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Title: Cell type-specific qualitative and quantitative analysis of saikosaponins in three *Bupleurum* species using laser microdissection and liquid chromatography–quadrupole/time of flight-mass spectrometry

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ABSTRACT: Cell type-specific metabolite analysis is a promising method for understanding plant metabolite production, function, transport and storage. In the present study, laser microdissection (LMD) and ultra-high performance liquid chromatography quadrupole/time of flight-mass spectrometry are combined to determine where secondary metabolites are accumulated in the roots of *Bupleurum scorzonerifolium* Willd, *B. chinense* DC. and *B. falcatum* L.. Four tissues, namely cork, cortex, phloem and xylem, were microdissected by laser microdissection, and their chemical profiles were analyzed. The main metabolites are saikosaponins. Different tissues contained different saikosaponins. Generally, the cork and cortex from all three species contained more types of saikosaponins and higher contents of saikosaponins a, c and d than did the phloem and xylem. Interestingly, in the roots of *B. scorzonerifolium* and *B. falcatum,* the cork contained much higher contents of saikosaponins a, c and d than did the cortex; while in the root of *B. chinense*, the cortex contained higher contents of saikosaponins a, c and d than the cork. Explanation and application of the results are discussed. The present findings yield valuable insights into the quality evaluation of Bupleuri Radix by morphological features.

Keywords: Bupleurum, Tissue specific, Saikosaponin, Metabolite profiling, Laser microdissection, UHPLC-QTOF-MS

1. Introduction

Plants produce a high diversity of secondary metabolites, and many of these have been used by mankind for thousands of years as therapeutic agents. These compounds are synthesized in a tissue-, organ- and developmental-specific way by specific biosynthesis enzymes, and are then stored, sometimes in high concentrations in the plants producing them. If sites of synthesis are not the sites of storage, then long-distance transport occurs-- by xylem, phloem, or apoplast [1, 2]. Analyzing single-cell or tissue chemical profiling is a promising method for understanding secondary metabolite production, function, transport and storage in plants.

To detect the metabolites in single cells or tissues, various techniques have been developed [3-6]. Among these techniques, laser microdissection (LMD) can isolate specific single cells, while mass spectrometry-based methods are the most straightforward way to identify metabolites in a single cell or tissue. The method combining LMD and ultra-high performance liquid chromatography quadrupole/time of flight-mass spectrometry (UHPLC-QTOF-MS) can achieve both qualitative and quantitative analysis of metabolites in a single cell or tissue [7, 8]. However, the method has not been applied to analyze the tissue-specific chemical profiles in different species from the same genus.

Currently, the quality evaluation of herbal medicines mostly depends on analytical techniques with modern instruments for determination the contents of marker compounds. However, when the public goes to buy herbal medicines in the retail shops, it is not possible for them to evaluate the quality by modern instruments. In practice, characteristics of herbal medicines such as shape, size, color, texture, and cross-section are usually used to evaluate their quality. But the morphological investigation for evaluating the quality of herbal medicines lacks

objective data and hasn't been validated by modern analytical methods. Bupleuri Radix is a commonly used herbal medicine for the treatment of fevers and colds, malaria, cholecystitis, hepatitis, pancreatitis, and menstrual disorders in China. According to the Chinese Pharmacopoeia (2010 edition), Radix Bupleuri is derived from the dried roots of *Bupleurum scorzonerifolium* Willd or *B*. *chinense* DC. [9]. In the Japanese Pharmacopoeia (16th edition), the official botanical origin of Bupleuri Radix (pronounced "saiko" in Japanese) is the roots of *B*. *falcatum* L. [10]. In the traditional morphological descriptions used for quality evaluation of Bupleuri Radix, samples of larger sizes are considered to have higher quality for medicinal use. However, how the pharmaceutical quality being linked to the sizes is not known.

The bioactive components of herbal medicines generally belong to the category of plant secondary metabolites. For Bupleuri Radix, saponins, especially saikosaponins a and d have been demonstrated to be responsible for its main pharmacological effects [11]. To date, more than 100 saikosaponins have been isolated and elucidated from *Bupleurum* species [12]. A previous histochemical study on the species of *B*. *chinense* has demonstrated that saikosaponins are mainly distributed in the pericycle and primary phloem of young roots but in the vascular cambium and secondary phloem of mature roots [13]. Another similar study of *B*. *chinense* also indicated that saikosaponins were abundant in the cortex outside the cambium but rare in the xylem of the root [14]. The two reports localized the position of saikosaponins using 5% vanillin-glacial acetic acid-perchloric acid solution and 99% ethanol-sulphuric acid solution, for the cortex and xylem, respectively. However, these studies couldn't definitively elucidate the chemical profiles of various tissues. Moreover, no comparative investigation of the various specific tissues of the three official *Bupleurum* species has been carried out. In the present study, the combination of LMD and UHPLC-QTOF-MS was used for analyzing the chemical profiles

of cork, cortex, phloem and xylem from the roots of *B. scorzonerifolium*, *B. chinense* and *B. falcatum*. The study reveals where different saikosaponins accumulate in these three *Bupleurum* species. This information is directly relevant to the quality evaluation of Bupleuri Radix by morphological features.

2. Experimental

2.1. Plant materials

Six batches of the roots of *B. scorzonerifolium*, *B. chinense* and *B. falcatum* were collected. Details of the samples are shown in Table 1. The diameter of roots for tissue preparation was about 0.7 cm. All the herbal samples were authenticated by Dr. Liang Zhitao from the School of Chinese Medicine, Hong Kong Baptist University and deposited in the Bank of China (Hong Kong) Chinese Medicines Centre of Hong Kong Baptist University.

2.2. Chemicals and reagents

Chemical markers of saikosaponins a, c and d were isolated by the author [15]. Ginsenoside Rb_1 (GS) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, People's Republic of China). The purity of each chemical was above 98%, as determined by high performance liquid chromatography (HPLC) analysis. The

106 solvents, acetonitrile and methanol, were of HPLC grade from E. Merck (Darmstadt, Germany).
107 Formic acid with a purity of 96% was also of HPLC grade (Tedia, U.S.A.). Water was obtained
108 from a Milli-Q water purification system (Millipore, Bedford, MA, U.S.A.).

109

110 *2.3. Preparation of tissue and sample solutions*

111

112 After softening by wrapping with water-soaked non-cellulose paper, the roots were cut into
113 small sections, embedded in cryomatrix^{TM} (Thermo Shandon Limited, U.K.), and then placed on
114 a cutting platform in the cryobar of a cryostat (Thermo Shandon As620 Cryotome, U.K.) at -20

115 ℃. Serial slices of 40 μm in thickness were cut at -10 ℃. Each sectioned tissue slice was

116 mounted directly to a non-fluorescent PET microscope steel frame slide (76 mm × 26 mm, 1.4
117 μm thick, Leica Microsystems, Germany). The slide was observed with a Leica LMD 7000
118 microscope system (Leica, Benshein, Germany) in fluorescence mode with a dichromatic mirror.
119 Microdissection was conducted by a DPSS laser beam at 349 nm wavelength, aperture of 10,
120 speed of 12 and power of 50-60 μJ under a Leica LMD-BGR fluorescence filter system at 10X
121 magnification. Tissue parts within an area of approximately 1×10^6 μm^2 were determined as the
122 investigated size and dissected separately under fluorescence inspection mode. The

123 microdissected tissues fell into caps of 500 μL microcentrifuge tubes (Leica, Germany) by
124 gravity.

125 The separated tissue part in each cap was transferred to the bottom of the tube through
126 centrifugation (Centrifuge 5415R, Eppendorf, Hamburg, Germany) at 10,000 rpm for 5 minutes.
127 100 μL methanol was added into each microcentrifuge tube. The tube was sonicated for 30 min
128 (CREST 1875HTAG ultrasonic processor, USA). The microcentrifuge tube was centrifuged again
129 for 10 min at 10,000 rpm, and 4 ℃. 90 μL of the supernatant was transferred to a glass insert with
130 plastic bottom spring (400 μL, Grace, HK) in a 1.5 mL brown HPLC vial (Grace, HK) and stored
131 at 4℃ for analysis.

132

133 *2.4. UHPLC-QTOF-MS analysis*

134

135 UHPLC-QTOF-MS analysis was performed on an Agilent 6540 ultra-high definition accurate
136 mass quadrupole time-of-flight spectrometer with UHPLC (UHPLC-QTOF-MS, Agilent

Technologies, USA). A UPLC C_{18} analytical column (2.1mm × 100 mm, I.D. 1.7μm, ACQUITY UPLC® BEH, Waters, U.S.A.) was used for separation, coupled with a C_{18} pre-column (2.1mm × 5mm, I.D. 1.7μm, VanGuardTM BEH, Waters, U.S.A.) at room temperature of 20 ℃. The mobile phase was a mixture of water (A) and acetonitrile (B), both containing 0.1% formic acid, with an optimized linear gradient elution as follows: 0–5 min, 10–35 % B; 5–25 min, 35–55 % B; 25–28 min, 55–85 % B; 28–30 min, 85–100 % B. The injection volume was 4 μL. The flow rate was set at 0.35 mL/min. The mass spectra were acquired in negative mode by scanning from 100 to 1700 in mass to charge ratio (*m/z*). The MS analysis was performed under the following operation parameters: dry gas temperature 300℃, dry gas (N_2) flow rate 5 L/min, nebulizer pressure 30 psi, Vcap 3000, nozzle voltage 500V, and fragmentor voltage 200V.

Data analysis was performed on Agilent MassHunter Workstation software-Qualitative Analysis (version B.04.00, Build 4.0.479.5, Service Pack 3, Agilent Technologies, Inc. 2011).

2.5. Method validation

Linearity, repeatability, stability, recovery, limits of detection (LODs), and limits of quantification (LOQs) were carried out under the above mentioned conditions. Chemical markers of saikosaponins a, c and d were accurately weighed and dissolved individually in methanol to produce standard stock solutions. Working solutions were prepared by diluting the stock solutions with methanol to yield a series of standard solutions in the concentration range of 60–3000 ng/mL, 30-1500 ng/mL and 110-5500 ng/mL for saikosaponins a, c and d, respectively. The internal standard ginsenoside Rb_1 was dissolved in methanol and added in sample solution with a concentration of 185 ng/mL.

The cortex was selected for testing the method repeatability, stability and recovery. Repeatability was evaluated by three replicated analyses of cortex at locations close to each other in the same tissue slice. Stability testing was performed on a sample solution after it had stood, at room temperature, for 0, 2, 4, 8, 12, 24 and 48h. Recovery study was conducted on a sample spiked with approximately 100% of known amounts of saikosaponins a, c and d in the samples, with three replicated analyses.

3. Results and discussion

3.1. Laser microdissection of tissues

171 The anatomical features of the roots from *B*. *scorzonerifolium*, *B*. *chinense* and *B*. *falcatum*
172 were found to be fundamentally identical, being composed of cork, cortex, phloem, cambium
173 and xylem. Cork consisted of several layers of flat cells. Cortex was narrow; a few oil canals
174 could be found in *B*. *chinense*. Phloem showed scattered oil canals, often with clefts. Cambium
175 was in a ring. Xylem was broad, occupying more than half of the radius of the root; vessels were
176 single or grouped; xylem fibres were well developed. Investigated by fluorescence mode, the
177 cork showed brown or reddish-brown fluorescence. The cortex, phloem and xylem sometimes
178 showed the same fluorescence, such as sample CH-12 of *B*. *scorzonerifolium* showing slight blue
179 fluorescence. Sometimes they showed different fluorescence. For example, sample CH-15 of *B*.
180 *chinense*, cortex showed bluish-yellow fluorescence; phloem showed slight bluish-purple
181 fluorescence and xylem showed yellowish-green fluorescence (Fig. 1). According to their tissue
182 structures and fluorescence characteristics, we separated cork, cortex, phloem and xylem tissues
183 for analysis of cellular metabolites.

184

185 *3.2. Identification of saikosaponins in microdissected tissues*

186

187 The chemical profiles of microdissected tissues were analyzed by UHPLC-QTOF-MS. The
188 representative total ions current (TIC) chromatograms of microdissected tissues from *B*. *chinense*
189 (CH-13) are showed in Fig.2. The TIC chromatograms of microdissected tissues from other
190 herbal samples were showed in the supplementary material (Fig. S1-S6). Sixty chromatographic
191 peaks were detected (Table 2). Peaks of saikosaponins, as the major type of secondary
192 metabolites in *Bupleurum* species, can be easily recognized by their generated molecular ions of

193 $[M-H]^-$ and/or $[M+HCOO]^-$. Peaks 3, 6 and 15 were identified as saikosaponins a, c and d,
194 respectively, by their accurate mass weight and corresponding mass ions; identifications were
195 confirmed by comparison with weight, mass ions and retention times of chemical markers. The
196 molecular ions of saikosaponins a (779.4566 *m/z*, $[M-H]^-$ and 825.4678 *m/z*, $[M+HCOO]^-$) , c
197 (971.5245 *m/z*, $[M+HCOO]^-$) and d (779.4589 *m/z*, $[M-H]^-$ and 825.4651 *m/z*, $[M+HCOO]^-$)
198 were detected in standard and sample solutions. Other detected peaks were tentatively identified
199 by their accurate mass data in comparison with reported references [16-20]. The detailed
200 information is shown in Table 2.

201 In the TIC chromatograms of microdissected tissues, the chemical profiles of the three
202 *Bupleurum* species varied (Table 3). For examples, peaks 5, 10, 14 were unique to *B*.
203 *scorzonerifolium* and peaks 52-60 were only detected in *B*. *falcatum*. Nevertheless, it is distinct
204 and consistent for all that cork and cortex contain more chromatographic peaks. For example,
205 sample CH-15, seventeen, sixteen, three and one peaks were detected in the cork, cortex, phloem
206 and xylem, respectively. Peaks 3, 6, 12, 15, 17 and 18, corresponding to saikosaponin c (peak 3),
207 saikosaponin a (peak 6), O-acetyl-saikosaponin a or O-acetyl-saikosaponin b1 (peak 12),
208 saikosapoin d (peak 15) and O-acetyl-saikosaponin d or O-acetyl-saikosaponin b_2 (peak 17&18)
209 were detected in the cork from all samples. Peaks 3, 6, 11 and 15 were found in the cortex of all
210 samples but no common chromatographic peaks were found in the phloem or xylem. A few
211 chromatographic peaks identified as saikosaponins were found in the phloem, especially from *B*.
212 *chinense*. For example, peaks 3, 4, 6, 11, 15, 17, 18, 26 and 37 were detected in the phloem from
213 sample CH-13. For *B*. *scorzonerifolium* and *B*. *falcatum*, in samples CH-11 and CH-TL2,
214 respectively, no saikosaponin peaks were detected; the peaks of saikosaponin a and saikosaponin
215 c were detected in sample CH-12; and saikosaponin a was found in sample CH-TL1. For the

xylem, although a few saikosaponin peaks could be found in the xylem from *B*. *chinense*, no saikosaponin peak was detected in the samples of *B*. *scorzonerifolium* and *B*. *falcatum*.

From Fig. 2, peaks of saikosaponins a (peak 3), c (peak 6) and d (peak 15) appear in chromatograms of many tissues. Their chemical structures with their calculated accurate mass weights are showed in Fig. 3. Also, peaks 3, 6 and 15 were found as the common peaks of cork and cortex. The results distinctly demonstrate that saikosaponins a, c and d are the main components in the tissues of roots from these three *Bupleurum* species. Therefore, more specific determinations of saikosaponins a (peak 3), c (peak 6) and d (peak 15) in the four specific tissues (cork, cortex, xylem, phloem) were carried out by UHPLC-QTOF-MS.

3.3. Method validation

Method validation parameters included linearity, repeatability, stability, recovery, limits of detection (LODs), and limits of quantification (LOQs) (Table 4). Linearity was examined with selected concentration range with 6 levels. The calibration curves were constructed by plotting the peak areas (y axis) of the analytes versus the concentration (x axis, ng/ml). Satisfactory linearity for the analysis of each component was obtained with correlation coefficients of determination (R^2), all greater than 0.9946. Repeatability was investigated by analyzing three individual samples on the same day; the relative standard deviation (RSD) of the results was 8.40% for saikosaponin a, 8.11% for saikosaponin c, and 7.34% for saikosaponin d. Stability testing was performed on a sample solution at time intervals of 0, 2, 4, 8, 12, 24 and 48h. The results showed that the RSD of saikosaponins a, c and d was 4.47%, 5.91% and 6.90% (n=7),

238 respectively. The average recovery was 77.81% for saikosaponin a, 79.5% for saikosaponin c
239 and 75.15% for saikosaponin d, within RSDs of 14.7%, 9.40%, and 12.2% for each analyte,
240 respectively. The LODs of these analytes, calculated by a signal-to-noise (S/N) of 3, were 6.00,
241 10.0, and 11.0 ng/mL for saikosaponins a, c and d, respectively; while the LOQs of these
242 analytes, calculated by the S/N of 10, were 73.65, 79.86, and 177.26 ng/mL, respectively.

243 When the tissue was dissected by LMD, a few tissue parts will be burned to induce the loss
244 of tissue which influences the microdissected areas of each tissue. Sometimes, the clefts in cortex
245 varied in different regions of transverse section. These factors influence the values of RSD in the
246 test of repeatability and recovery. In our previous study on the determination of alkaloids in
247 microdissected tissues and cells of the stem of *Sinomenium acutum*, the recovery ranges were
248 also vast from 69.99 to 134.39% with RSDs from 9.00% to 11.08% [7]. Thus, considering the
249 tiny sampling for analysis and the above factors, the present method validation for determination
250 of saikosaponins in various tissues is acceptable.

251 For quantitative analysis of herbal samples, it is hard to get the "blank samples" without
252 detected components and with similar sample background for evaluating the matrix effect. In the
253 present study, an internal standard substance ginsenoside Rb_1 with the same concentration was
254 added in the analyzed sample solutions for investigating the matrix effect. The result showed that
255 the RSD of detected peak area of ginsenoside Rb_1 was 2.64%, which indicated that the matrix
256 effect can be neglected.

257

258 *3.4. Comparison of the contents of saikosaponins a, c and d in various tissues*

259

The contents of saikosaponins a, c and d in cork, cortex, phloem and xylem from various samples were determined using UHPLC-QTOF-MS (Table 5). Sample solutions of laser microdissected tissues were diluted within linearity before injection. The contents of saikosaponins a, c and d in various tissues from three *Bupleurum* species varied greatly. For example, saikosaponins a, c and d couldn't be detected in the phloem and xylem from sample CH-11 and the cork contained much higher contents of saikosaponins a, c and d than those of cortex. In detail, the cork from sample CH-11 contained saikosaponins a, c and d as 403.53 ng, 208.10 ng and 269.93 ng in each unit area of about 10000 μm^2 while the cortex contained 43.82 ng, 23.21 ng and 27.18 ng per unit area, respectively. For the same tissue from different samples, the contents of saikosaponins a, c and d also varied greatly. For example, for cork tissue from three *Bupleurum* species, saikosaponins a, c and d were detected in amounts varying from 235.44ng to 1028.96 ng, 129.58 ng to 1567.82 ng and 138.11 ng to 1091.51 ng per unit area, respectively.

The amount of each saikosaponin in various tissues is graphed in Fig. 4. Generally, the contents of saikosaponins a, c and d in the cork and cortex were much higher than in phloem and xylem. For *B. scorzonerifolium* and *B. falcatum*, the cork contained more of saikosaponins a, c and d than did the cortex; while for *B. chinense*, the cortex contained higher contents of saikosaponins a, c and d than did the cork. Notably, the main site of accumulation of saikosaponins in *B. chinense* was different than in *B. scorzonerifolium* and *B. falcatum*. In other words, saikosaponins are stored in the cortex in *B. chinense* and in the cork in *B. scorzonerifolium* and *B. falcatum*. Overall, in the four tissues investigated here, the total content of saikosaponins a, c and d was higher in *B. chinense* and *B. falcatum* than in *B. scorzonerifolium*.

Specially, the sample of *B*. *chinense* from the Lingqiu County produces plants with the most saikosaponins.

3.5. Different accumulated and stored sites of saikosaponins in Bupleurum species

Samples CH-11 and CH-12 of *B*. *scorzonerifolium* were cultivated for two years and three years, respectively. Samples CH-13, CH-15 and CH-17 of *B*. *chinense* were collected from the same cultivation place with the same age yet were genetically different. Two representative samples from CH-TL of *B*. *falcatum* were also analyzed. All the different sample sources were collected for analyzing the accumulated site of saikosaponins in various tissues. The herbal samples of *B*. *scorzonerifolium* and *B*. *chinense* were collected from the same cultivation site; thus, their growing conditions were quite similar. The classic plant taxonomy based on morphological features of the plants has demonstrated that *B*. *scorzonerifolium* and *B*. *falcatum* have a close relationship [21, 22]. The recent studies on cell type-specific transcriptome profiles have demonstrated that complex gene regulatory networks occur at the cellular level and that cell type-specific expression of many proteins is involved in secondary metabolism, many of which were masked during whole-organ analysis [23]. From above, it can be deduced that the roots of *B*. *scorzonerifolium* and *B*. *falcatum* may have the similar cell type-specific transcriptional profiles. The site of saikosaponin synthesis may be the cork in *B*. *scorzonerifolium* and *B*. *falcatum* but the cortex in *B*. *chinense*. Thus, the sites where saikosaponins are accumulated in the roots of *B*. *scorzonerifolium* and *B*. *falcatum* are the same but differ from *B*. *chinense*. In further study, cell

type-specific transcriptional profiling of various tissues from *B*. *scorzonerifolium*, *B*. *falcatum* and *B*. *chinense* will be carried out for further revealing the difference.

3.6. Guidelines for quality evaluation of Radix Bupleuri by morphological features

Mapping of the active components in the root tissues of three officially used *Bupleurum* species can correlate the morphological features and active components of Radix Bupleuri. Morphological features of Radix Bupleuri include shape, size, color, and texture, both external and internal (e.g., cross-sections). The cross-section is a key point for morphological features of Radix Bupleuri as it reveals tissue structures and arrangement. For example, the root of *B*. *scorzonerifolium*, in the surface, cork showing as outside bark; cambium showing as a ring; radiated structures form from rays showing as radiated striations; the bark narrow as the cortex and phloem narrow; the wood part wide due to a broad xylem, occupying more than half of the radius of the root; and so on (Fig. 5). The present study has revealed that saikosaponins mainly accumulate in cork and cortex. Therefore, Radix Bupleuri with thinner main root and more lateral roots would contain higher active components, thus be of better quality in clinical application. Recently, an HPLC method for determination of saikosaponins a and d in the roots of *B*. *chinense* also showed that the lateral roots contained higher contents of saikosaponin a and d than those of the main root [24]. Farmers will be able to adjust their cultivation techniques and harvest time to produce the most potent herbs. When processing Radix Bupleuri, the lateral roots should be kept for medicinal use.

4. Conclusions

In the present study, an approach by the combination of LMD and UHPLC-QTOF-MS was established to profile the chemicals in tissues of the roots of *B. scorzonerifolium*, *B. falcatum* and *B. chinense*. The exact tissues were separated by LMD and analyzed using UHPLC-QTOF-MS with characterization and determination of saikosaponins. The study showed that the cork and cortex from all three species contained more types of saikosaponins and higher contents of saikosaponins a, c and d than did the phloem and xylem. The interesting result is that cork is the site of the highest accumulation of saikosaponins in *B. scorzonerifolium* and *B. falcatum*; while cortex is the site of highest concentration of saikosaponins in *B. chinense* among the four tissues studied here. Results revealed the relationship between saikosaponins and herbal tissues, which indicated that Radix Bupleuri with thinner main root and more lateral roots would contain higher active components, thus be of better quality in clinical application. The established relationship also provides the valuable information for the cultivation, harvest and processing of *Bupleurum* spp. for production of Bupleuri Radix.

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References

[1] M. Wink, Functions of plant secondary metabolites and their exploitation in biotechnology, CRC Press (Taylor & Francis Group), Boca Raton, USA, 1999.

[2] H.W. Heldt, B. Piechulla, Plant biochemistry, Academic Press, San Diego, USA, 2010.

[3] A. Oikawa, K. Saito, Metabolite analyses of single cells, Plant J. 70 (2012) 30-38.

[4] A. Amantonico, P.L. Urban, R. Zenobi, Analytical techniques for single-cell metabolomics: state of the art and trends, Anal. Bioanal. Chem. 398 (2010) 2493-2504.

[5] S. Kueger, D. Steinhauser, L. Willmitzer, P. Giavalisco, High-resolution plant metabolomics: from mass spectral features to metabolites and from whole-cell analysis to subcellular metabolite distributions, Plant J. 70 (2012) 39-50.

[6] Y.J. Lee, D.C. Perdian, Z.H. Song, E.S. Yeung, B.J. Nikolau, Use of mass spectrometry for imaging metabolites in plants, Plant J. 70 (2012) 81-95.

[7] L. Yi, Z.T. Liang, Y. Peng, X. Yao, H.B. Chen, Z.Z. Zhao, Tissue-specific metabolite profiling of alkaloids in Sinomenii Caulis using laser microdissection and liquid chromatography-quadrupole/time of flight-mass spectrometry, J. Chromatogr. A 1248 (2012) 93-103.

[8] Z.T. Liang, T.T. Sham, G.Y. Yang, L. Yi, H.B. Chen, Z.Z. Zhao, Profiling of secondary metabolites in tissues from Rheum palmatum L. using laser microdissection and liquid chromatography mass spectrometry, Anal. Bioanal. Chem. 405(2013) 4199-4212.

[9] State Pharmacopoeia Committee, Pharmacopoeia of the People's Republic of China, China Medical Science and Technology Press, Beijing, China, 2010, p181.

[10] The Society of Japanese Pharmacopoeia, The Japanese Pharmacopoeia, 16th edition (English Version), The Ministry of Health, Labor and Welfare, Tokyo, Japan, 2011, pp. 1613-1614.

[11] Z.Z. Zhao, P.G. Xiao, Encyclopedia on Contemporary Medicinal Plants, Vol. 1 (English version), World Publishing Corporation, Shanghai, China, 2009, pp. 156-159.

[12] S.L. Pan, Bupleurum species-scientific evaluation and clinical applications, CRC Press (Taylor & Francis Group), Boca Raton, USA, 2006.

[13] L.L. Tan, X. Cai, Z.H. Hu, X.L. Ni, Localization and dynamic change of saikosaponin in root of *Bupleurum chinense*, J. Integr. Plant Biol. 50 (2008) 951-957.

[14] X.W. Du, M.Y. Liu, A histochemical study of saikosaponins, China J. Chin. Mat. Med. 17 (1992) 261-263.

[15] Z.T. Liang, M.J. Qin, Z.T. Wang, Study on the constituents of the roots of *Bupleurum marginatum*, J Chin. Pharm. Univ. 34 (2003) 305-358.

[16] S.L. Pan, Q.S. Shun, Q.M. Pai, X.S. Bao, The coloured atlas of the medicinal plants from genus Bupleurum in China. Shanghai Science and Technology press, Shanghai, China, 2002.

[17] J.J. Zhou, G.R. Xie, X.J. Yan, Chemical components of source plants in traditional Chinese medicine, Science press, Beijing, China, 2009.

[18] H.Q. Huang, X. Zhang, M. Lin, Y.H. Shen, S.K. Yan, W.D. Zhang, Characterization and identification of saikosaponins in crude extracts from three *Bupleurum* species using LC-ESI-MS, J. Sep. Sci. 31 (2008) 3190-3201.

[19] Y.Y. Yang, Y.Z. Tang, C.L. Fan, H.T. Luo, P.R. Guo, J.X. Chen, Identification and determination of the saikosaponins in Radix Bupleuri by accelerated solvent extraction combined with rapid-resolution LC-MS, J. Sep. Sci. 33 (2010) 1933-1945.

[20] Z.T. Liang, J. Zhang, G.Y. Yang, H.B. Chen, Z.Z. Zhao, Chemical profiling and histochemical analysisof *Bupleurum marginatum* roots from different growing areas of Hubei province, Acta Pharm. Sin. B 3 (2013) 53-64.

[21] R.H. Shan, M.L. She, Umbelliferae, in: Committee of Flora of China (Eds), Flora of China, Vol. 55, Science press, Beijing, China, 1979, pp. 215-236.

[22] P. Su, C.Q. Yuan, M.L. She, Y.Z. Liu, B.R. Xiang, D.K. An, Numerical taxonomy of medicinal *Bupleurum* species in China. Acta Bot. Boreal –Occident Sin.18 (1998) 277-283.

[23] E.D. Rogers, T. Jackson, A. Moussaieff, A. Aharoni, P.N. Benfey, Cell type-specific transcriptional profiling: implications for metabolite profiling, Plant J. 70 (2012) 5-17.

[24] J. Meng, X.F. Chen, W.Y. Yang, R.Y. Yao, J.H. Song, Z.F. Li, X.W. Yang, Determination of saikosaponin a and d in taproot and lateral root of *Bupleurum chinense* DC., Chin. J. Pharma. Anal. 33 (2013) 1218-1222.

Figure Legends

Fig. 1. Microscopic characteristics of the roots of *Bupleurum*. *scorzonerifolium* Willd. (CH-12, A), *B. chinese* DC. (CH-15, B) and *B. falcatum* L. (CH-TL1, C). i: Under normal light

407 microscope; ii: Under fluorescence mode with dichromaticmirror. CK: cork; CT: cortex; PH:
408 phloem; XY: xylem; OC: oil canals.

409 **Fig. 2.** The representative total ions current (TIC) chromatograms of microdissected tissues
410 from *B. chinense* (CH-13). The peak No. referred to Table 2. GS: ginsenoside Rb_1.

411 **Fig. 3.** The chemical structures of saikosaponin a, c and d.

412 **Fig. 4.** The changing trend of saikosaponin a (Ssa), c (Ssc) and d (Ssd) in various tissues of
413 *Bupleurum* species.

414 **Fig. 5.** The cross-section of the root of *Bupleurum scorzonerifolium* revealing the relationship
415 between morphological features and tissue structures.

416

417 **Table Legends**

418 **Table 1** Sample information for the analysis.

419 **Table 2** Characteristics of secondary metabolites of various tissues from the roots of *Bupleurum*
420 *scorzonerifolium*, *B. chinense* and *B. falcatum* by UHPLC-Q-TOF/MS.

421 **Table 3** The distribution of second metabolites in various tissues from different herbal samples.
422 **Table 4** Method validation data of the detected chemicals.

423 **Table 5** Contents of saikosaponin a (Ssa), c (Ssc) and d (Ssd) in the laser dissected tissues from
424 *Bupleurum scorzonerifolium*, *B. chinense* and *B. falcatum*.

425

426

Table 1

Sample information for the analysis.

Species	Sample No.	Source	Date of collection
Bupleurum scorzonerifolium Willd.	CH-11	Species from Mingshui county, Hei Long Jiang Province, cultivated for two years in Taigu county , Shan Xi Province	2010.09.13
	CH-12	Species from Mingshui county, Hei Long Jiang Province, cultivated for three years in Taigu county , Shan Xi Province	2010.09.13
Bupleurum chinense DC.	CH-13	Species from Lingqiu county, Shan Xi Province, cultivated for three years in Taigu county , Shan Xi Province	2010.09.13
	CH-15	Species from Zuoquan county, Shan Xi Province, cultivated for three years in Taigu county , Shan Xi Province	2010.09.13
	CH-17	Species from Lingchuan county, Shan Xi Province, cultivated for three years in Taigu county , Shan Xi Province	2010.09.13
Bupleurum falcatum L.	CH-TL	Plantation of Shenzhen Tsumura Medicine Co., LTD	2010.06.15

455

456 **Table 2**

457 Characteristics of secondary metabolites of various tissues from the roots of *Bupleurum scorzonerifolium*, *B. chinense* and *B. falcatum* by
458 UHPLC-Q-TOF/MS.

Peak No.	Retention Time (min)	Molecular formula	$[M-H]^-$ (*m/z*) (Mass accuracy, ppm)	$[M+HCOO]^-$ (*m/z*) (Mass accuracy, ppm)	Identification
1*	2.80	$C_{21}H_{36}O_{11}$	463.2187 (0.2)	509.2244 (0.4)	Unknown
2	6.82	$C_{42}H_{68}O_{14}$	795.4546 (1.0)	841.4603	Bupleuroside VI
3	7.52	$C_{48}H_{78}O_{17}$		971.5245 (2.4)	**Saikosaponin c**
4	7.63	$C_{48}H_{80}O_{17}$		973.5380 (0.2)	Saikosaponin f
5	8.28	$C_{42}H_{68}O_{14}$	795.4566 (3.0)	841.4605 (1.2)	Bupleuroside VI isomer
6	10.52	$C_{42}H_{68}O_{13}$	779.46 (1.3)	825.4678 (3.6)	**Saikosaponin a**
7	11.60	$C_{44}H_{70}O_{14}$		867.4740 (-0.8)	O-acetyl-saikosaponin a or O-acetyl-saikosaponin b_1
8	11.72	$C_{44}H_{70}O_{14}$	821.4702 (0.9)	867.4748 (0)	O-acetyl-saikosaponin a or O-acetyl-saikosaponin b_1
9	12.25	$C_{42}H_{68}O_{13}$	779.4588 (-0.1)	825.4678 (3.6)	Saikosaponin b_2
10	12.73	$C_{47}H_{76}O_{16}$	895.5048 (-1.3)	941.5130 (1.5)	Rotundioside G
11	13.22	$C_{42}H_{68}O_{12}$		809.4705 (1.2)	Saikosaponin e
12	14.12	$C_{44}H_{70}O_{14}$	821.4690 (-0.3)	867.4748 (0)	O-acetyl-saikosaponin a or O-acetyl-saikosaponin b_1
13	14.76	$C_{17}H_{22}N_9O_{13}$	558.1188 (0.2)		Unknown
14	15.20	$C_{47}H_{76}O_{17}$	911.5015 (0.5)	957.5080 (1.5)	Rotundifolioside V
15	15.58	$C_{42}H_{68}O_{13}$	779.4589 (0.2)	825.4651 (0.9)	**Saikosaponin d**
16	16.95	$C_{45}H_{70}O_{16}$	865.4586 (-0.5)		Malonylsaikosaponin d
17	17.30	$C_{44}H_{70}O_{14}$	821.4700 (0.7)	867.4737 (-1.1)	O-acetyl-saikosaponin d or O-acetyl-saikosaponin b_2
18	19.81	$C_{44}H_{70}O_{14}$	821.4699 (0.6)	867.4764 (1.6)	O-acetyl-saikosaponin d or O-acetyl-saikosaponin b_2
19	29.52	$C_{24}H_{29}O_7$	427.1784 (5.7)		Unknown
20	29.69	$C_{23}H_{24}O_5$	379.1588 (3.7)		Unknown
21	15.63	$C_{18}H_{34}O_4$	313.2379 (-0.5)		Unknown
22	22.15	$C_{18}H_{32}O_3$	295.2278 (0.1)		Unknown
23	22.51	$C_{18}H_{33}O_3$	295.2278 (0.1)		Unknown

24	9.92	$C_{48}H_{78}O_{18}$	941.5098 (-1.7)	987.5169 (-0.1)	3β,16α,28,30-tetrahydroxy-olean-11, 13(18)-dien-3β-yl-β-D-glucopyranosyl-(1→6)-β-D-[α-L-rhamnopyranosyl-(1→4)]-β-D-glucopyranoside
25	14.30	$C_{48}H_{78}O_{18}$	941.5123 (0.8)	987.5171 (0.1)	3β,16β,23-trihydroxy-olean-13, 28-epoxy-olean-11-en-3β-yl-[β-D-glucopyranosyl-(1→2)]-[β-D glucopyranosyl-(1→3)]-β-D-fucopyranoside
26	4.64	$C_{42}H_{66}O_{15}$	809.4369 (4.0)	855.4465 (8.1)	Saikosaponin x
27	5.12	$C_{56}H_{77}O_{16}$	1003.5081 (-5.8)		Unknown
28	5.89	$C_{42}H_{70}O_{14}$	797.4776 (8.3)	843.4789 (4.1)	Hydroxysaikosaponin a
29	6.12	$C_{42}H_{70}O_{14}$	797.4757 (6.4)	843.4794 (4.6)	Hydroxysaikosaponin d
30	6.30	$C_{48}H_{78}O_{18}$		987.5241 (7.1)	Saikosaponin n
31	8.02	$C_{48}H_{78}O_{17}$		971.5351 (12)	Saikosaponin BK_1 isomer
32	11.68	$C_{45}H_{70}O_{16}$	865.4690 (0.9)		malonylsaikosaponin b_1
33	13.01	$C_{44}H_{70}O_{14}$		867.4811 (6.3)	O-acetyl-saikosaponin a or O-acetyl-saikosaponin b_1
34	14.26	$C_{44}H_{70}O_{14}$	821.4749	867.4809 (6.1)	O-acetyl-saikosaponin a or O-acetyl-saikosaponin b_1
35	16.28	$C_{42}H_{66}O_{13}$	777.4391 (-4.0)	823.4438 (-4.7)	Unknown
36	10.98	$C_{17}H_{26}O_4$	293.1794 (3.6)		Unknown
37	3.65	$C_{53}H_{86}O_{24}$	1105.5424 (-1.2)	1151.5484 (-0.7)	Saikosaponin v
38	3.83	$C_{47}H_{76}O_{19}$	943.4923 (1.5)	989.4968 (0.5)	Saikosaponin v1
39	4.90	$C_{42}H_{68}O_{14}$	795.4514 (-2.2)	841.4595 (0.4)	Saikosaponin l
40	5.40	$C_{48}H_{78}O_{17}$	925.4802 (36.4)		Saikosaponin BK_1
41	5.58	$C_{23}H_{45}O_{16}$	576.2509 (12.6)		Unknown
42	6.57	$C_{48}H_{78}O_{18}$		987.5170 (0)	Rotundioside N
43	7.33	$C_{47}H_{72}O_{19}$	939.4605 (1.0)		Unknown
44	17.56	$C_{44}H_{70}O_{14}$	821.4696 (0.3)	867.4751 (0.3)	O-acetyl-saikosaponin d
45	22.34	$C_{46}H_{72}O_{15}$	863.4769 (-2.9)	909.4861 (0.8)	diacetyl- saikosaponin d
46	21.67	$C_{46}H_{72}O_{15}$		909.4860 (0.7)	diacetyl- saikosaponin a
47	3.88	$C_{25}H_{24}O_{12}$	515.1186 (-0.9)		Unknown
48	4.20	$C_{25}H_{24}O_{12}$	515.1185 (-0.8)		Unknown
49	4.36	$C_9H_{16}O_4$	187.0973 (-0.3)		Unknown
50	7.28	$C_{18}H_{34}O_5$	329.2333 (0)		Unknown
51	7.95	$C_{51}H_{80}O_{20}$	1011.5170 (0)		Unknown
52	8.97	$C_{43}H_{72}O_{14}$	811.4848 (-0.1)	857.4905 (-0.1)	Saikosaponin b_4

53	12.22	$C_{36}H_{58}O_8$		663.4106 (-0.8)	Prosaikogenin F
54	17.58	$C_{44}H_{70}O_{14}$	821.4681 (-1.2)	867.4744 (-0.4)	O-acetyl-saikosaponin d
55	17.89	$C_{36}H_{58}O_8$		663.4112 (-0.2)	Prosaikogenin D
56	4.64	$C_{42}H_{68}O_{14}$	795.4550 (1.4)	841.4597 (0.6)	Saikosaponin l isomer
57	7.15	$C_{42}H_{68}O_{14}$	795.4537 (0.1)	841.4602 (1.1)	Bupleuroside VI isomer
58	9.10	$C_{50}H_{80}O_{18}$		1013.5329 (0.2)	O-acetyl-saikosaponin c
59	9.55	$C_{42}H_{68}O_{13}$		825.4646 (0.4)	Saikosaponin b_1
60	13.57	$C_{44}H_{70}O_{13}$	805.4755 (1.1)	851.4801 (0.3)	O-acetyl-saikosaponin e

* The peak numbers referred to Figure 2.

Table 3 The distribution of second metabolites in various tissues from different herbal samples

Sample No.	Herbal Tissues / Peak No. [*1]			
	Cork	Cortex	Phloem	Xylem
CH-11	Peaks 1-20	Peaks 1, 3, 4, 6, 11, 13-15, 19, 20	Peaks 1, 13, 19-23	Peaks 1, 13, 19-23
CH-12	Peaks 1, 3-20, 24	Peaks 1, 3, 4, 6-8, 10-20, 24	Peaks 1, 3, 6, 13, 19, 20	Peaks 13, 19, 20
CH-13	Peaks 3, 4, 6, 9, 11-13, 15-18, 26-35, 47	Peaks 3, 4, 6, 9, 11-13, 15-18, 26-36, 47	Peaks 3, 4, 6, 11, 13, 15, 17, 18, 26, 36	Peaks 3, 4, 6, 13, 15, 26, 36
CH-15	Peaks 3, 4, 6-9, 11, 12, 15-18, 29, 30, 33-35	Peaks 3, 4, 6-9, 11, 12, 15, 17, 18, 29, 30, 33, 35, 36	Peaks 6, 15, 36	Peaks 36
CH-17	Peaks 1, 3, 4, 6-9, 11, 12, 15, 17, 18, 26, 30, 34, 37-45	Peaks 1, 3, 4, 6-9, 11, 12, 15, 17, 18, 26, 30, 34-37, 43-46	Peaks 3, 4, 6-9, 11, 15, 17, 18, 36	Peaks 3, 4, 6-8, 11, 15, 17, 18, 36
CH-TL1	Peaks 1, 3, 6-8, 11, 12, 15-18, 26, 28-30, 35-37, 39, 42, 47-55	Peaks 3, 6-8, 11, 12, 15, 17, 18, 29, 30, 36, 50, 53	Peaks 6, 50	Peak 50
CH-TL2	Peaks 2, 3, 6-9, 11, 12, 15-18, 20, 28-30, 34, 35, 37, 39, 50, 51, 54, 56-60	Peaks 2, 3, 6-9, 11-13, 15-20, 35, 50, 60	Peaks 13, 19, 20, 50	Peaks 13, 22, 23, 19, 20, 50

[*1] The peak numbers referred to Table 2 and Figure 2.

487 **Table 4**

488 Method validation data of the detected chemicals.

Chemicals	Calibration curve	R^2	Repeatability (n=3, RSD, %)	Stability (48h, RSD, %)	Recovery (n=3, %, Mean±SD)	LODs (ng mL^{-1})	LOQs (ng mL^{-1})
Saikosaponin a	y=204.32x+1446.1	0.9983	8.40	4.47	77.81±11.44	6.00	73.65
Saikosaponin c	y=735.47x-14488	0.9946	8.11	5.91	79.50±7.47	10.00	79.86
Saikosaponin d	y=146.63x+2780.1	0.9984	7.34	6.90	75.15±9.19	11.00	177.26

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Table 5

Contents of saikosaponin a (Ssa), c (Ssc) and d (Ssd) in the laser dissected tissues from *Bupleurum scorzonerifolium*, *B. chinense* and *B. falcatum*.

Sample No.	Tissue	Total microdissected areas (μm^2)	Ssa (ng)	Ssc (ng)	Ssd (ng)
CH-11	Cork	1032791	403.53	208.10	269.93
	Cortex	1183872	43.82	23.21	27.18
	Phloem	1302152	-*	-	-
	Xylem	1281726	-	-	-
CH-12	Cork	1164513	235.44	204.59	138.11
	Cortex	1190628	53.18	99.45	47.75
	Phloem	1315233	-	9.11	-
	Xylem	1364065	-	-	-
CH-13	Cork	1026844	411.26	373.31	459.29
	Cortex	1181483	758.16	535.78	962.38
	Phloem	1014707	195.24	152.07	337.46
	Xylem	1034935	28.46	24.00	21.35
CH-15	Cork	1058668	402.75	173.49	442.74
	Cortex	1071292	461.83	219.36	510.45
	Phloem	1118554	17.21	8.24	-
	Xylem	1043006	-	-	-
CH-17	Cork	1040131	367.95	129.58	352.02
	Cortex	1065687	718.87	205.42	894.51
	Phloem	1049056	118.27	25.31	173.60
	Xylem	1179142	83.33	21.63	101.97
CH-TL1	Cork	1051569	718.87	650.37	1091.51
	Cortex	1007260	316.01	206.21	336.69
	Phloem	1031779	115.24	9.90	-
	Xylem	1002570	-	-	-
CH-TL2	Cork	1150312	1028.96	567.82	981.80
	Cortex	1210182	267.77	113.90	255.92
	Phloem	1202215	-	-	-
	Xylem	1293177	-	-	-

*under detection limit

Highlights

- Tissue-specific metabolite profiles from different species by characterization and determination of main components
- Tissue-specific metabolite profiling correlates morphology with quality of Bupleuri Radix
- The correlation can be applied for evaluating the qualtiy of Bupleuri Radix by morphological features

Figure(s)

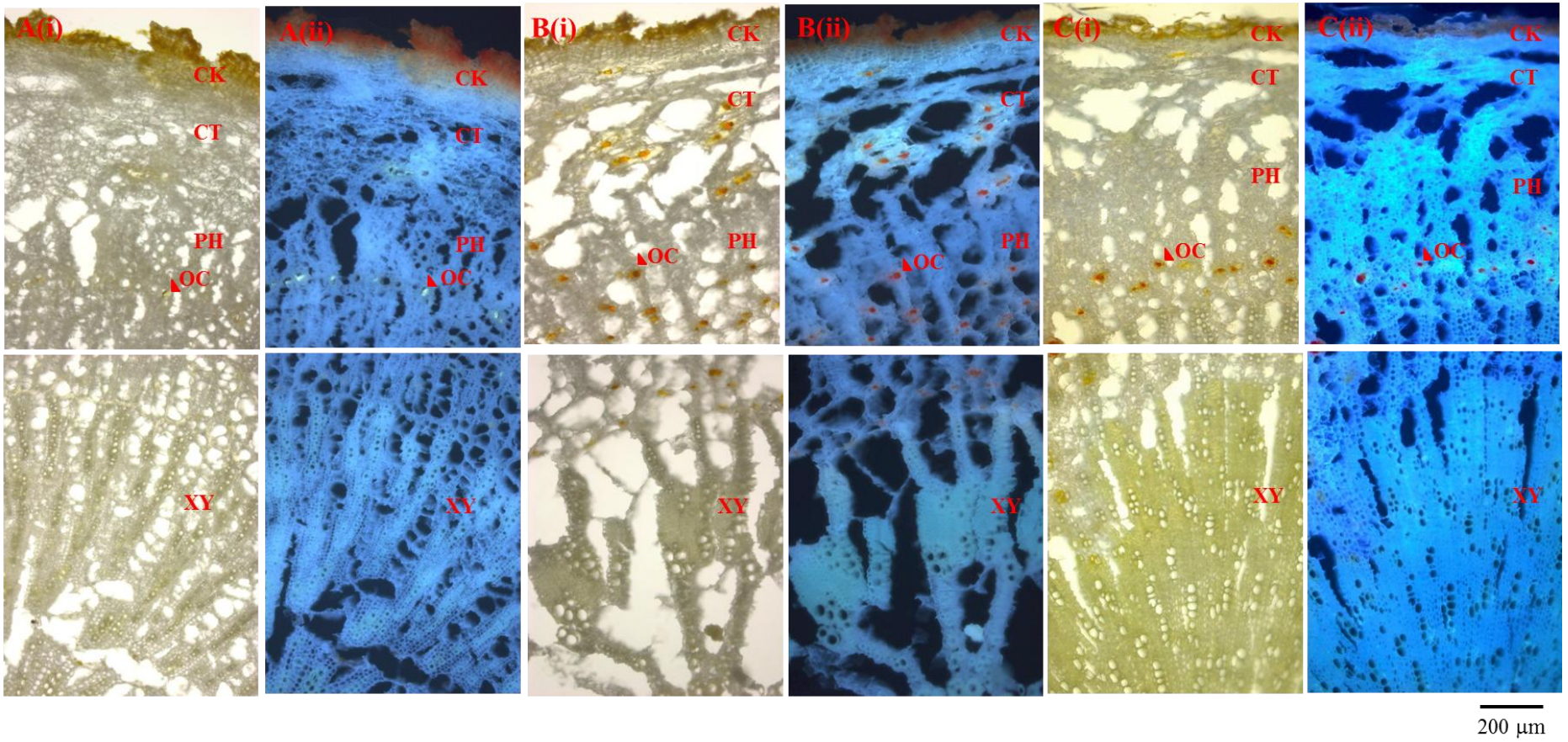


Fig. 1. Microscopic characteristics of the roots of *Bupleurum*. *scorzonerifolium* Willd.(CH-12, A), *B. chinese* DC. (CH-15, B) and *B. falcatum* L. (CH-TL1, C). i: Under normal light microscope; ii: Under fluorescence mode with dichromaticmirror. CK: cork; CT: cortex; PH: phloem; XY: xylem; OC: oil canals.

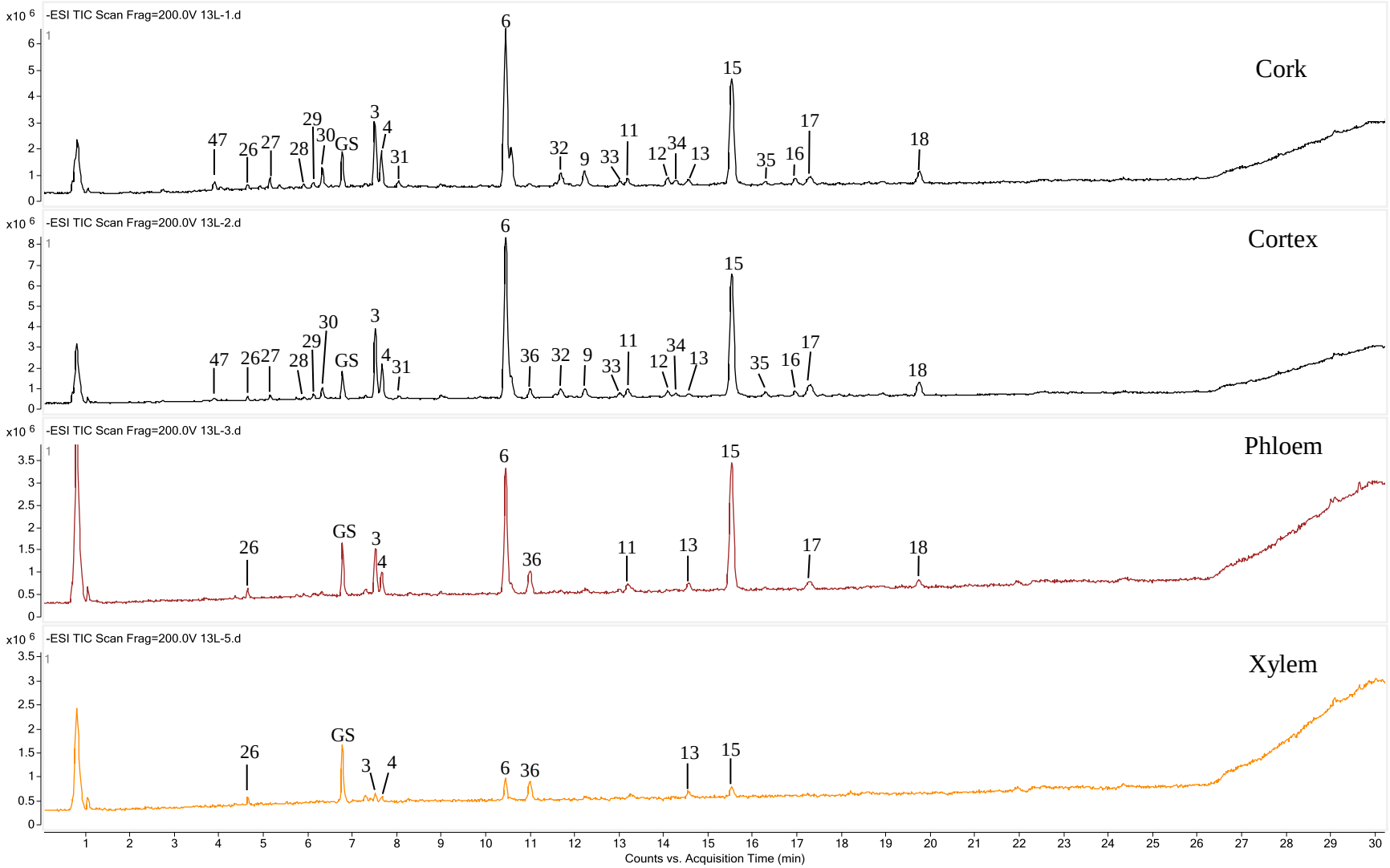
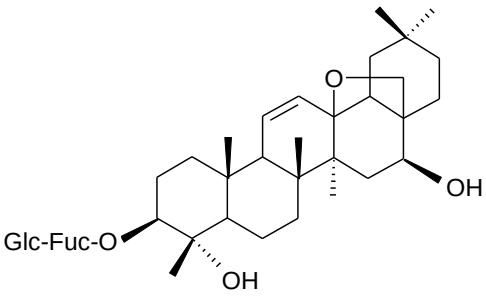
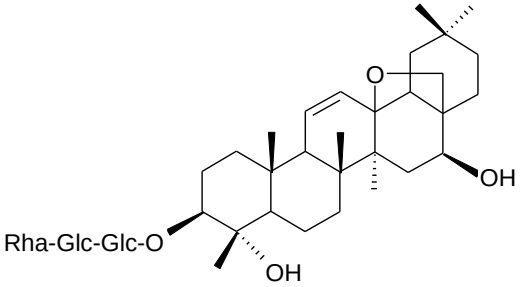


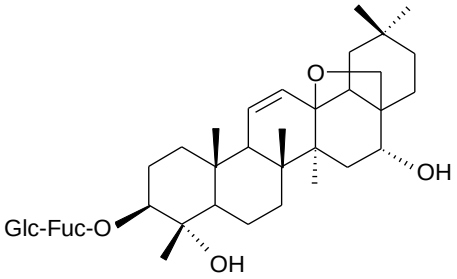
Fig. 2. The representative total ions current (TIC) chromatograms of microdissected tissues from *B. chinense* (CH-13). The peak No. referred to Table 2. GS: ginsenoside Rb_1.



saikosaponin a
$C_{42}H_{68}O_{13}$
MW 780.4660



saikosaponin c
$C_{48}H_{78}O_{17}$
MW 926.5239



saikosaponin d
$C_{42}H_{68}O_{13}$
MW 780.4660

Fig. 3. The chemical structures of saikosaponin a, c and d.

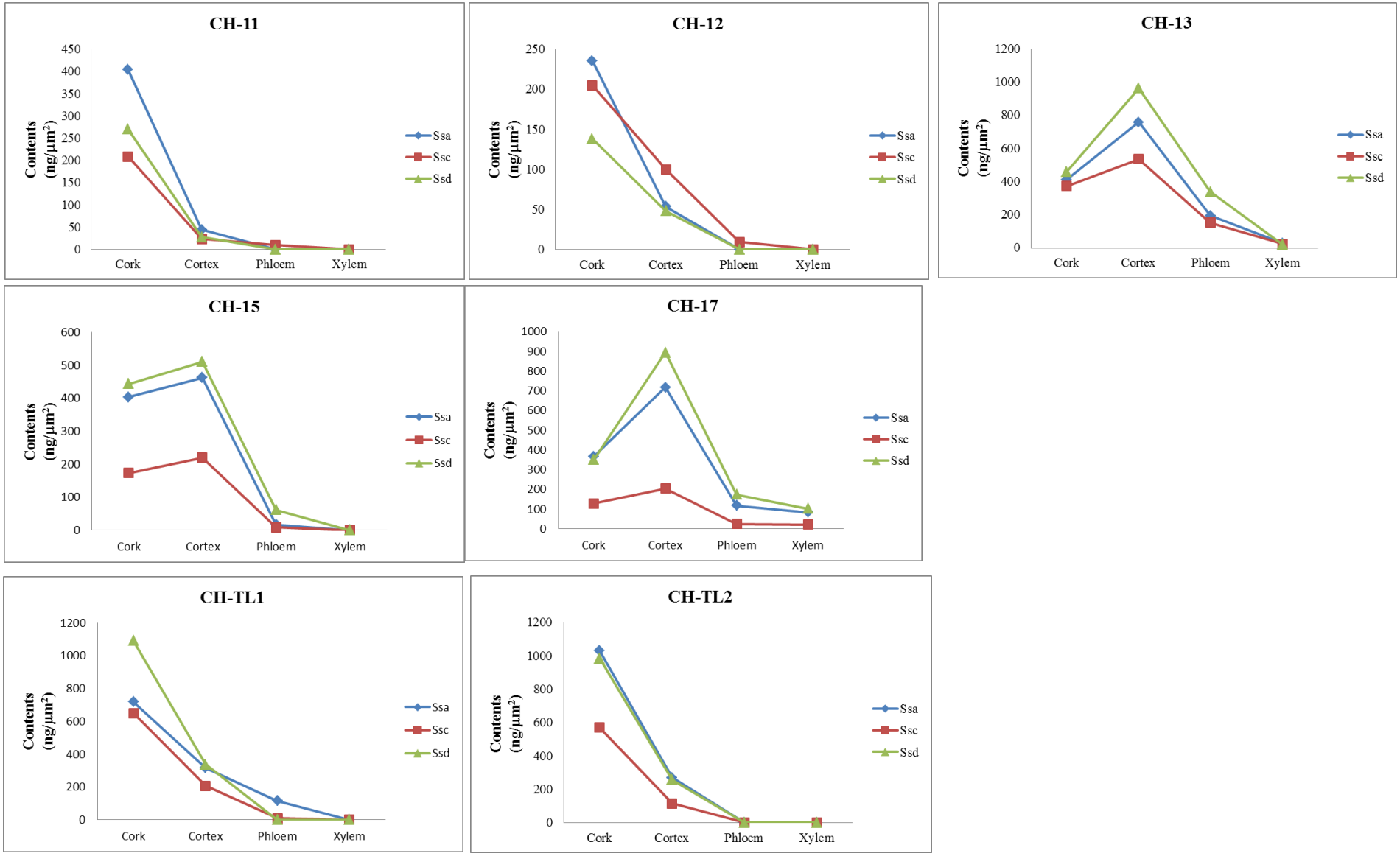


Fig. 4. The changing trend of saikosaponins a (Ssa), c (Ssc) and d (Ssd) in various tissues of *Bupleurum* species.

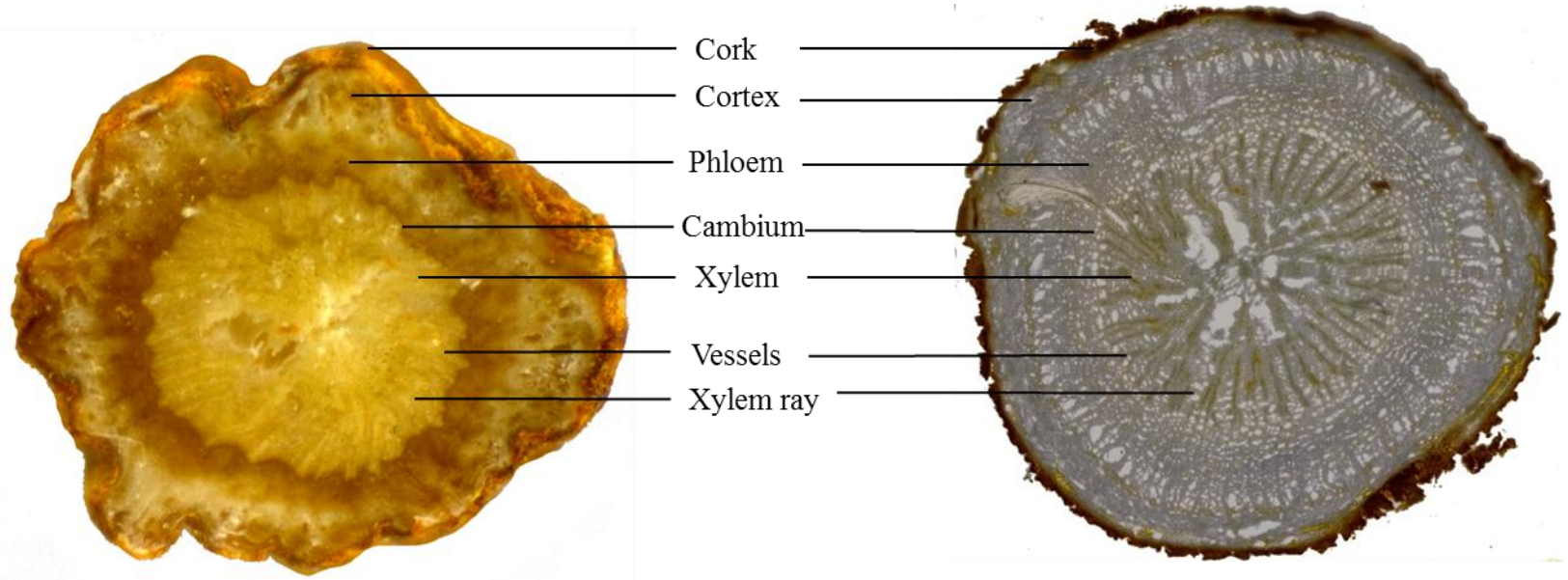


Fig. 5. The cross-section of the root of *Bupleurum scorzonerifolium* revealing the relationship between morphological features and tissue structures.