncoated (for capsule and tablet), powdered (for tablet), and extracted as described above. Since the contents of each analyte could vary considerably among different products, the extract solutions were diluted to appropriate concentrations to fit the validated calibration range for HPLC analysis. The extract was filtered through a 0.45 μm membrane and then 10 μl of the filtrate was analyzed by LC.

2.4. HPLC–DAD–ESI-MS system

The HPLC system consisted of an Agilent 1100 series HPLC with a Diode Array Detector. The column was an Alltima C_{18} (250 mm × 4.6 mm i.d., 5 μm, Alltech, USA) maintained at 30 °C. The eluents were acetonitrile (A) and water–formic acid (100:0.1, v/v) (B) at a flow rate of 1 ml/min. The following multi-step linear gradient was applied: 0–40 min, 5–30% A; 40–65 min linear increased to 55% A; 65–85 min linear increased to 100% A, and then maintained at that level for another 5 min. Total time of analysis was 90 min. The DAD spectra were recorded between 190 and 400 nm and the chromatographic profiles were recorded at 254 nm for qualitative analysis, while 231 and 275 nm for quantitative analysis.

The above HPLC system was interfaced with an Agilent 1100 LC/MSD Trap XCT ESI (Agilent Technologies, MA, USA). The same conditions were used during the HPLC–MS analysis. The ESI-MS spectra were acquired in both negative and positive ionization modes recorded on a mass range of m/z 100–800. Capillary voltage was 3500 V. Drying gas temperature was set at 350 °C with a flow rate of 9.0 l/min and nebulising pressure was of 40 psi. Data were processed by LC/MSD Trap Software 4.2 and Data Analysis 2.2.

2.5. Qualitative analysis of peaks

Identification of constituents in DSS extract was carried out by HPLC/DAD and LC/ESI/MS analysis. In order to obtain MS fragmentation patterns of constituents, standards and samples were analyzed by LC/ESI/MS/MS in both negative and positive ion modes. In the full scan mass spectra, most of the authentic compounds exhibited quasi-molecular ions $[M+H]^+$ in positive mode or $[M-H]^-$ in negative mode and more detailed structural information could be obtained via collision-induced dissociation (CID). The fragmentation patterns were proposed and they were helpful for the structural identification of constituents.

2.6. Validation of quantitative analysis

The prepared mixed standard stock solution containing 14 analytes was diluted to a series of appropriate concentrations for the construction of calibration curves. Six different concentrations of the mixed standard solution were injected in triplicate. The LODs and LOQs were determined at signal-to-noise ratios (S/N) of 3 and 10, respectively.

The precision of the method was determined for intra- and inter-day variations. The intra-day variability was performed in triplicate on the same sample extracted during a single day, while the inter-day precision was carried out in triplicate in another independent sample extracted on three different days. The ratios of observed concentration and nominal concentration of the mixed standard

Table 1
HPLC–DAD–ESI-MS identification of the constituents in DSS extract.

Peak no.	T_R (min)	Positive ions (m/z)	Negative ions (m/z)	λ_{max} (nm)	Identification	Crude drug
1*	7.6	–	169[M−H]$^-$, 125[M-H-CO_2]$^-$, 97[M-H-CO_2-CO]$^-$	214, 270	Gallic acid	1
2	9.9	–	–	283	Unidentified	1
3*	12.6	–	153[M−H]$^-$, 109[M-H-CO_2]$^-$	310	Protocatechuic acid	3
4	13.4	725, 591, 563, 545, 383, 325, 261, 197	705, 659, 583, 543, 421, 381, 259	231, 274	Unidentified	1
5	14.2	545[M+H]$^+$, 401[M+H+H_2O-Glc]$^+$, 383[M+H-Glc]$^+$, 285[BA+Glc+H]$^+$, 261[M+H-BA-Glc]$^+$, 197[M+H-BA-Glc-SO_2]$^+$	543[M−H]$^-$, 497[M-H-HCOOH]$^-$, 421[M-H-BA]$^-$, 375[M-H-HCOOH-BA]$^-$ 259[M-H-BA-Glc]$^-$, 215[M-H-CO_2-BA-Glc]$^-$, 177[aglycone-H-H_2O]$^-$	232, 274	Paeoniflorin sulfonate	1
6	16.1	–	495[M−H]$^-$, 451[M-H-CO_2]$^-$, 357[M-H-*p*OHBA]$^-$, 333[M-H-Glc]$^-$, 281[Glc+*p*OHBA-H-H_2O]$^-$, 195[aglycone-H]$^-$, 167[aglycone-H-CO]$^-$, 137[*p*OHBA-H]$^-$	258	Oxypaeoniflorin	1
7*	18.4	–	289[M−H]$^-$, 271[M-H-H_2O]$^-$, 245[M-H-C_2H_4O]$^-$, 227[M-H-C_2H_4O-H_2O]$^-$	278	(+)-Catechin	1
8*	19.3	–	–	277	Phthalic acid	2
9*	20.1	–	167[M−H]$^-$, 137[M-H-HCHO]$^-$, 123[M-H-CO_2]$^-$	260, 292	Vanillic acid	2, 3
10	22.4	661[M+H+H_2O]$^+$, 643[M+H]$^+$, 625[M+H-H_2O]$^+$, 539[M+H+H_2O-BA]$^+$, 485[M+H-2H_2O-BA]$^+$, 463[M+H-H_2O-Glc]$^+$, 341[M+H-H_2O-Glc-BA]$^+$, 325[2Glc+H]$^+$, 301[M+H-H_2O-2Glc]$^+$	687[M+HCOO]$^-$, 611[M-H-HCHO]$^-$, 623[M-H-H_2O]$^-$, 593[M-H-H_2O-HCHO]$^-$, 519[M-H-BA]$^-$, 489[M-H-HCHO-BA]$^-$, 445[M-H-aglycone]$^-$, 323[2Glc-H]$^-$, 283[BA+Glc-H]$^-$, 269[M-H-H_2O-HCHO-2Glc]$^-$	232, 274	Isomaltopaeoniflorin	1
11*	22.8	481[M+H]$^+$, 359[M+H-BA]$^+$, 319[M+H-Glc]$^+$, 197[M+H-BA-Glc]$^+$, 161[M+H-BA-Glc-2H_2O]$^+$, 133[M+H-BA-Glc-2H_2O-CO]$^+$,	525[M+HCOO]$^-$, 479[M−H]$^-$, 435[M-H-CO_2]$^-$, 357[M-H-BA]$^-$, 327[M-H-HCHO-BA]$^-$, 283[BA+Glc-H]$^-$, 195[aglycone-H]$^-$	231, 273	Albiflorin	1
12	23.6	–	695, 649, 573, 525, 463, 395, 313	222, 274	Unidentified	1
13*	24.7	503[M+Na]$^+$, 463[M+H-H_2O]$^+$, 319[M+H-Glc]$^+$, 301[M+H-Glc-H_2O]$^+$, 179[M+H-Glc-BA-H_2O]$^+$, 161[M+H-Glc-BA-2H_2O]$^+$	525[M+HCOO]$^-$, 479[M−H]$^-$, 449[M-H-HCOH]$^-$, 327[M-H-HCOH-BA]$^-$, 121[BA-H]$^-$	232, 274	Paeoniflorin	1
14	27.9	–	787[M−H]$^-$, 635[M-H+H_2O-GA]$^-$, 617[M-H-GA]$^-$, 573[M-H-GA-CO_2]$^-$, 465[M-H+H_2O-2GA]$^-$, 447[M-H-2GA]$^-$, 313[M-H+2H_2O-3GA]$^-$, 295[M-H+H_2O-3GA]$^-$, 169[GA-H]$^-$, 125[GA-H-CO_2]$^-$	220, 270	Tetragalloyglucose	1
15*	30.5	195[M+H]$^+$, 177[M+H-H_2O]$^+$, 145[M+H-H_2O-CH_3OH]$^+$, 117[M+H-H_2O-CO-CH_3OH]$^+$	–	295, 322	Ferulic acid	2, 3
16	32.3	–	939[M−H]$^-$, 769[M-H-GA]$^-$, 617[M-H+H_2O-2GA]$^-$, 447[M-H+H2O-3GA]–, 169[GA]$^-$, 125[GA-CO_2]$^-$	220, 280	Pentagalloylglucose	1
17	33.9	633[M+H]$^+$, 471[M+H-Glc]$^+$, 349[M+H-Glc-BA]$^+$, 301[M+H-Glc-GA]$^+$, 197[aglycone+H]$^+$, 153[GA+H-H_2O]$^+$	631[M−H]$^-$, 613[M-H-H_2O]$^-$, 509[M-H-BA]$^-$, 491[M-H-BA-H_2O]$^-$, 479[M-H-gallic carbonyl]$^-$ 313[GA+Glc-H-H_2O]$^-$, 169[GA-H]$^-$, 125[GA-H-CO_2]$^-$	220, 279	Galloylpaeoniflorin	1
18*	34.4	–	–	238, 272	Benzoic acid	1
19	35.8	481[M+H]$^+$, 435[M+H-HCOOH]$^+$, 359[M+H-BA]$^+$, 319[M+H-Glc]$^+$, 197[aglycone+H]$^+$, 179[aglycone+H-H_2O]$^+$, 161[aglycone+H-2H_2O]$^+$, 133[aglycone+H-2H_2O-CO]$^+$	525[M+HCOO]$^-$, 479[M−H]$^-$, 357[M-H-BA]$^-$, 283[BA+Glc-H]$^-$, 195[aglycone-H]$^-$	228	Mudanpioside I	1
20*	37.5	247[M+Na]$^+$, 225[M+H]$^+$, 207[M+H-H_2O]$^+$, 189[M+H-2H_2O]$^+$, 179[M+H-H_2O-CO]$^+$, 165[M+H-H_2O-C_3H_6]$^+$	–	270	Senkyunolide I	2, 3
21*	37.9	485[M+Na]$^+$, 481[M+H+H_2O]$^+$, 463[M+H]$^+$, 341[M+H-BA]$^+$, 301[M+H-Glc]$^+$, 273[M+H-Glc-CO]$^+$, 179[M+H-BA-Glc]$^+$, 151[M+H-BA-Glc-CO]$^+$	507[M+HCOO]$^-$, 461[M−H]$^-$, 443[M-H-H_2O]$^-$, 431[M-H-HCOH]$^-$, 371[M-H-HCOH-HOAc]$^-$, 339[M-H-BA]$^-$, 308[M-H-BA-CH_3O]$^-$, 299[M-H-Glc]$^-$, 283[M-H-$C_6H_{10}O_6$]$^-$, 195[$C_6H_9O_6$+H_2O]$^-$, 177[$C_6H_9O_6$]$^-$, 159[$C_6H_9O_6$-H_2O]$^-$	234	Lactiflorin	1
22	39.3	–	–	275	Unidentified	2, 3
23*	49.6	585[M+H]$^+$, 463[M+H-BA]$^+$, 319[M+H-BG]$^+$, 267[M+H-aglycone-BA]$^+$, 197[M+H-BA-BG]$^+$, 165[aglycone+H-CH_3OH]$^+$, 105[benzoyl]$^+$	583[M−H]$^-$, 553[M-H-HCHO]$^-$, 461[M-H-BA]$^-$, 387[M-H-aglycone]$^-$	238	Benzoylpaeoniflorin	1

Table 1 (*Continued*)

Peak no.	T_R (min)	Positive ions (m/z)	Negative ions (m/z)	λ_{max} (nm)	Identification	Crude drug
24	54.5	505$[M+H]^+$, 487$[M+H-H_2O]^+$, 469$[M+H-2H_2O]^+$, 415$[M+H-H_2O-C_4H_8O]^+$, 397$[M+H-2H_2O-C_4H_8O]^+$	–	244	16-Oxoalisol A	6
25	56.8	–	–	246	Unidentified	2, 3
26*	63.1	–	193$[M-H-C_{10}H_{10}O_2]^-$, 178$[M-H-C_{10}H_{10}O_2-CH_3]^-$, 149$[M-H-C_{10}H_{10}O_2-CO_2]^-$, 134$[M-H-C_{10}H_{10}O_2-CH_3-CO_2]^-$	268, 317	Coniferyl ferulate	2, 3
27	64.4	487$[M+H]^+$, 469$[M+H-H_2O]^+$, 451$[M+H-2H_2O]^+$, 397$[M+H-H_2O-C_4H_8O]^+$, 353$[M+H-H_2O-C_4H_8O-CO_2]^+$	–	–	Alisol C	6
28*	64.5	489$[M+H]^+$, 471$[M+H-H_2O]^+$, 453$[M+H-2H_2O]^+$, 399$[M+H-H_2O-C_4H_8O]^+$	–	–	Alisol F	6
29*	65.2	249$[M+H]^+$, 231$[M+H-H_2O]^+$, 213$[M+H-2H_2O]^+$, 203$[M+H-H_2O-CO]^+$, 175$[M+H-H_2O-2CO]^+$, 163$[M+H-H_2O-C_5H_8]^+$, 135$[M+H-H_2O-C_5H_8-CO]^+$, 117$[M+H-H_2O-C_5H_8-HCOOH]^+$, 107$[M+H-H_2O-C_5H_8-2CO]^+$, 79$[M+H-H_2O-C_5H_8-2CO-C_2H_4]^+$	–	235	Atractylenolide III	4
30*	66.1	193$[M+H]^+$, 175$[M+H-H_2O]^+$, 147$[M+H-H_2O-CO]^+$	–	280	Senkyunolide A	2, 3
31*	67.6	191$[M+H]^+$, 173$[M+H-H_2O]^+$, 145$[M+H-H_2O-CO]^+$	–	230, 276	3-Butylphthalide	2, 3
32	69.8	471$[M+H]^+$, 453$[M+H-H_2O]^+$, 443$[M+H-CO]^+$, 435$[M+H-2H_2O]^+$, 395$[M+H-H_2O-CO-HCHO]^+$, 381$[M+H-H_2O-C_4H_8O]^+$,	–	–	11-Deoxyalisol C	6
33	70.2	191$[M+H]^+$, 173$[M+H-H_2O]^+$, 155$[M+H-2H_2O]^+$	–	280, 326	E-Ligustilide	3
34*	70.8	529$[M+H]^+$, 511$[M+H-H_2O]^+$, 469$[M+H-HOAc]^+$, 451$[M+H-H_2O-HOAc]^+$, 433$[M+H-2H_2O-HOAc]^+$	–	247	Alisol C 23-acetate	6
35	71.3	195$[M+H]^+$, 177$[M+H-H_2O]^+$, 149$[M+H-H_2O-CO]^+$, 125$[M+H-C_5H_{10}]^+$, 121$[M+H-H_2O-CO-C_2H_4]^+$ 107$[M+H-H_2O-CO-C_3H_6]^+$, 93$[M+H-H_2O-CO-C_4H_8]^+$	–	–	Cnidilide	3
36*	72.1	191$[M+H]^+$, 173$[M+H-H_2O]^+$, 163$[M+H-CO]^+$, 155$[M+H-2H_2O]^+$, 149$[M+H-C_3H_6]^+$, 145$[M+H-H_2O-CO]^+$, 117$[M+H-H_2O-CO-C_2H_4]^+$, 105$[M+H-H_2O-CO-C_3H_4]^+$, 91$[M+H-H_2O-CO-C_4H_6]^+$	–	281, 328	Z-Ligustilide	2, 3
37*	72.2	473$[M+H]^+$, 455$[M+H-H_2O]^+$, 383$[M+H-H_2O-C_4H_8O]^+$, 365$[M+H-2H_2O-C_4H_8O]^+$	–	–	Alisol B	6
38*	72.7	189$[M+H]^+$, 171$[M+H-H_2O]^+$, 153$[M+H-2H_2O]^+$, 128$[M+H-H_2O-CO-CH_3]^+$, 115$[M+H-H_2O-CO-C_2H_4]^+$	–	262, 313	Z-Butylidenephthalide	2, 3
39*	73.8	233$[M+H]^+$, 215$[M+H-H_2O]^+$, 205$[M+H-CO]^+$, 187$[M+H-H_2O-CO]^+$, 177$[M+H-C_4H_8]^+$, 151$[M+H-C_4H_8-C_2H_2]^+$	–	220	Atractylenolide II	4
40*	78.0	231$[M+H]^+$, 203$[M+H-CO]^+$, 185$[M+H-H_2O-CO]^+$, 175$[M+H-2CO]^+$, 143$[M+H-H_2O-CO-C_3H_6]^+$	–	276	Atractylenolide I	4
41	80.0	381$[M+H]^+$, 191$[C_{12}H_{15}O_2]^+$, 173$[C_{12}H_{15}O_2-H_2O]^+$	–	280	Riligustilide	2, 3
42	80.3	381$[M+H]^+$, 191$[C_{12}H_{15}O_2]^+$, 173$[C_{12}H_{15}O_2-H_2O]^+$	–	281	Tokinolide B	2, 3
43*	80.7	381$[M+H]^+$, 191$[C_{12}H_{15}O_2]^+$, 173$[C_{12}H_{15}O_2-H_2O]^+$, 163$[C_{12}H_{15}O_2-CO]^+$, 155$[C_{12}H_{15}O_2-2H_2O]^+$, 145$[C_{12}H_{15}O_2-CO-H_2O]^+$, 135$[C_{12}H_{15}O_2-CO-C_2H_4]^+$	–	276	Levistolide A	2, 3
44	81.1.	383$[M+H]^+$, 365$[M+H-H_2O]^+$ 355$[M+H-CO]^+$, 191$[C_{12}H_{15}O_2]^+$	–	226, 282	Senkyunolide P	2, 3
45*	84.0	515$[M+H]^+$, 497$[M+H-H_2O]^+$, 479$[M+H-2H_2O]^+$, 455$[M+H-HOAc]^+$, 437$[M+H-H_2O-HOAc]^+$, 419$[M+H-2H_2O-HOAc]^+$, 381$[M+H-H_2O-HOAc-C_4H_8]^+$, 365$[M+H-H_2O-HOAc-C_4H_8O]^+$, 339$[M+H-H_2O-HOAc-C_4H_8O-C_2H_2]^+$		–	Alisol B 23-acetate	6

46*	84.6	–	563[M+Cl]$^-$, 527[M−H]$^-$, 481[M-H-HCOOH]$^-$, 467[M-H-HOAc]$^-$, 465[M-H-H_2O-CO_2]$^-$, 449[M-H-HOAc-H_2O]$^-$, 397[M-H-HOAc-C_5H_{10}]$^-$, 355[M-H-H_2O-$C_9H_{14}O_2$]$^-$, 313[M-H-HOAc-$C_9H_{14}O_2$]$^-$, 287[M-H-HOAc-$C_9H_{14}O_2$-C_2H_2]$^-$	242	Pachymic acid	5

1. Radix *Paeoniae Alba*, 2. Radix *Angelica sinensis*, 3. Rhizoma *Chuanxiong*, 4. Rhizoma *Atractylodis macrocephalae*, 5. *Poria cocos*, 6. Rhizoma *Alismatis*. BA: Benzoic acid; GA: gallic acid; Glc: glucosyl group; BG: glucosyl group with benzoate group on C-6.

* Compared with authentic compounds.

solutions were calculated as accuracy. The accuracy of this method was further evaluated by recovery test. The analogs were added at three concentration levels (approximately equivalent to 0.8, 1.0 and 1.2 times of the amount of the matrix) with two parallels at each level and then extracted and analyzed as described in Section 2.3.

3. Results and discussion

3.1. Optimization of HPLC–DAD–ESI-MS conditions

The great structural diversity of compounds in the formula makes it difficult to give good responses to all chemical components in MS analysis. Monoterpene glucosides could be analyzed in both positive and negative ionization. However, most organic acids were detected only in negative ion mode, and the signals of phthalides, lactones and triterpenes were obvious in the positive ion mode. Therefore, both positive and negative ion modes had to be employed to identify the corresponding signals. In addition, trace amount of formic acid was also added in the mobile phase to improve the ionization responses for some compounds.

The quantification of constituents in DSS was achieved at 231 and 275 nm, where the UV spectra of the 14 analytes exhibited maximum absorbance, in which better response and less interference could be accomplished. High-gradient slope and aqueous formic acid in the mobile phase were applied to acquire good resolution within reasonable time as well. Compounds 1, 11, 13 and 15 showed better peak forms when 0.5% formic acid was included, while 0.1% formic acid was utilized to ameliorate the baseline drift at 231 nm. 50%, 75% and 100% ethanol and methanol were tested as extraction solvents, and the extraction time was also investigated. One gram DSS powder was extracted three times with 20 ml of each solvent system under ultrasonic for 15, 30 and 60 min, respectively. The results showed that all the 14 components were almost completely extracted by ultrasonication with 75% methanol three times for 30 min each.

3.2. Identification of constituents in DSS by HPLC–DAD–ESI-MS

The authentic compounds could be classified into five groups according to their chemical structures and their dominant fragmentation pathways were studied. Most of the authentic compounds exhibit $[M-H]^-$ and/or $[M+H]^+$ ions of sufficient abundance that could be subjected to MS^n analysis. MS/MS and MS^n data were obtained by collision-induced dissociation (CID), and utilized for the structural identification of compounds with similar fragmentation patterns. Twenty-six peaks in the HPLC–DAD and HPLC–MS (TIC) chromatograms were unequivocally identified by comparisons of their retention times, MS data and UV spectra with those of authentic compounds. The other 15 peaks were identified tentatively by comparing their UV spectrum, molecular weight and structural information from MS^n spectra with reference data from literature. Table 1 listed the retention time (RT), UV λ_{max}, MS data and the most characteristic fragments of the reference compounds and identified peaks. Their chemical structures are shown in Fig. 1. The UV chromatograms at 254 nm and MS TIC chromatograms of DSS extract were presented in Fig. 2a–c, respectively.

For most of the constituents in the MS, ions of $[M+H]^+$, $[M+Na]^+$, $[M-H]^-$, $[M+Cl]^-$, and $[M+HCOO]^-$ were observed. Peaks 5, 6, 10, 11, 13, 17, 19, 21 and 23 were identified as monoterpene glucosides in *Paeoniae alba*, which showed similar fragmentation patterns such as losses of a benzoic acid (122 Da), a glucosyl group (162 Da) and their combined loss (284 Da). The aglycone ions at *m*/*z* 195 or 197 could be observed occasionally and their fragmentations of losing H_2O and CO were also detected in some cases such as compounds 5, 6, 13, 19 and 23 [35–38]. Additionally, fragment ions of the compounds

with substitutive groups and their corresponding losses were often observed in MS^2 or MS^3 spectra, which provided very useful information for structural identification. Peak 5 yielded $[M+H]^+$ and $[M-H]^-$ at m/z 545 and 543, respectively. Besides the characteristic UV spectrum and MS fragmentations of paeoniflorin derivates, loss of SO_2 was also observed in positive ion mode. Compared with literature data, it was identified as paeoniflorin sulfonate, an artifact generated in the processing of white peony root by sulfur dioxide [32]. Peak 4 exhibited a molecular mass of 706, the similar fragmentations with the ions of 162 Da larger than that of compound 5, and the fragments of losing 2 glucosyl ($706 \rightarrow 503 \rightarrow 381$), indicating a product of a glucosyl linked to paeoniflorin sulfonate, which was not found previously. Another analog of peak 10 gave the $[M+H]^+$ at m/z 643 and fragment pattern resembled paeoniflorin with the

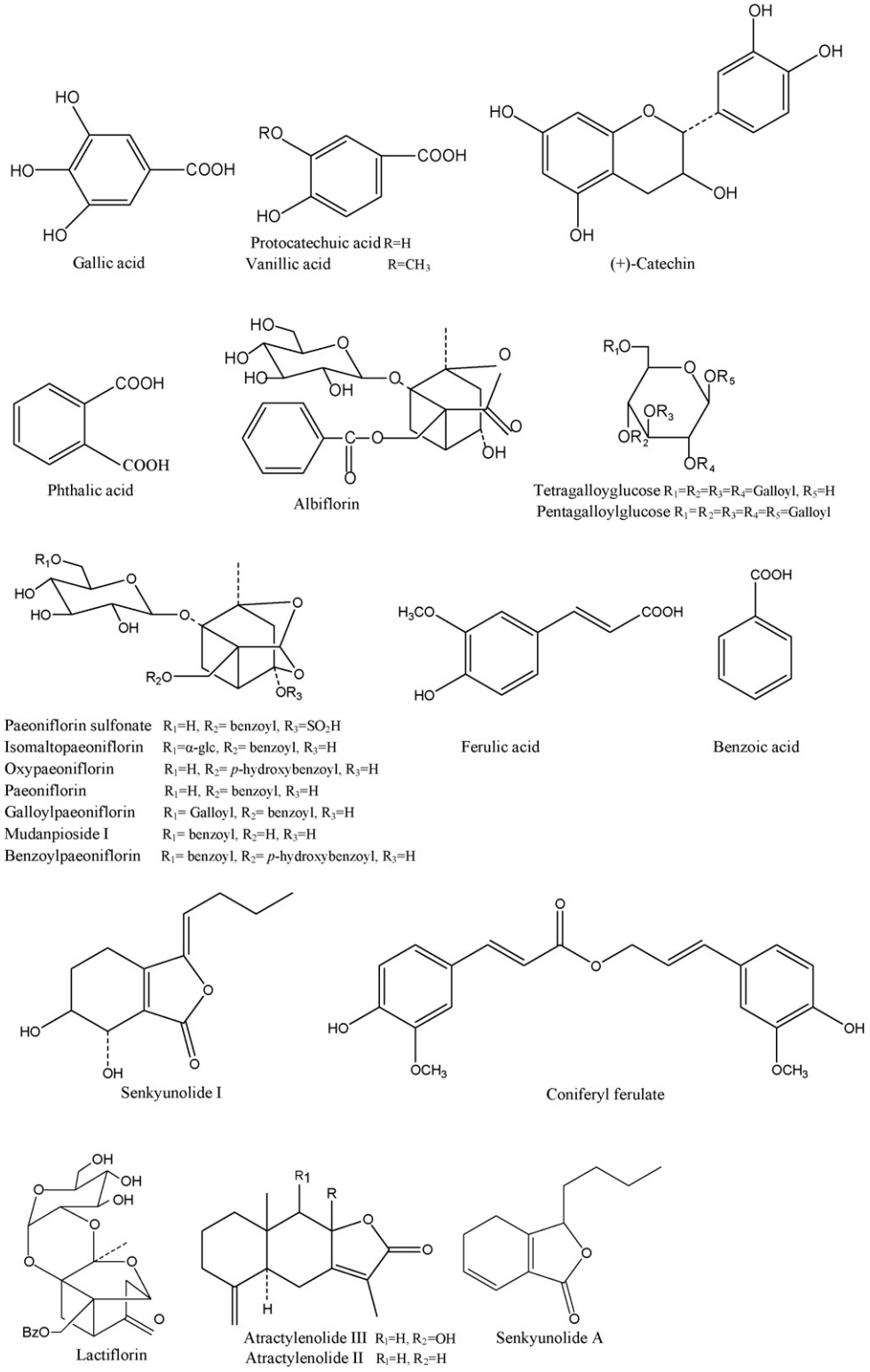

Fig. 1. Chemical structures of compounds identified in DSS extract.

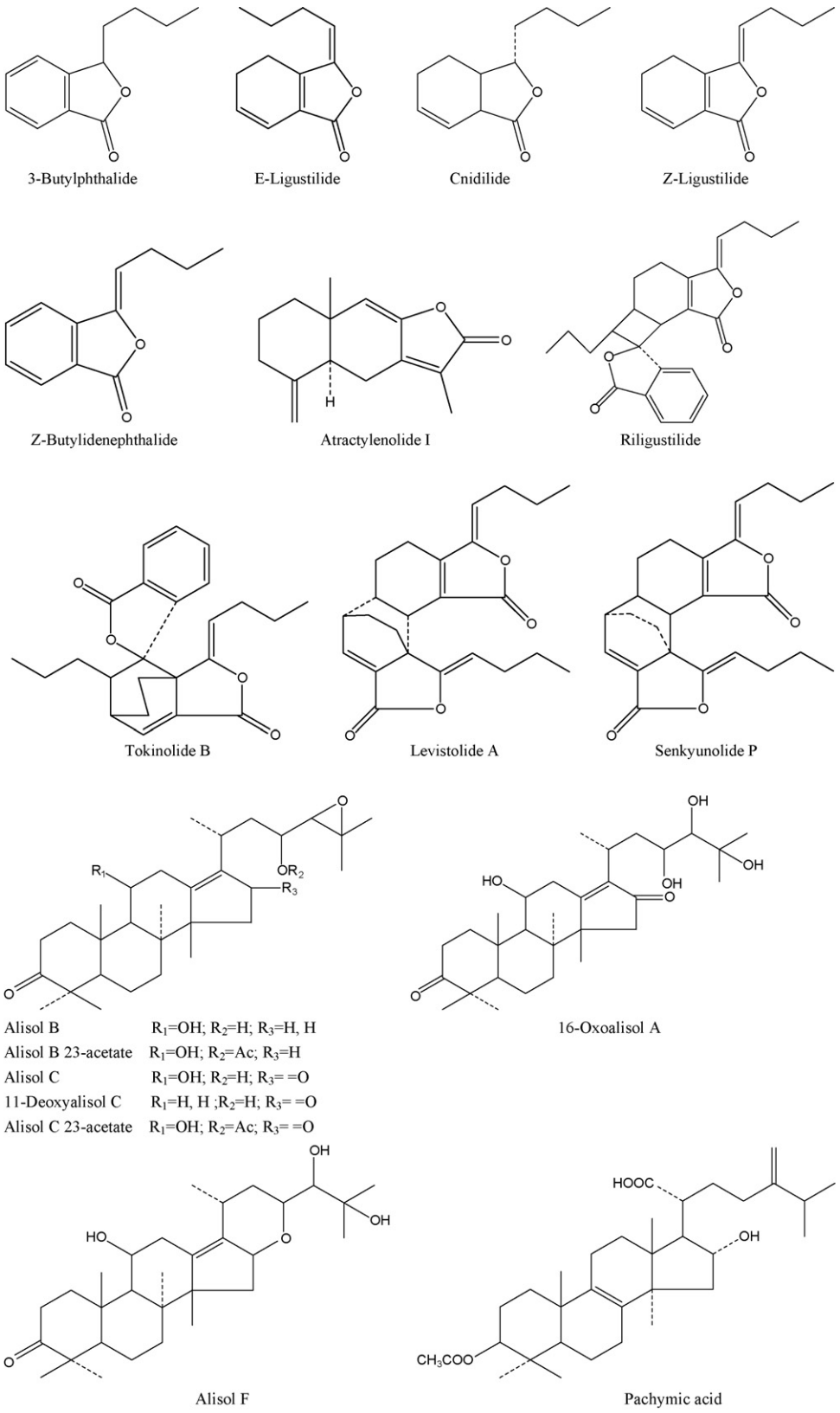

Fig. 1. (*Continued*).

appearance of $[2Glc+H]^+$ at m/z 325 and $[M+H-H_2O-2Glc]^+$ at m/z 301, showing a glucose linked to paeoniflorin, which is in agreement with the structure of isomaltopaeoniflorin [39]. Peaks 17 and 23 showed typical fragmentations of substituted paeoniflorin, as well as the diagnostic ions produced by substituent groups [2]. For instance, peak 17 afforded the fragment ions at m/z 479 by losing gallic carbonyl from the $[M-H]^-$ ion, together with the ions at m/z 313, 169 and 125, which could be easily identified as galloylpaeoniflorin [2,36]. Peaks 14 and 16 displayed intense UV absorptions in 270 nm and successive losses of 170 Da, with the presence of the

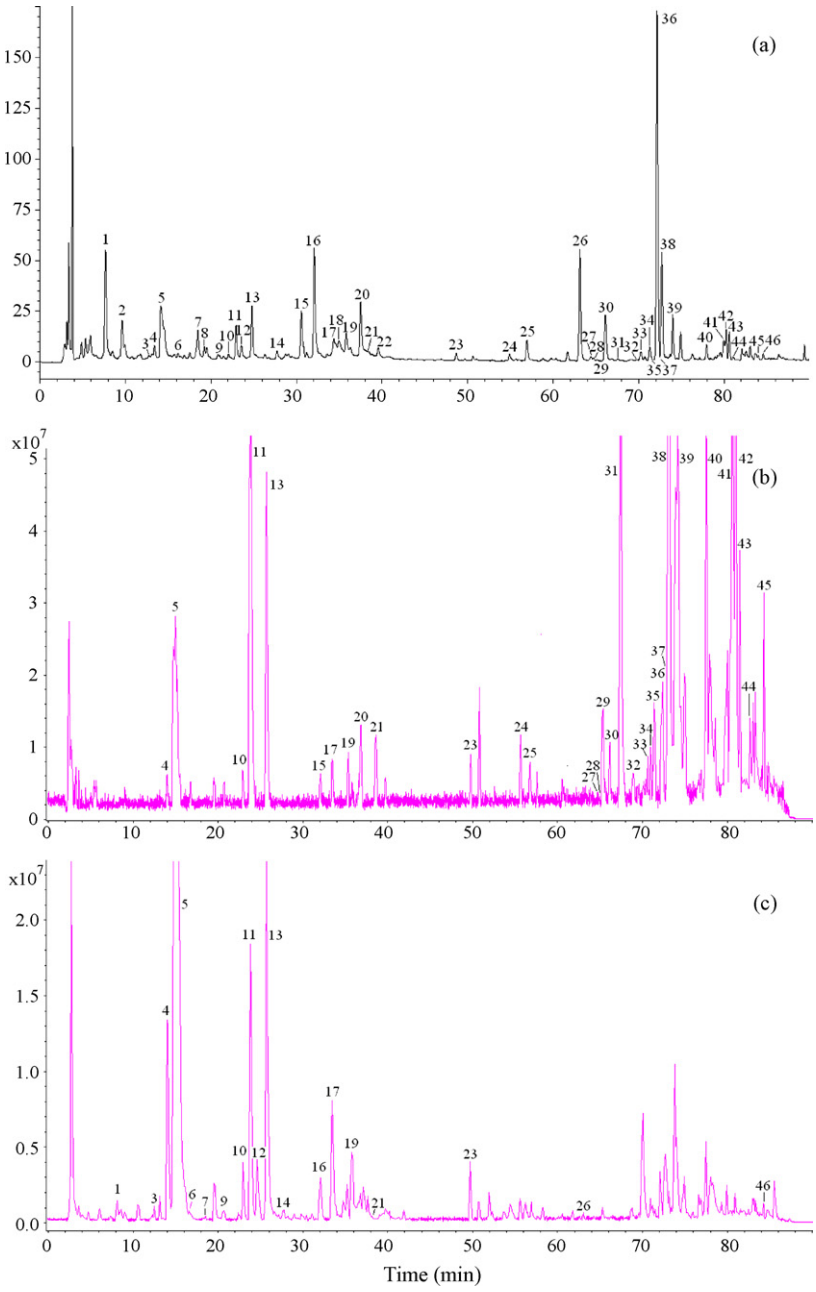

Fig. 2. HPLC–DAD–ESI-MS chromatograms of DSS extract. (a) HPLC–DAD chromatogram at 254 nm. (b) TIC chromatogram in positive ion mode. (c) TIC chromatogram in negative ion mode.

ions at m/z 125 $[GA\text{-}CO_2]^-$ and 161 $[Glc\text{-}H]^-$, suggesting that they might be glucoses attached by several GA units in their structures. Actually, they were regarded respectively as tetragalloylglucose and pentagalloylglucose, two components in *Paeoniae alba* previously reported in literature [36].

The characteristic ions of organic acids including gallic acid, protocatechuic acid, phthalic acid, vanillic acid, ferulic acid and benzoic acid were mainly formed by the losses of CO_2, CO, HCHO, CH_3OH and their combinations. Peak 26 was an ester between ferulic acid and ciniferol with a dominant fragment ion of m/z 193, which could be attributed to the loss of coniferyl [40]. It should be mentioned that organic acids and their esters showed low responses under this MS condition, especially phthalic acid and benzoic acid, which could only be identified according to the retention time and UV characteristics in comparison with those of their standards. On the contrary, intense quasi-molecule ions $[M+H]^+$ were found for phthalides, whose fragments were usually generated by the losses of H_2O (18 Da), CO (28 Da) and side chains such as CH_4 (16 Da), C_2H_4 (28 Da) and C_3H_6 (42 Da), etc. [41,42]. Moreover, protonated dimeric ions $[2M+H]^+$ were also observed for phthalide monomers. Peaks 33 and 36 had the same UV and MS behaviors, indicating that they should be typical phthalide isomers. After the structure of peak 36 was identified as Z-ligustilide compared with authentic standard, peak 33 was assigned as E-ligustilide, the *cis*-isomer of Z-ligustilide [41,42]. Peaks 41–43 exhibited similar mass spectra with the protonated molecular ions $[M+H]^+$ at m/z 381 as well as obvious fragment ions at m/z 191, indicating that they were dimeric phthalide with the same molecular mass of 380 Da. With authentic standard, peak 43 was identified as levistolide A, and the other two were tentatively assigned to be riligustilide and tokinolide B by the comparison of their UV λ_{max} with reported data [43,44]. Three lactones in R. *Atractylodis macrocephalae* were elucidated as

atractylenolide I (peak 40), II (peak 39) and III (peak 29), respectively, based on comparisons with standard compounds. Similar to phthalides, the basic fragment ions of these compounds were $[M+H-H_2O]^+$, $[M+H-CO]^+$ and $[M+H-H_2O-CO]^+$, with other characteristic ions produced by the cleavage of lipid rings.

Compounds 24, 27, 28, 32, 34, 37, and 45 were all protostane-type triterpenoids from R. *Alismatis*. Their common fragmentation pattern was the loss of H_2O followed by a cleavage of the side chain C_4H_8O (72 Da). Meanwhile, loss of CH_3COOH (60 Da) might occur in acetylated alisols. Their structures were determined by reference substances while compounds 24, 27 and 32 were deduced by literature data [33]. A triterpenoid in *Poria cocos* named pachymic acid (peak 46) was detected with $[M+Cl]^-$ and $[M-H]^-$ ions at *m*/*z* 563 and 527, respectively. The MS spectrum with negative mode for this compound also afforded diagnostic ions at *m*/*z* 481, 467, 465, 355 and 313, in which the product ions at *m*/*z* 355 and 313 were originated by losing the side chains, which was compatible with the typical fragmentation of lanostane triterpene [45]. However, some peaks, such as peaks 2, 22 and 25, showed good UV responses but no signals in MS spectrum.

3.3. Quantitative determination of constituents in DSS by HPLC–DAD

Fourteen peaks in chromatogram of DSS with reasonable heights and good resolution were chosen as mark substances, including gallic acid (GA), albiflorin (AF), paeoniflorin (PF), ferulic acid (FA), benzoic acid (BA), senkyunolide I (SI), coniferyl ferulate (CF), senkyunolide A (SA), 3-butylphthalide (BP), Z-ligustilide (LL), Z-butylidenephthalide (BE), atractylcnolide II (AII), atractylcnolide I (AI) and levistolide A (LA). They were generally considered as active components except benzoic acid, which was regarded as a toxic component. Additionally, some characteristic compounds in the herbs, such as alisol B 23-acetate, atractylcnolide III and pachymic acid were not determined in the preparations for their poor ultraviolet absorption or extremely low content. Fig. 3 displayed representative HPLC profiles of DSS and standard substances detected at 231 and 275 nm. Owing to the great polarity difference of these components, gradient elution and the long HPLC run time were applied for the complete separation of the marker components.

As shown in Table 2, the HPLC–DAD method provided a good reproducibility for the quantification of the analytes, with intra- and inter-day precision of less than 1.64% and 2.27%, respectively. The intra- and inter-day accuracy was in the range of 95.32–104.30%. The LODs and the LOQs for the analytes were less than 215 and 684 ng/ml, respectively. The overall recoveries ranged from 96.8% to 103.3%, with the R.S.D. ranging from 1.63% to 4.78%. These results demonstrated that the quantitative method was precise, accurate, and sensitive for the determination of the 14 components in DSS samples.

3.4. Application

The established method of quantification has been applied to the analysis of 9 kinds of commercial DSS products including powder, granula, tablets and capsules from 6 different manufacturers. As listed in Table 3, the 14 constituents were comprised in most preparations and their total amounts varied from 8.28 to 32.43 mg/g. Among these compounds, PF was found to be an abundant and essential component, which showed the highest amount in most samples with the relative contents ranged from 16.11–56.54%. GA, AF, SA and LL were also quite common in the drug products. In addition, products from the same manufacturer have a definite ratio of components for using the same intermediate. For instance, the contents of 14 analytes in sample 3 (intermediate) were about twice as much as in sample 4, while they were similar in samples 6 and 7.

According to Table 3, the contents of the 14 constituents varied remarkably from different dosage forms and manufacturers. For example, samples 1 and 2 were DSS powder produced in China and Taiwan, respectively, while their contents of components differed remarkably because both the sources of crude drugs and formula were different in the two regions. Even in the products of same dosage form from one region such as samples 5 and 6, their contents could be various for the discrepancy in raw materials and processing procedures. The extraction process was another key factor affecting the contents of ingredients. Sample 9 showed high amount of most ingredients, indicating that the procedure of cap-

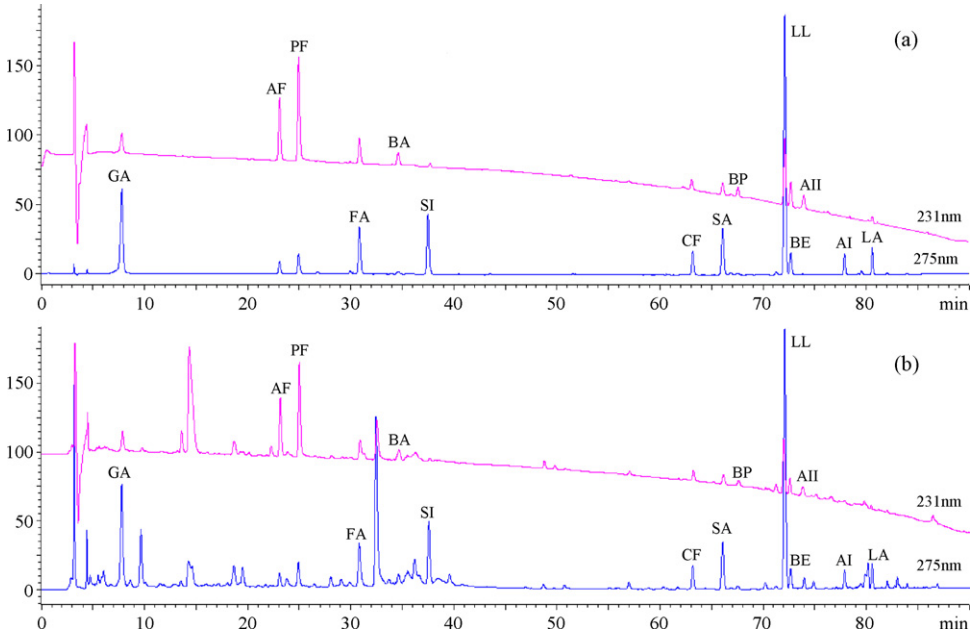

Fig. 3. HPLC–DAD chromatograms of 14 analytes in DSS extract at 231 and 275 nm. (a) Chromatogram of the mixed standard. (b) Chromatogram of DSS extract.

Table 2
Detection wavelength, linear regression data, LOD, LOQ, precision and accuracy of 14 constituents in DSS extract ($n = 6$).

Analyte	λ (nm)	Regression equation[a]	R^2	Linear range (μg/ml)	LOD (ng/ml)	LOQ (ng/ml)	Precision R.S.D. (%)		Accuracy (%)	
							Intra-day	Inter-day	Intra-day	Inter-day
Gallic acid	275	$y = 27264x + 1373.3$	1.0000	2.00–80.00	20	74	0.73	0.74	99.49	100.12
Albiflorin	231	$y = 10348x + 18289$	0.9997	8.00–320.0	215	684	0.22	1.51	100.57	104.30
Paeoniflorin	231	$y = 12092x + 23453$	0.9997	11.60–464.0	176	459	0.42	1.31	96.34	99.76
Ferulic acid	275	$y = 24045x - 13.491$	1.0000	1.08–43.20	44	203	0.25	0.80	100.18	99.61
Benzoic acid	231	$y = 36830x + 8162.3$	0.9990	0.52–20.80	52	169	1.64	1.78	100.98	98.90
Senkyunolide I	275	$y = 19981x - 2023.7$	1.0000	1.60–64.00	105	338	0.19	0.60	100.99	99.70
Coniferyl ferulate	275	$y = 2352.4x - 1331.9$	0.9996	11.2–224.0	190	589	0.52	0.96	100.61	98.93
Senkyunolide A	275	$y = 2912.8x + 3656.1$	0.9999	9.24–369.6	117	332	0.58	1.61	101.64	103.01
3-Butylphthalide	231	$y = 6058x - 351.54$	0.9993	1.50–60.0	145	425	1.62	2.27	98.86	98.60
Z-Ligustilide	275	$y = 11662x + 7176.5$	0.9999	10.8–432.0	100	353	0.47	1.22	100.07	99.85
Z-Butylidenephthalide	275	$y = 13982x + 1772.9$	0.9986	0.75–30.0	85	273	0.76	1.89	99.66	95.32
Atractylenolide II	231	$y = 26174x + 156.67$	0.9991	0.26–4.14	61	208	1.38	2.04	100.22	101.19
Atractylenolide I	275	$y = 49975x + 2004.7$	0.9997	0.18–7.20	22	72	0.15	0.85	103.52	99.65
Levistolide A	275	$y = 8540.4x + 189.47$	1.0000	1.09–43.52	178	633	0.30	0.86	100.05	99.73

[a] In the regression equation $y = ax + b$, x refers to the concentration (μg/ml), y indicates the peak area, and R^2 is the correlation coefficient of the equation.

Table 3
The contents (mg/g) of 14 constituents in DSS preparations ($n = 3$).

Products	GA	AF	PF	FA	BA	SI	CF	SA	BP	LL	BE	AII	AI	LA
Danggui-Shaoyao-San[a]	0.48	1.86	2.69	0.25	0.12	0.90	0.64	2.14	0.35	2.54	0.16	0.01	0.04	0.26
Tang Kuei Shao Yao San[b]	0.22	0.82	1.97	0.18	0.14	0.38	2.38	0.60	0.08	1.07	0.15	0.03	0.04	0.21
Toki Shakuyaku San Ex[c]	0.85	4.07	11.32	0.36	1.01	0.20	0.18	1.01	0.74	0.52	0.04	–	0.06	0.53
Toki Shakuyaku San Granula[d]	0.51	2.40	6.58	0.21	0.52	0.12	–	0.48	0.27	0.26	0.02	0.01	0.02	0.25
DSS Conc. Granula[e]	1.24	1.63	1.65	0.14	1.25	0.92	0.63	0.81	0.52	0.99	0.10	0.07	0.07	0.25
DSS Conc. Granula[f]	1.51	3.49	15.21	0.49	0.32	1.45	2.73	2.40	0.75	1.87	0.23	0.01	0.01	0.64
DSS Conc. Tablet[g]	1.47	4.30	16.77	0.53	0.33	1.35	2.02	2.09	0.42	1.74	0.21	0.01	0.01	0.62
Guishao Tiaojing Tablet[h]	4.43	9.69	15.05	0.14	1.60	0.38	–	0.02	0.86	0.01	–	–	–	0.24
Danggui Shaoyao Capsule[i]	1.43	4.81	13.75	0.57	0.32	1.85	0.83	4.67	0.68	3.26	0.31	0.05	0.05	0.80
DSS Description Granula[j]	3.11	6.39	15.30	0.93	0.43	0.43	0.24	0.44	–	0.18	0.01	–	–	0.04

"–": below the LOD.
[a] Provided by author's laboratory.
[b] Provided by Cheng Yung Pharmaceutical Co., Taoyuan, Taiwan. Batch No. AI006014.
[c] Provided by Tsumura Pharmaceutical Co., Tokyo, Japan. Batch No. 2070023010.
[d] Provided by Tsumura Pharmaceutical Co., Tokyo, Japan. Batch No. B10371.
[e] Provided by Tong Yang Pharmaceutical Co., Taichung, Taiwan. Batch No. 550397.
[f] Provided by Sun Ten Pharmaceutical Co., Taichung, Taiwan. Batch No.131642.
[g] Provided by Sun Ten Pharmaceutical Co., Taichung, Taiwan. Batch No. 271841.
[h] Provided by Sanjiu Nankai Pharmaceutical Co., Hunan, China. Batch No. 20070802.
[i] Provided by Huquan Pharmaceutical Co., Hubei, China. Batch No. 071101.
[j] Provided by Jianyin Tianjiang Pharmaceutical Co., Jiansu, China. Batch No. 0808177.

sule could comparably yield high extraction of most of its bioactive components. As to samples 8 and 10, the content of PF fulfilled the national standard of China, but the levels of other components such as SA, LL, BE, AII and AI were obviously lower than those of other products, which might result from the low extraction efficiency of lipid-soluble constituents in extraction process. In addition, the relative contents of BA were less than 5% in all products except 5, in which it reached to 12.15%. Although its amount in product 5 was in the safe range for clinical applications, monitoring this component and choosing appropriate herbs are still recommended in order to increase the safety of the drug product. These results suggested that the occurrence and contents of the compounds in commercial preparations depend on the raw materials and are also influenced by dosage form, extraction process and formula. To ensure its stability, safety and efficacy for clinical use, guidelines and quality control for commercial products of DSS should be standardized.

4. Conclusion

A reliable and simple analytical method was developed for the qualitation and quantitation of components in DSS by using LC–DAD–MS/MS. Forty-one components including monoterpene glycosides, phenolic compounds, phthalides, sesquiterpenoids and triterpenes in the formula were successfully identified based on retention time, UV and MS spectra compared with those of authentic compounds or literature data. Fourteen components were simultaneously determined by LC–DAD at two different wavelengths. The developed method was validated to have good precision, accuracy, and repeatability, thus could be used to evaluate the quality of the drug products. The results demonstrated that the present method could readily provide full-scale qualitative and quantitative information for the quality evaluation of DSS intermediates and final preparations. Furthermore, the method can be modified to analyze the ingredients of individual herbal medicine in DSS and some other complex prescriptions containing these herbs.

Acknowledgements

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