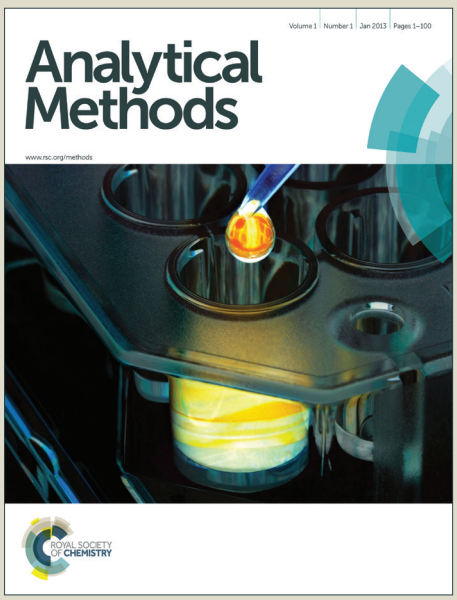
Analytical Methods

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/methods

Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

ARTICLE TYPE

Simultaneous analysis of coumarin derivatives in extracts of Radix angelicae pubescentis (Duhuo) by HPLC-DAD-ESI-MS^n technique

Bin Wang,* Xianhua Liu, An Zhou, Mei Meng and Qinglin Li

Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX

DOI: 10.1039/b000000x

Radix angelicae pubescentis (Duhuo) is a traditional Chinese herbal medicine (TCM) used for the treatment of rheumatic diseases. The coumarin derivatives in the Duhuo extracts are important bioactive compounds. In this work, a high performance liquid chromatography–diode array detection–electrospray ionization tandem mass spectrometry (HPLC-DAD-ESI-MS^n) method was established for the rapid separation and characterization of coumarin compounds from the Duhuo extracts. A total of 24 coumarin compounds were detected, of which 7 compounds were unambiguously characterized by comparing their retention time (t_R), UV and MS spectra with those of the reference standards, and the others were tentatively identified based on their tandem mass spectrometry fragmentation data obtained in the positive ion mode on line. Importantly, five of these characterized coumarin compounds were reported from this plant for the first time.

Introduction

Coumarins, naturally occurring compounds with substituents on benzene and pyrone rings, are widely distributed in traditional Chinese herbal medicine (TCM).[1] Recent pharmacological studies showed that the coumarin derivatives possess a wide range of bioactivities.[2] For example, the osthole,[3] one type of the coumarin derivatives, was found to be the most active in anti-inflammatory, anti-allergenic, anticancer and neuroprotective field; Umbelliferone[4] showed antidiabetic, antioxidant and neuroprotective effects; Psoralen,[5]xanthotoxin[6] and bergapten,[7] the three linear furanocoumarins, possessed immunomodulatory, antioxidant and anticancer effects. Moreover, the angular dihydrofurocoumarins, columbianadin[8] and columbianetin,[9] have been shown to possess anti-microbial and cytotoxic activity. Because of the important bioactivity, the coumarin derivatives were considered to be one of the main herbal active ingredients in TCM, and commonly act as reference indicators in identification and quality evaluation of these TCM.[10] Therefore, it is extremely important to determine the coumarin constituents in TCM so as to assess its safety, efficacy and therapeutic reproducibility.

Radix angelicae pubescentis (Duhuo) is a typical TCM which is widely employed for the treatment of rheumatic disease in Chinese clinics. The pharmacological investigations[11] have shown that extractives of Duhuo displayed anti-tumor, anti-angiogenic, anti-platelet aggregation, anti-inflammation and immunoregulatory activities. In addition, the research indicated that the coumarin derivatives may be the main and characteristic markers constituents in the Duhuo extractive. However, the exact structures of the coumarin derivatives in Duhuo extractive were not well characterized, thus it is high importance to develop a rapid and efficient method to determine the coumarin derivatives in the Duhuo extract.

To date, a number of analytical methods such as thin-layer chromatography (TLC),[12] UV-Vis spectroscopy,[13] high-performance liquid chromatography (HPLC),[14]high-speed counter-current chromatography (HSCCC),[15] pressurized capillary electrochromatography (pCEC),[16] gas chromatography in combination with mass spectrometry (GC-MS),[17] and liquid chromatography-mass spectrometry (LC/MS)[18] were developed for determination of coumarin derivatives. Among these methods, the TLC and UV-Vis spectroscopy showed either low sensitivity or inadequate separation. Though the pCEC and HSCCC methods have high separation and high resolution, the selection of proper internal standard for the former or appropriate solvent system for the latter makes the two methods time-consuming. The high separation, high sensitivity and excellent resolution could be achieved by GC-MS, but this method requires that the analyzed coumarins were volatile. Some other techniques like HPLC coupled with ultraviolet or photodiode array detection (HPLC-UV or HPLC-DAD) was demonstrated to provide convincing and satisfactory results. However, these techniques only can be applied to identify known compounds. In the complex Duhuo matrices, plenty of important bioactive constituents have not identified yet. Therefore, a more efficient method is urgently demanded. The LC-MS technique, especially HPLC combined with photodiode-array detection and electrospray ionization multiple-stage mass spectrometry (HPLC-DAD-ESI-MS^n), makes the determination possible. The HPLC-DAD-ESI-MS^n technique[19] is suitable for achieving high sensitivity and providing adequate fragmentation patterns to simultaneously analyse and identify much more active constituents in complex TCM systems. In contrast to the

traditional methodologies on TCM separation and determination, it is a unique approach to solve this problems encountered. However, to the best of our knowledge, the qualitative research of the multiple coumarin derivatives in Duhuo extractive by HPLC-DAD-ESI-MS^n has not been reported yet in the literature.

Herein, we investigated the determination of coumarin derivative in Duhuo extractive by HPLC-DAD-ESI-MS^n technique. Basing on the fragmentation patterns and reference to the literatures, we firstly developed a simple and accurate HPLC-DAD-ESI-MS^n technique for determination of eleven simple coumarins, six linear furanocoumarins and seven angular dihydrofurocoumarin in Duhuo. Their structures are listed in Fig. 1. Proposed MS fragmentation mechanism of some coumarin compounds，especially three pairs of coumarin isomers，were tentatively identified basing on the fragmentation patterns and reference to the literatures. The proposed determination method was proved to be robust and reliable for the quality control of Duhuo.

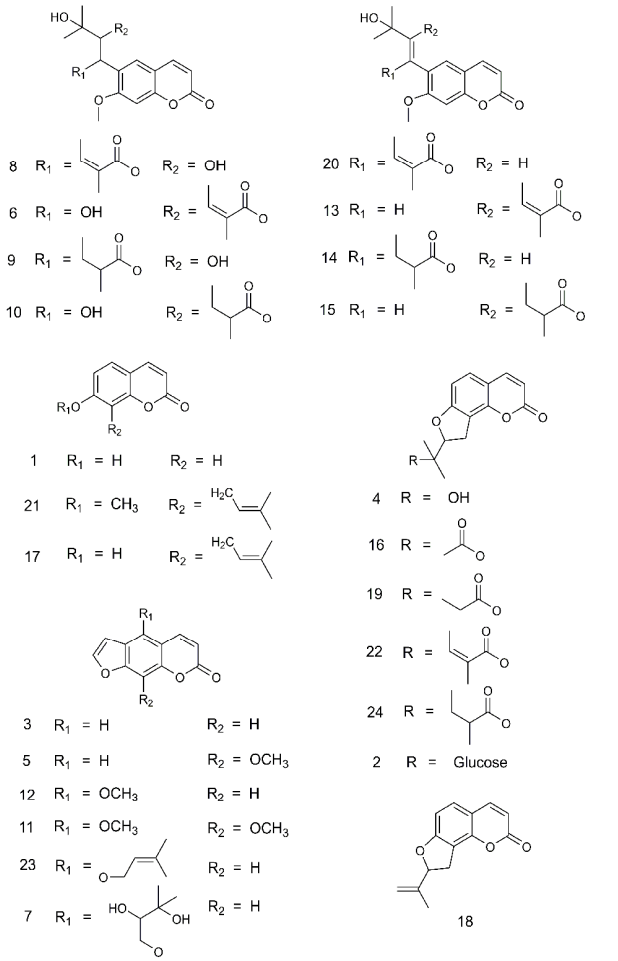


Fig. 1 Structures of the constituents identified or tentatively characterized from the Radix angelicae pubescentis

Results and discussion

Optimization of extraction conditions

In order to obtain satisfactory extraction efficiency for the coumarin compounds in Duhuo, variables involved in the procedure including extraction solvents and extraction times were assessed based on single factor experiment. Six different solvent systems (petroleum ether, chloroform, methanol, acetonitrile, chloroform-methanol (1:1, v/v) and chloroform-acetonitrile (1:1, v/v)) and three various extraction times (10 min, 30 min and 60 min) were selected for extracting the coumarin compounds by ultrasonic technique. Furthermore, the peak area of osthole in HPLC chromatogram and ratio of Duhuo extracts to sample were also recorded. As a result, optimal extraction condition of Duhuo was obtained by ultrasonication extraction with chloroform-methanol (1:1, v/v) for 30 min. The optimal result was listed in Table S1.

Optimization of HPLC-DAD-ESI-MS^n system

To obtain optimized elution conditions, four trials with elution systems of acetonitrile-water (a), methanol-water (b), methanol-0.1% acetic acid aqueous solution (c) and methanol-0.1% formic acid aqueous solution (d) in various proportions were performed. It was shown that the resolution and the separation were poor by using elution system (a), (b) and (c). Good one was achieved by using elution system (d), especially when the similar coumarin components such as xanthotoxin (**5**) and bergapten (**12**), etc. were to be analyzed. As a result, elution system (d), consisting of methanol-0.1% formic acid aqueous solution, was finally employed, which produced high resolution and good separation. DAD detection was employed at the wavelength range of 200-400 nm. The wavelength for detection was set at 246 nm, 274 nm and 320 nm, at which most coumarin components can be measured sensitively. The ESI-MS^n spectra were detected in both positive and negative ion modes, while remarkably lower intensity was obtained in negative ion mode. Therefore, positive ion mode was selected for the analysis. In the positive mode, coumarin compounds generally produced molecular adduct ions such as $[M+H]^+$ and $[M+Na]^+$ when collision energy was not applied. The HPLC chromatograms of a mixture of standard compounds and Duhuo extracts are shown in Fig. S1 and Fig. S2, respectively. The ESI-MS^n chromatograms of the samples are shown in Fig. 2.

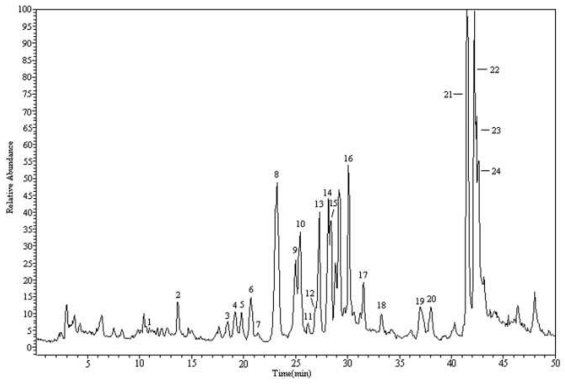


Fig. 2 Mass Spectrometer-Total Ion Chromatogram (MS-TIC, in positive ion mode) of Duhuo extracts

Identification of coumarin compounds

Coumarin compounds occurring in Duhuo extracts were separated by HPLC and UV spectra was obtained using a diode-array detector, and subsequently analysed by ESI-MS^n. HPLC-DAD-ESI-MS^n technique could provide comprehensive

information including real-time, UV spectra, molecular mass and CID fragmentations. Based on the information from literatures about Duhuo and HPLC-DAD-ESI-MS^n analysis, a total of 24 coumarin compounds were identified or tentatively identified (Table S2).

Among them, seven peaks were unambiguously identified as umbelliferone (**1**), psoralen (**3**), xanthotoxin (**5**), bergapten (**12**), osthole (**21**), columbianadin (**22**) and isoimperatorin (**23**) by co-elution with reference standards according to the t_R and MS spectra. For those compounds not commercially available, referring to previous reports and analyzing their UV spectra and fragmentation behaviors in ESI-MS^n spectra would be a powerful means for their characterisation. Accordingly, the peaks **2**, **4**, **6-11**, **13-20** and **24** were ascribed to β-D-glucosyl- Columbianetin (**2**), columbianetin (**4**), angelol A (**6**), oxypeucedanin hydrate (**7**), isoangelol (**8**), anpubesol (**9**), angelol C (**10**), isopimpinellin (**11**), angelol A dehydration (**13**), anpubesol dehydration (**14**), angelol C dehydration (**15**), columbianetin acetate (**16**), osthenol (**17**), angenomalin (**18**), columbianetin propionate (**19**), Isoangelol dehydration (**20**) and dihydrocolumbianadin (**24**). Among them, components **13**, **14**, **15**, **20** and **24** were first reported from the species.

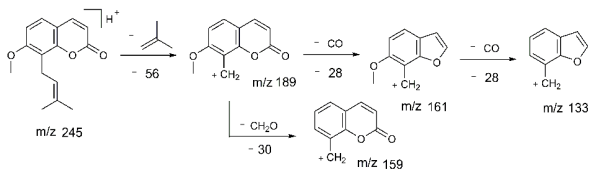
In this article, Peaks **1**, **6**, **8**, **9**, **10**, **13**, **14**, **15**, **17**, **20** and **21** were collectively called simple coumarin. This class of simple coumarins would be known as Type I for convenience. Peak **3**, **5**, **7**, **11**, **12** and **23** were linear furocoumarins (Type II), and Peaks **2**, **4**, **16**, **18**, **19**, **22** and **24** were angular dihydrofurocoumarins (Type III).

Identification of Type I simple coumarins

The fragmentation behavior of simple-type coumarins of Duhuo extracts in ESI-MS^n spectra was investigated. Although substituent positions and substituent groups in coumarin ring were different, their fragmentation patterns contained certain rules.

If substituent group was hydroxy and substituent position was at C-7, such as umbelliferone (Peak **1**, t_R=11.21 min), the ESI-MS spectrum produced a $[M+NH_4]^+$ molecular adduct ion of m/z 180 and exhibited a $[M+H]^+$ ion of m/z 163 as the base peak. When applied to an collision energy of 35 %, the $[M+H]^+$ ion at m/z 163 produced a prominent ion at m/z 119, which should be attributed to the loss of neutral CO_2 (Δm=44). The m/z 119 ion was further subjected to MS^3 analysis giving a signal at m/z 91, which should be attributed to the loss of the carbonyl group (CO, Δm=28) (Scheme S1). Then ion at m/z 91 was subjected to MS^4 analysis while no fragmentation information was obtained. The reason was probably that coumarin compounds in ESI-MS^n spectrum was inclined to lose CO or CO_2 moiety until there were no oxygen atoms in entire molecule.

If methoxyl group was at C-7 together with isopentenyl at C-8, such as osthole (Peak **21**, t_R=41.45 min), The MS spectra exhibited an abundant parent ion $[M+H]^+$ at m/z 245. The ion at m/z 245 was further fragmented by loss of C_4H_8 (Δm=56) in MS^2 analysis, which could only be rationalized by neutral loss of a isobutenylmoiety leading to the formation of a predominant ion at m/z 189. Ion at m/z 189 was then subjected to MS^3 analysis and yielded ions at m/z 161 and m/z 159, signaling loss of CO (Δm=28) and CH_2O (Δm=30), respectively. The ion at m/z 161, further produced signal at m/z 133, signaling loss of the carbonyl group (CO, Δm=28) in MS^4 spectrum (Scheme 1). However, the ion of neutral loss of CO_2 was not observed.



Scheme 1. Proposed MS fragmentation pathway for the $[M+H]^+$ ion of Peak **21**

Peak **6** (t_R=20.72 min) and Peak **8** (t_R=23.21 min) gave the same mass number ions at m/z 377 $[M+H]^+$ and m/z 359 $[M+H-H_2O]^+$. Thus, they were a pair of isomers. By examining known coumarins in Duhuo,[20] they were plausibly identified as angelol A and isoangelol, which were distinguished by the substituent position of hydroxy group. The ion at m/z 359 of Peak **6** or Peak **8**was subjected to MS^2, MS^3 and MS^4 analysis and yielded the same ions at m/z 301, m/z 219 and m/z 191, signaling loss of CH_3COCH_3 (Δm=58), C_5H_7O (Δm=83) and CO (Δm=28), respectively. Interestingly, the $[M+H]^+$ ion of Peak **6** or Peak **8** was subjected to MS/MS analysis (Fig. S3) and also yielded the same ions at m/z 345, m/z 301, m/z 277 and m/z 205. The mechanism (Scheme S2 and Scheme S3) was probably that the $[M+H]^+$ ions of Peak **6** and Peak **8** were fragmented and firstly produced ion at m/z 277 (**A**) and m/z 277 (**F**) by losing angelic acid moiety, respectively. The ion **A** or ion **F** subsequently converted into ion **B** (m/z 277) and then the ion **B** converted into ion **C** (m/z 277). With the loss of weight of 72 Da, a base peak ion at m/z 205 was yielded from the ion **C**. The ion at m/z 205 was further subjected to MS^3 and MS^4 analysis and yielded a prominent ion at m/z 175 (Δm=30) and m/z 147 (Δm=28), which should attributed to loss of neutral CH_2O and CO molecule respectively. In particular, the relative abundances of ion at m/z 301 and m/z 345 were larger in Peak **6** than in Peak **8**. We speculated that this was probably attributed to different substituent position of hydroxy group. If the compound possessed hydroxy group at C-9, like angelol A, it could form structure of transition state of six-member-ring[21] with methoxyl group at C-7 in overall molecular structure. However, if the hydroxy group was at C-10, like isoangelol, it couldn't form six-member-ring structure transition state with methoxyl group at C-7 in overall molecular. It was most likely easy for six-member-ring structure transition state losing a methanol moiety produce ion at m/z 345(**D**). Thus, the abundance of ion at m/z 345 in Peak **6** was boosted. Because of inductive effect of electron withdrawing group being at C-6 in the m/z 345 ion(**D**), the carbon-oxygen bond at C-8a and at O-1 was weaken and it can produce ion at m/z 301(**E**) by the loss of CO_2. So, the abundance of ion at m/z 301 was also boosted. In conclusion, we tentatively identified Peak **6** and Peak **8** as angelol A and isoangelol, respectively.

Peak **9** (t_R=25.02 min) and Peak **10** (t_R=25.44 min) gave the same mass number ions at m/z 379 $[M+H]^+$ and m/z 361 $[M+H-H_2O]^+$. Thus, they were also a pair of isomers. Their $[M+H]^+$ ions yielded very similar MS^2, MS^3 and MS^4 spectra as Peak **6** and Peak **8**, with m/z 205, m/z 175 and m/z 147 ions as the base peak, respectively. Their $[M+H-H_2O]^+$ ion also yielded very similar MS^2, MS^3 and MS^4 spectra as Peak **6** and Peak **8**, with m/z 303, m/z 219 and m/z 191 ions as the base peak, respectively. Thus,

we speculated that these ions of Peak **9** and peak **10** were produced by the similar mechanism as Peak **6** and Peak **8**. By analysing above ESI-MS fragmentation information and examining known coumarins, Peak **9** and Peak **10** was plausibly identified as Anpubesol[20(b)] and Angelol C[22], respectively.

The pseudo-molecular ion at m/z 231 of Peak **17** (t_R =31.56 min) gived the same loss of C_4H_8(Δm=56) as Peak **21** from the parent ion, with m/z 175 ion, suggesting the presence of a isobutenyl moiety. Its MS^3 and MS^4 spectra yielded prominent ions at m/z 147 (loss of CO, Δm =32) and m/z 103 (loss of CO_2, Δm=44), respectively. By comparing known coumarin compound[23] with molecular weight of 231 Da had been isolated from Duhuo, Peak **17** was tentatively identified as osthenol.

Two peaks, Peak **13** (t_R=27.29 min) and Peak **20** (t_R=37.89 min), in the ESI-MS total ion current chromatogram gave the same quasi-molecular ion at m/z 359. No coumarin with molecular weight of 359 Da had been reported from Duhuo, hence they should be new constituents. The two ions at m/z 359 produced very similar MS^2, MS^3 and MS^4 spectra as the two $[M+H-H_2O]^+$ ion of Peak **6** and Peak **8**, with the ions at m/z 301, m/z 219 and m/z 191 as the base peak, respectively. By comparing their t_R, UV and ESI-MS spectra with Peak **6** and Peak **8**, Peak **13** and Peak **20** was preliminarily characterized as angelol A dehydration and isoangelol dehydration, respectively.

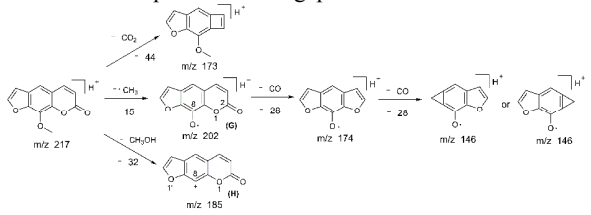
The new fragmentation behavior for Peak **13** and Peak **20** was also observed in Peak **14**(t_R=28.14 min) and Peak **15** (t_R=28.35 min). They represented a pair of isomers, both giving a $[M+H]^+$ ion at m/z 361. The ions at m/z 361 of Peak **14**yielded very similar MS/MS and MS^3 spectra as the $[M+H-H_2O]^+$ ion at m/z 361 of Peak **9**, with m/z 303 and m/z 219 ions as the base peak, respectively. The ion at m/z 219 further yielded ion at m/z 191(Δm=28), which should result from the loss of a carbonyl group. Interestingly, the Peak **15** yielded very similar MS/MS, MS^3 and MS^4 spectra as the $[M+H-H_2O]^+$ ion at m/z 361 of Peak **10**. Thus, according to their t_R, UV and ESI-MS spectra and comparing with Peak **9** and Peak **10**, Peak **14** and Peak **15** was preliminarily characterized as Anpubesol dehydration and Angelol C dehydration.

Identification of Type II linear furocoumarins

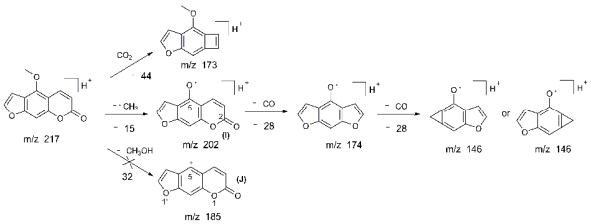
Relatively fewer linear furocoumarins were detected from Duhuo. Substituent groups of the linear-type furocoumarins in Duhuo were mainly oxysubstituent groups, and substituent positions were at C-5 or C-8. According to the fragmentation information in ESI-MS^n spectra and referring to previous reports,[24] we proposed the fragmentation mechanism of several coumarin compounds, which showed in the following paragraphs.

Peak **3** (t_R=19.01 min) was unambiguously identified as psoralen by comparing retention time and MS^n spectra with the authentic standard. Its $[M+H]^+$ ion (m/z 187) was subjected to MS/MS and MS^3 analysis, which yielded the ions at m/z 143 (Δm=44) and m/z 115 (Δm=28), which should attributed to the loss of CO_2 and CO, respectively (Scheme S4). Although the m/z 115 ion was subjected to MS^4 analysis, its fragmentation information was not obtained. The reason was also probably that coumarin compounds in ESI-MS^n spectrum was inclined to lose CO or CO_2 moiety until there were no oxygen atoms in entire molecule.[24(a)]

A pair of isomers were unambiguously identified as xanthotoxin (Peak **5**, t_R=19.78 min) and bergapten (Peak **12**, t_R=26.95 min) by comparing retention times and MS^n spectra with their authentic standards (Fig. S4 and Fig. S5). The ESI-MS^n spectra of Peak **5** and Peak **12** were displayed in Fig. S6-9. When applied to an collision energy of 35 %, the $[M+H]^+$ ion (m/z 217) of xanthotoxin(Fig. S7(A) and Scheme 2) produced two major ions at m/z 202 (Δm=15) and m/z 185 (Δm=32), which should attributed to loss of methyl radical and CH_3OH moiety, and one minor ion at m/z 173 (Δm=44), suggesting loss of CO_2 moiety, respectively. However, bergapten(Fig. S7(B) and Scheme 3) only yielded one major ion at m/z 202 (Δm=15) and one minor ion at m/z 173 (Δm=44) by similar loss as xanthotoxin. The ion at m/z 202 of xanthotoxin further yielded ion at m/z 174 (Δm=28) and m/z 146 (Δm=28+28) in their MS^3 and MS^4 spectrum, respectively. Those of two ions should be attributed to the successive loss of neutral CO. However, the ion at m/z 202 of bergapten also yielded very similar MS^3 and MS^4 spectra as xanthotoxin. In particular, the m/z 185 ion was not observed and the relative abundance of ion at m/z 202 was boosted in MS^2 spectrum of bergapten. We speculated that this was probably attributed to the stability of ion at m/z 202 and m/z 185. Owing to conjugation effect between the oxygen atom at C-2 and C-8, the stability of free radical ion at m/z 202 (**I**) was boosted. However, owing to lack of conjugation effect in entire free radical ion at m/z 202 (**G**), its stability was reduced. Thus, the relative abundance of m/z 202 (I) was larger than that of m/z 202 (**G**); In the m/z 185 ion (**H**), the positive charge resides mainly at C-8 and its stability was enhanced by electric field effect of oxygen lone pair electrons at O-1 and O-1′. However, in the m/z 185 ion (**J**), the positive charge resides mainly at C-5 and its stability was largely reduced for lack of electric field effect.[26] As a result, the m/z 185 ion was not observed in MS^2 spectrum of bergapten.



Scheme 2. Proposed MS fragmentation pathway for the $[M+H]^+$ ion of Peak **5**



Scheme 3. Proposed MS fragmentation pathway for the $[M+H]^+$ ion of Peak **12**

Peak **11** (t_R=25.81 min) produced the protonated ion at m/z 247 in its ESI-MS spectrum. In MS^2 and MS^3 spectra, the ions at m/z 232[(Δm=15) and m/z 217(Δm=15+15) were observed due to successive elimination of methyl radical moiety. Then the MS^4 spectrum displayed ions at m/z 189 (Δm=28) and m/z 161 (Δm=28+28) resulting from successive loss of neutral CO. By

referring to previous reports,[27] Peak **11** was tentatively identified as isopimpinellin.

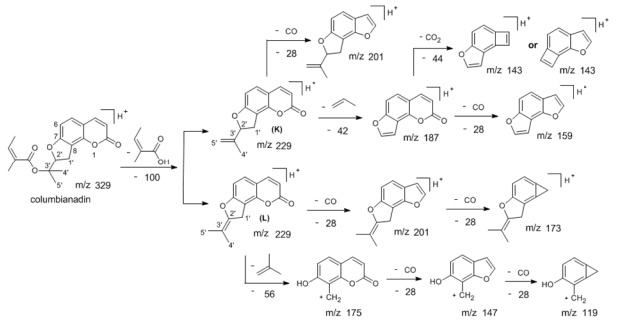
Peak **23** (t_R=42.48 min) produced a $[M+H]^+$ ion at m/z 271. It was unambiguously identified as isoimperatorin by comparing the authentic standard. The ion at m/z 271 was further fragmented by loss of C_5H_{10} (Δm=68) from the parent ion, which could only be rationalized by neutral loss of a rearranged isopentenyl moiety leading to the formation of ion at m/z 203.[24(a)] Ion at m/z 203 was then subjected to MS^3 analysis and yielded ion at m/z 159, signaling loss of CO_2 (Δm=44). The m/z 159 ion further gave signal ion at m/z 131 in MS^4 spectrum, which should be attributed to the loss of the carbonyl group (CO, Δm=28).

Peak **7** (t_R=21.38 min) gave a $[M+H]^+$ ion at m/z 305. The m/z 305 ion was subjected to MS/MS analysis and yielded ion at m/z 203, signaling loss of a molecular weight of 102 Da. The m/z 203 ion yielded very similar MS^3 and MS^4 spectra as the m/z 203 ion of Peak **23**, with m/z 159 (Δm=44) and m/z 131 (Δm=28) ions as the base peak, suggesting loss of CO_2 and CO. By referring to previous reports,[21] Peak **7** was tentatively identified as oxypeucedanin hydrate.

Identification of Type III angular dihydrofurocoumarins

One angular dihydrofurocoumarin was identified and six were tentatively identified from this species. By loss of substituent groups of angular dihydrofurocoumarins, all the parent ions produced characteristic ion at m/z 229.

Peak **22** (t_R=42.20 min) gave a $[M+H]^+$ ion at m/z 329 and a $[M+Na]^+$ ion at m/z 351. It was unambiguously identified as columbianadin by comparing the authentic standard. The $[M+H]^+$ ion at m/z 329 were introduced to MS/MS experiment (Fig. S10(A)) and yielded the m/z 229 ion (Δm=100) as the base peak, signaling the loss of a molecule of angelic acid. The MS^3 experiment gave ions at m/z 187 as the base peak, showing the loss of a molecule of propylene (Δm=42). Then the m/z 187 ion in MS^4 experiment yielded ions at m/z 159 and m/z 143, signaling loss of CO (Δm=28) and CO_2 (Δm=44), respectively. The proposed fragmentation mechanism of columbianadin showed in the Scheme 4. We inferred that the columbianadin was fragmentated mainly producing the major ion at m/z 229 (**K**) and the minor ion at m/z 229 (**L**) because the base peak ion of m/z 187 in MS^3 spectra was only produced from ion **K.**



Scheme 4. Proposed MS fragmentation pathway for the $[M+H]^+$ ion of Peak **22**

Peak **4** (t_R=19.21 min) gave [M+H]+ ion at m/z 247. Its MS/MS spectrum (Fig. S10(B)) yielded prominent ions at m/z 229$[M+H-H_2O]^+$ and m/z 175$[M+H-H_2O+2H-C_4H_8]^+$, and excluded the possibility of the known isopimpinellin (Peak **11**). The m/z 229 ion was introduced to MS^3 and MS^4 experiments and yielded ions at m/z 201 and m/z 173, signaling successive loss of CO(Δm=28), respectively. Interestingly, the ion abundance of m/z 175 was very high in MS/MS spectrum. When applied to an successive collision energy of 35 %, the m/z 175 ion gave ions at m/z 147 and m/z 119 in MS^3 and MS^4 experiments, respectively. This was only rationalized by successive loss of CO moiety from the precursor ion. By comparing known compounds in Duhuo,[9] Peak **4** was tentatively identified as columbianetin. So, it should be another angular dihydrofurocoumarin. In particular, the ion abundance of m/z 175 in MS^2 spectra reached 95 % (after the m/z 229 ion). It was explained as a consequence of the m/z 175 ion being only produced from the m/z 229 ion (**L**) (Scheme S5). Thus, we inferred the $[M+H]^+$ ion of columbianetin was fragmentated producing major ion **L** and producing minor ion **K**. However, this is opposite to that of columbianadin. This was probably attributed to space steric effect.[28]

Peak **24** (t_R=42.61 min) gave two ions at m/z 331 $[M+H]^+$ and m/z 353 $[M+Na]^+$. No coumarin with molecular weight of 331 Da had been reported from Duhuo, hence it should be a new constituent. The $[M+H]^+$ ion at m/z 331 was introduced to MS/MS experiment and yielded very similar spectra as columbianadin, with m/z 229 ion (Δm=102) as the base peak. The MS^3 and MS^4 spectra of Peak **24** were also very similar as columbianadin, with m/z 187 and m/z 159 ions as the base peak, respectively. Thus, it should be columbianadin analogue. By referring to t_R and UV spectrum of columbianadin, the 102 mass units was identified as dihydroangelic acid. So, Peak **24** was preliminarily characterized as dihydrocolumbianadin.

All of the $[M+H]^+$ ion of Peak **16** (t_R=30.09 min, m/z 289) and Peak **19** (t_R= 36.98 min, m/z 303) yielded prominent ions at m/z 229, m/z 187 and m/z 159 as the base peak in MS/MS, MS^3 and MS^4 spectra, respectively. These fragmentation behaviors in ESI-MS^n spectra were very similar as columbianadin. By referring to previous reports[23,29] and the loss of 60 and 74 mass units from their each parent ions, Peak **16** and Peak **19** were preliminarily characterized as columbianetin acetate, columbianetin propionate, respectively.

The pseudo-molecular ion (m/z 409) of Peak **2** (t_R =13.67 min) produced ion at m/z 247(Δm=162) in MS/MS spectra, suggesting the loss of a glucose moiety. Its MS^3 spectra yielded very similar prominent ions as columbianadin, with m/z 229 and m/z 175 as the base peak. The two ions, m/z 229 and m/z 175, further produced in MS^4 spectrum signals at m/z 187 and m/z 147, respectively. These fragmentation behaviors in ESI-MS^n spectra were also analogous to columbianadin. By comparing known coumarin compound[20(a)] with molecular weight of 409 Da had been isolated from Duhuo, Peak **2** was tentatively identified as β-D-glucosyl-columbianetin.

Peak **18** (t_R=33.25 min) gave a $[M+H]^+$ ion at m/z 229. Its MS/MS and MS^3 spectrum yielded prominent ions at m/z 187 and m/z 159, respectively. The ion at m/z 159 was further introduced to MS^4 experiment and yielded ion at m/z 131, which should be attributed to the loss of the carbonyl group (CO, Δm=28). According to previous reports[20(a)] togather with ESI-MS^n spectra, Peak **18** was tentatively identified as angenomalin.

Conclusions

In this study, 24 coumarin compounds were identified or tentatively identified in Radix angelicae pubescentis using HPLC-DAD-ESI-MS^n in positive ion mode. Coumarin compounds seem to be the major constituents in Duhuo according to our investigation. Four simple-type coumarins and one angular were identified from this species for the first time. Some fragmentation rules of coumarin compounds were summarized: (1) Coumarin compounds in ESI-MS^n spectrum was inclined to lose CO or CO_2 moiety until there were no oxygen atoms in entire molecule; (2) Simple-type coumarins (Type I) containing hydroxy at C-7 were inclined to lose CO or CO_2 moiety and but containing methoxyl at C-7 were inclined to lose CO moiety producing fragmentation ion; (3) linear-type furocoumarins (Type II) containing methoxyl at C-5 or C-8 were inclined to produce the ion at m/z 202 by loss of methyl radical and containing greater substituent group such as isopentenyl were inclined to produce the ion at m/z 203 by loss of rearranged moiety; (4) Angular dihydrofurocoumarins (Type III) were inclined to produce characteristic ion at m/z 229 by loss of substituent groups.

This newly established method was successfully applied to simultaneously identify the coumarin constituents in Duhuo. The results were mostly consistent to other phytochemical analyses, but it's timesaving and simple compared with the traditional phytochemical method. Moreover, with the high sensitivity of the mass spectrum detector, some components with trace amounts were also identified, and thus a full-scale chemical profile could be obtained. The results enriched the chemical knowledge of umbelliferae family, and provided valuable ground knowledge for further pharmacological research of coumarin compounds in TCM. Further quantitative analysis method of those components should be developed for the quality control of this medicinal herb.

This work was supported by the Natural Science Fund of Anhui University of Chinese Medicine, China (No. 2013zr016).

Notes and references

Key Laboratory of Xin'an Medicine, Ministry of Education, Anhui Province Key Laboratory of R&D of Traditional Chinese Medicine, Anhui University of Traditional Chinese Medicine, Hefei, Anhui, 230038, P. R. China. Fax: +86 551 65169371. E-mail: wangbin5654@163.com

† Electronic Supplementary Information (ESI) available: Experimentaldetails; synthesis and characterization of compounds; spectroscopic data.See DOI: 10.1039/b000000x/

1 (a) H. Chen,W. Zhang, J. Yuan, Y. Li, S. Yang, W. Yang, *J. Pharm. Biomed. Anal.,* 2012, **59**, 90-95; (b)Y. Li, S. Jiang, Y. Guan, X. Liu, Y. Zhou, L. Li, S. Huang, H. Sun, S. Peng, Y. Zhou, *Chromatographia,* 2006, **64**, 405-411; (c) J. Su, C. Zhang, W. Zhang, Y. Shen, H. Li, R. Liu, X. Zhang, X. Hu , W. Zhang, *J.Chromatogr.A,* 2009, **1216**, 2111-2117.

2 (a) A. Messer, A. Nieborowski, C. Strasser, C.Lohr, D. Schrenk, *Food Chem. Toxicol*, 2011, **49**, 3224-3221; (b) L. Wan, Y. Sun, Q. Yu, Y. Huo, J. Zhu, Y. Li, C. Guo,*Fitoterapia*, 2013, **85**, 144-153; (c) T.O. Neill, J. A.Johnson, D. Webster, C. A.Gray,*J. Ethnopharmacol*, 2013, **147**, 232–237; (d) Q. Luo, C. Wang, J. Li, W. Ma, Y. Bai, L. Ma, X. Gao, B. Zhang, Y. Chang, *J. Ethnopharmacol*,2013, **150**,175-180; (e) B. Girennavar, S. M. Poulose, G. K. Jayaprakasha,N. G. Bhat, B. S. Patil,*Bioorg. Med. Chem.*, 2006, **14**, 2606-2612.

3 (a) J.Li, W. Chan, *J. Pharm. Biomed. Anal.,* 2013, **74**, 156-161; (b) K. Wang, L. Yao, Y. Du, J. Xie, J. Huang, Z. Yin,*Parasitol. Res*, 2011,**108**, 195-200.

4 (a) B. Ramesh, P. Viswanathan, K. V. Pugalendi, *Eur. J. Pharmacol*, 2007, *566*, 231-239; (b) S. R. Subramaniam, E. M. Ellis, *J. Neurosci. Res*, 2013,**91**, 453-461.

5 Y. Wang, Y. Liu, W. Xiong, D. Yan, Y. Zhu, X. Gao, Y. Xu, A. Qi, *J. Ethnopharmacol*, 2014, **151**, 609-617.

6 T. L. Harrison, A. R. Zangerl, M. A. Schuler, M. R. Berenbaum, *Arch. Insect. Biochemi*, 2001, **48**, 179-189.

7 Y. M. Lee, T. H. Wu, S. F. Chen, J. G. Chung, *Toxicol. In Vitro*, 2003,**17**, 279-287.

8 J. Zaugg, E. Eickmeier, D. C. Rueda, S. Hering, M. Hamburger, *Fitoterapia*, 2011, **82**, 434-440.

9 Y. Chang, Z. Zhu, J. Li, Q. Zhang, Y. Deng, L. Kang, B. Zhang,X.Gao, *Chromatographia*, 2011, **74**, 639-643.

10 J. Xu, Q. Han, S. Li, X. Chen, X. Wang, Z. Zhao,H. Chen, *Phytochem. Rev*, 2013,**12**, 341-367.

11 (a) S. Wang, M. Sun, Y. Zhang, J. Zhang, L. He, *Sci. China. Chem*, 2010,**53**, 2357-2362; (b) Y. Liu, F. Wang, G. Wang, J. Han, Y. Wang, Y. Wang, *Parasito*l. Res, 2010,106, 1233-1239.(c) Y. Chang, Q. Zhang, J. Li , L. Zhang, X. Guo, J. He, P. Zhang, L. Ma, Y. Deng, B. Zhang, X. Gao, J. Pharm. Biomed. Anal., 2013, 77, 71-75.

12 X. Guo, Y. Zhang, *Acta. Pharm. Sin*, 1983, **18**,446-452.

13 K. Zhang, M. Li, *Chin. J. Inform. TCM*, 2010, **17**, 45-46.

14 F. Maggi, L. Barboni, G. Caprioli, F.Papa, M. Ricciutelli, G. Sagratini, S. Vittori, *Fitoterapia*, 2011,**82**, 1215-1221.

15 O. Sticher, *Nat. Prod. Rep*, 2008, **25**, 517-554.

16 X. Illa, W. D. Malsche, J. Bomer, H. Gardeniers, J. Eijkel, J. R. Morante, R. R. Albert, G. Desmet, *Lab. Chip*, 2009, **9**, 1511-1516.

17 I. Masuck, C. Hutzler, A. Luch, *Anal. Methods*, 2013, **5**, 508-515.

18 (a) M. Ren, H. Li, L. Sheng, P. Liu, P. Li, *J. Sep. Sci*, 2009, **32**, 3988-3995; (b) S. Krieger, H. Hayen, O. J. Schmitz, *Anal. Bioanal. Chem*, 2013, **405**, 8337-8345.

19 (a)W. Liu , C. Zhou, C. Yan, S. Xie, F. Feng, C. Wu, N. Xie, *Chin. J. Natural. Med*, 2012, **10**, 0456−0463; (b) M. Liu, Y. Li, F. Zhang, L. Yang, G. Chou, Z. Wang, Z. Hu, *J. Sep. Sci.,* 2007, **30**, 2256-2267; (c) G. Zhang, F. Zhang, L. Yang, E. Zhu, Z. Wang, L. Xu, Z. Hu, *Analytica. Chimica. Acta*, 2006, **571**, 17–24; (d) F. Gao, Y. Hu, X. Ye, J. Li, Z. Chen, G. Fan, *Food Chem.*, 2013, **141**,1962-1971; (e) L. Barros, C. T. Alves, M. Duenas, S. Silva, R. Oliveira, A. M. Carvalho, M. Henriques, C. S. Buelga, I. C. F. R. Ferreira, *Ind. Crop. Prod*, 2013, **44**, 104-110.

20 (a) X. Yang, Q. Guo, C. Zhang, B. Zhang, *Pharm. J. Chin. PLA*,2008, **24**, 389-392; (b) J. Pen, K. Lamy, B. Arison, J. Smith, G.Han, *Acta. Pharm. Sin*, 1987, **22**, 380-384.

21 M. V. Stipdonk, M. Kullman, G. Berden, J. Oomens,*Int.J. Mass. Spectrom*, 2012, **330-332**, 134-143.

22 J. Liu, S. Xu, X. Yao, *J. Shenyang. Pharm. Univ*,1994, **11**, 143-150.

23 Y. Xie, Y. Chen, M. Lin, J. Wen, G. Fan, Y. Wu, *J. Pharm. Biomed. Anal.*, 2007, **44**, 166-172.

24 (a) J. Kang, L. Zhou, J. Sun, J. Han, D. Guo, *J. Pharm. Biomed. Anal.*, 2008, **47**, 778-785; (b) Y. Chen, G. Fan, Q. Zhang, H. Wu, Y. Wu, *J. Pharm. Biomed. Anal.*, 2007, **43**, 926-936.

25 T. Zhao, Y. Jiang, L. Rong, J. Yang, Y. Hu, H. Hu, *Chem. J. Chin. Univ*, 1987, **8**, 145-148.

26 F. Celestini, G.. Kirstetter, *Soft Matter*, 2012, **8**, 5992-5995.

27 G. Rao, W. Dai, Y. Dai, F. Pu, Z. Lin, H. Sun, *Nat. Prod. Res. Dev*, 1993, **5**, 47-49.

28 G. F. Manbeck, M. C. Kohler, M. R. Porter,R. A. Stockland, *Dalton. Trans*, 2011, **40**, 12595-12606.

29 J. Liu, S. Zschocke, R. Bauer, *Phytochemistry*, 1998, **49**, 211-213.