Simultaneous analysis of anthocyanins and flavonols in petals of lotus (Nelumbo) cultivars by high-performance liquid chromatography-photodiode array detection/electrospray ionization mass spectrometry

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\begin{abstract}
A fast and reliable HPLC method for the simultaneous separation of anthocyanins and flavonols in lotus petals was developed based on the study of four candidate solvent systems. Fifteen flavonoids were identified by high-performance liquid chromatography with photodiode array detection/mass spectrometry. Among them, two anthocyanins and nine flavonols were discovered in lotus petals for the first time. This work is valuable for both the hybrid breeding on lotus oriented to flower color and the utilization of lotus petals as functional food materials.
\end{abstract}

\section{Introduction}
Lotus, also known as Nelumbo, is a perennial aquatic herb, which consists of two species, \textit{N. nucifera} Gaertn. and \textit{N. lutea} (Willd.) Pers. based on the morphological characters. It is one of the most important ornamental and economic plants grown widely in Asia, Australia and North America. By 2005, the number of lotus cultivars had exceeded 600\textsuperscript{[1,2]}, Lotus flowers could be made into liquor and tea in folk diet\textsuperscript{[3,4]}. The petals have also been found useful in the therapies of hematemesis, eczema, weak spleen and stomach trouble\textsuperscript{[5]}. Anthocyanins and flavonols are two major subclasses of flavonoid compounds existing widely in flowers, fruits and vegetables. Many reports have explored the relationship between flower colors and pigments composition\textsuperscript{[6–9]}. Apart from their contribution in pigmentation, they have been receiving considerable attention due to their wide range of biological activities, including antioxidant, anti-inflammatory, antiallergic, antioxidant, and anticarcinogenic properties\textsuperscript{[10–13]}. The structure–activity relationship suggested that the \textit{ortho}-dihydroxyphenyl structure on the B-ring of anthocyanins was essential to the anticarcinogenic action in mouse JB6 cells and lipopolysaccharide (LPS)-activated murine macrophage RAW264 cells, which was only discovered in delphinidin and cyanidin\textsuperscript{[14,15]}. It was reported that malvidin exhibited the highest inhibitory activity against human cancer cell lines, AGS (stomach), HCT-116 (colon), MCF-7 (breast), NCI H460 (lung), and SF-268 (central nervous system, CNS) included\textsuperscript{[16]}. According to Dugas et al.\textsuperscript{[17]}, quercetin was the best scavenger of peroxyl radical out of seven common flavonoids. In view of the mass consumption of medicinal and functional food, it is of great importance to select lotus species or cultivars rich in these active components.

To our knowledge, there was no other detail information about the anthocyanins and flavonols composition in petals of lotus except the studies carried out by Rahman et al.\textsuperscript{[18]} and Masato et al.\textsuperscript{[19]}. The former work was a preliminary study performed by adsorption chromatography on magnesium trisilicate and just deduced one flavonol. The other one revealed five anthocyanins, but it lacked MS data to validate them. Thus, there is a solid need to use more advanced equipments to investigate the anthocyanins and flavonols composition and distribution in lotus petals. Considering the tremendous survey task on hundreds of cultivars, it is an urgent
requirement to establish a method by which anthocyanins and flavonols could be analyzed simultaneously with higher peak resolution and shorter time span. The two aims of the present work are to develop a fast and reliable HPLC method for the flavonoid analysis of lotus petals in large quantities and to illustrate the anthocyanins and flavonols composition by investigating several representative cultivars.

2. Experimental

2.1. Standards and solvents

Malvidin-3, 5-di-O-glucoside chloride (Mv3G5G) was purchased from Extrasynthese (Genay, France). Quercetin 3-O-rutinoside (rutin) was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Authentic samples of anthocyanins comprised cyanidin-3-O-glucoside (Cy3G), peonidin-3-O-glucoside (Pn3G), delphinidin 3-O-glucoside (Dp3G), petunidin 3-O-glucoside (Pt3G) and malvidin 3-O-glucoside (Mv3G), in which Cy3G and Pn3G were used as a raw mixture obtained from tree peony cultivars ‘Qing Long Wo Mo Chi’ and ‘Hohi’ [6,9], the others were extracted from Crape myrtle cultivar ‘Mei Gui Hong’ [7]. In the same way, the authentic sample of quercetin 3-O-galactoside (Qu3Gal) was obtained from a blueberry cultivar ‘Bluecrop’. Standards of quercetin, isorhamnetin and kaempferol 3-O-glucoside were generously offered by Professor Xiao Wang (Shandong Analysis and Test Center, Shandong Academy of Sciences, Shandong, China). Methanol and acetonitrile used for high-performance liquid chromatography-photoelectrodiode array detection/electrospray ionization multistage mass spectrometry (HPLC–DAD/ESI-MS²) analysis were of chromatographic grade and purchased from Alltech Scientific (Beijing, China). Trifluoroacetic acid (TFA; ≥99%) was obtained from Merck (Darmstadt, Germany). Methanol and formic acid were of analytical grade and purchased from Beijing Chemical Works (Beijing, China). HPLC-grade water was obtained from a Milli-Q System (Millipore, Billerica, MA, USA).

2.2. Plant materials

Petals of six lotus cultivars, ‘Yanyangtian’ (red purple), ‘Hongtaiyang’ (red), ‘Meiyuanxuise’ (purple violet), ‘Qianbanlian’ (pink), ‘Youyimudanlian’ (greenish-yellow) and ‘Dabilian’ (light yellow) included, were collected in 2007 at Beijing Botanical Garden, Institute of Botany, the Chinese Academy of Sciences (lat. 39° 48′ N long. 116° 28′ E, alt. 76 m). These cultivars were introduced from all over the world and have been planted in the same-sized containers (diameter, 40 cm; height, 30 cm) in Beijing Botanical Garden for more than 3 years under the same cultivated conditions regarding fertilization, irrigation and disease prevention. Full-blown petals were taken into the laboratory immediately after harvested, powdered in liquid nitrogen with mortars and pestles, and then stored at −40 °C for later analysis.

2.3. Preparation of standard solutions

Standards of Mv3G5G and rutin were accurately weighed, dissolved in 0.1% HCl–methanol and methanol, respectively, and then diluted to appropriate concentrations, containing 9.38–187.60 μg/mL for Mv3G5G and 9.17–184.30 μg/mL for rutin.

2.4. Extraction of anthocyanins and flavonols

Approximately 1 g of frozen petal powder was extracted with 5 mL 70% methanol aqueous solution containing 0.1% HCl (pH 2.08) at 4 °C in the dark for 24 h, shaken in a QL-861 vortex (Kylinbell Lab Instruments, Jiangsu, China) every 6 h. The liquid was separated from the solid matrix by filtration through sheets of qualitative filter paper (Hangzhou Special Paper Industry, Zhejiang, China). The filtrate was further passed through 0.22 μm reinforced nylon membrane filters (Shanghai ANPEL, Shanghai, China) before HPLC analysis. Three replicates were performed for each sample.

2.5. Acid hydrolysis of flavonols extraction

The filtered extract solution of ‘Dabilian’ was dried in a rotary evaporator (35 °C), re-dissolved in 4 mL of 1.5M HCl in a methanol–water solution (50:50, v/v), and then heated in a capped tube at 90 °C for 3 h [20]. The hydrolyte obtained was purified through a Supelclean ENVI-18 SPE Tube (Supelco, Bellefonte, PA, USA), which was conducted according to Giusti and Wrolstad [21] and Pirisi et al. [22].

2.6. Optimization of HPLC separation for anthocyanins and flavonols

The chromatographic separation was carried out on a Dionex (Sunnyvale, CA, USA) HPLC system equipped with a P680 HPLC pump, an UltiMate 3000 autosampler, a TCC-100 thermostated column compartment and a Dionex PDA100 photodiode array detector. The analytical column was a C18 column of ODS-80Ts QA (150 mm x 4.6 mm I.D., Tosoh, Tokyo, Japan) protected with a Transgenomic CARR Sep Coregel 87C Guard Cartridge (Transgenic, Omaha, NE, USA). An aliquot of 10 μL solution was injected for HPLC analysis. Chromatograms were acquired at 520 and 350 nm for anthocyanins and flavonols, respectively, and photodiode array spectra were recorded from 200 to 800 nm. Four candidate solvent systems, abbreviated as S I, S II, S III and S IV, were tested to optimize the simultaneous separation of anthocyanins and flavonols. In S I, 1.5% phosphoric acid was selected as phase A and phosphoric acid–acetic acid–acetonitrile–water (1.5:20:25:53.5, v/v/v/v) as phase B [19]. S II was the same as S I except acetic acid was substituted by formic acid [6.23]. S III was much simpler with 0.1% TFA in water as phase A and acetonitrile as phase B, in contrast with the former two [24]. With regard to S IV, TFA–formic acid–water (0.1:2.97:9.2, v/v/v) constituted solvent A and TFA–formic acid–acetonitrile–water (0.1:2.35:62.9, v/v/v/v) constituted solvent B [25].

2.7. Identification of anthocyanins and flavonols

2.7.1. Qualitative analysis of anthocyanins

Anthocyanins were primarily identified according to their HPLC retention times, elution order, UV–vis spectra and MS fragmentation pattern and by comparison with published data and authentic samples such as petal extracts of tree peony and crape myrtle [6,7,9]. HPLC–ESI-MS² analysis for anthocyanins was performed on an Agilent-1100 HPLC system coupled with a DAD system and a LC/MSD Trap VL electrospray ion mass spectrometer (Agilent Technologies, Palo Alto, CA, USA). The chromatographic separation conditions were those obtained in the optimization experiment (see Section 3.1 for details). The MS conditions were as follows: positive ion mode; gas (N₂) temperature, 325 °C; flow rate, 8.0 L/min; nebulizer pressure, 241.3 kPa; HV voltage, 3.5 kV; octopole RF amplitude, 150 Vpp; skim 1 voltage, 37.5 V; skim 2 voltage, 6.0 V; capillary exit, 111.8 V; cap exit offset, 74.3 V and scan range, m/z 100–1000 units.
3. Results and discussion

3.1. Optimization of chromatographic separation for anthocyanins and flavonols

The aims of optimization experiment for HPLC separation were not only to obtain higher separation efficiency and peak resolution of target compounds, but also to reserve shorter run-time. The optimization of HPLC conditions for lotus petals was achieved after many trials with four candidate elution systems in various proportions at different temperatures. S I was the one employed to analyze anthocyanins in lotus petals. S II and S III were widely used to separate flavonoids obtained from the acid hydrolysis were determined by correlation with standards and published data, and by comparison with standards and published data. Special attention has been given to MS fragmentation characteristics since they include valuable information in the position of the link between aglycone and sugar, the interglycosidic linkage type, as well as the molecular weight.

3.2. Method validation

To test the reliability and validity of the optimized method, the analytical parameters were determined for anthocyanins with Mv3G5G and flavonols with rutin, respectively [6–9,28].

The linearity calibration curves were constructed by five concentration assays of each standard in triplicate. Results of the regression analyses and the correlation coefficients ($r^2$) were listed in Table 1. The high correlation coefficients ($r^2 > 0.9993$) indicated good linearity in relatively wide concentration ranges for both Mv3G5G and rutin. The limits of detection (LODs) calculated as a signal-to-noise ratio of 3 were 0.7588 and 0.2290 µg/mL for Mv3G5G and rutin, respectively, while the limits of quantitation (LOQs) with signal-to-noise ratio of 10 were 2.5293 and 0.7635 µg/mL.

The precision of the proposed method was assessed considering repeatability (intra-day analysis, $n = 3$) and intermediate precision (inter-day analysis, $n = 6$) for major ingredients by preparing eight aliquots of a red cultivar (‘Meiyuanxiuse’) abundant in anthocyanins and eight aliquots of a yellow one (‘Dabilian’) rich in flavonols. The parameter discussed here is a recalculated content of the studied compounds, described in µg per 1 g fresh petals. The results (Table 2) showed that the relative standard deviations (RSDs) of eleven major compounds (five anthocyanins and six major flavonols) was less than 0.6% in intra-day test and less than 2.2% for inter-day analysis.

3.3. Qualitative analysis for anthocyanins

The HPLC–MS$^n$ analysis was performed in positive mode with the [M+H]$^+$ ion subjected to further fragmentation, which allowed the identification of the anthocyanidin core (aglycone). Table 3 shows the characteristics of anthocyanins separated from lotus petals. Five peaks were identified as delphinidin 3-O-glucoside (Dp3G, peak 1), cyanidin 3-O-glucoside (Cy3G, peak 2), petunidin 3-O-glucoside (Pt3G, peak 3), peonidin 3-O-glucoside (Pn3G, peak 4)
Table 1
Linearity of response for Mv3G5G and rutin using the optimized method. Calibration fitting: y = kx + m*.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Linear range (μg/mL)</th>
<th>Slope (k) (mean ± SD, n = 3)</th>
<th>Intercept (m) (mean ± SD, n = 3)</th>
<th>Regression r² (n = 5)</th>
<th>LOD (μg/mL)</th>
<th>LOQ (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mv3G5G</td>
<td>9.38–187.60</td>
<td>0.3756 ± 0.0035</td>
<td>0.4620 ± 0.0950</td>
<td>0.9993</td>
<td>0.759</td>
<td>2.530</td>
</tr>
<tr>
<td>Rutin</td>
<td>9.17–183.40</td>
<td>0.4453 ± 0.0006</td>
<td>–1.6414 ± 0.0304</td>
<td>0.9994</td>
<td>0.229</td>
<td>0.764</td>
</tr>
</tbody>
</table>

* In the regression equation y = kx + m, y refers to the peak area, x is concentration of the standard substances (μg/mL), r² is the correlation coefficient of the equation.

Table 2
Intra- and inter-day precision of eleven main flavonoids in the extract of lotus petals by HPLC-DAD.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Intra-day precision (n = 3)*</th>
<th>Inter-day precision (n = 6)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Content (μg/g)</td>
<td>RSD (%)</td>
</tr>
<tr>
<td>1</td>
<td>18.10</td>
<td>0.11</td>
</tr>
<tr>
<td>2</td>
<td>5.64</td>
<td>0.35</td>
</tr>
<tr>
<td>3</td>
<td>11.98</td>
<td>0.50</td>
</tr>
<tr>
<td>4</td>
<td>4.92</td>
<td>0.45</td>
</tr>
<tr>
<td>5</td>
<td>61.90</td>
<td>0.57</td>
</tr>
<tr>
<td>7</td>
<td>27.20</td>
<td>0.19</td>
</tr>
<tr>
<td>8</td>
<td>64.92</td>
<td>0.30</td>
</tr>
<tr>
<td>9</td>
<td>49.26</td>
<td>0.21</td>
</tr>
<tr>
<td>10</td>
<td>30.12</td>
<td>0.36</td>
</tr>
<tr>
<td>11</td>
<td>52.95</td>
<td>0.11</td>
</tr>
<tr>
<td>12</td>
<td>27.82</td>
<td>0.16</td>
</tr>
</tbody>
</table>

* Compounds 1–5 were separated from ‘Meiyuanxiushe’ and quantified by Mv3G5G, while compounds 7, 8, 10, 12–14 were obtained from ‘Dabilian’ and quantified by rutin. They were listed according to their elution orders on the C18 column.

3.4. Identification of flavonoids

It was more challenging to identify the flavonols in lotus petals due to lack of previous report. Acid hydrolysis was performed to investigate the aglycone composition. Three aglycones were identified as quercetin, kaempferol and isorhamnetin by co-chromatography with corresponding standards. The compound eluted between kaempferol and isorhamnetin was tentatively assigned as syringetin by comparing its UV–vis spectrum with published data [29] and MS data (the molecule ions at m/z 463 and the fragment at m/z 301 by loss of 162 mass units (u) indicated peak 4 was paeonidin attached with a hexose. Their final structures were confirmed by co-chromatography with Pt3G and Pn3G extracted from Crape myrtle and tree peony, respectively [6,7,9]. So did Dp3G, Mel3G and Cy3G. Fig. 2 illustrates clearly the MS spectrum (in PI mode) and UV–vis absorption spectra of P3G (peak 3) and Pn3G (peak 4) in lotus flowers.

Table 3
UV–vis absorption maxima and main ESI-MS⁺ peaks of anthocyanins and flavonols in lotus petals.

<table>
<thead>
<tr>
<th>No.</th>
<th>Identification</th>
<th>λ max (nm)</th>
<th>ESI-PI (m/z)</th>
<th>ESI-NI (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Delphinidin 3-O-glucoside</td>
<td>522.1, 277.2</td>
<td>465 [M+H]+, 303 [Y0+, M+H−162]</td>
<td>609 [M−H]−, 301 [Y0−H], M−H−308</td>
</tr>
<tr>
<td>2</td>
<td>Cyanidin 3-O-glucoside</td>
<td>516.0, 280.6</td>
<td>449 [M+H]+, 287 [Y0+, M+H−162]</td>
<td>463 [M−H]−, 301 [Y0−H], M−H−162</td>
</tr>
<tr>
<td>3</td>
<td>Petunidin 3-O-glucoside</td>
<td>526.8, 276.0</td>
<td>479 [M+H]+, 317 [Y0+, M+H−162]</td>
<td>507 [M−H]−, 447 [M−H−162], 301 [Y0−H], M−H−162</td>
</tr>
<tr>
<td>4</td>
<td>Peonidin 3-O-glucoside</td>
<td>517.3, 279.8</td>
<td>463 [M+H]+, 301 [Y0+, M+H−162]</td>
<td>593 [M−H]−, 447 [M−H−162], 301 [Y0−H], M−H−162</td>
</tr>
<tr>
<td>5</td>
<td>Malvidin 3-O-glucoside</td>
<td>527.0, 277.4</td>
<td>493 [M+H]+, 331 [Y0+, M+H−162]</td>
<td>531 [M−H]−, 463 [M−H−162], 331 [Y0−H], M−H−308</td>
</tr>
<tr>
<td>6</td>
<td>Quercetin 3-O-rutinoside</td>
<td>353.9, 256.6</td>
<td>633 [M+Na]+, 465 [M+H−146]+, 303 [Y0+, M+H−308]</td>
<td>609 [M−H]−, 301 [Y0−H], M−H−308</td>
</tr>
<tr>
<td>7</td>
<td>Quercetin 3-O-galactoside</td>
<td>353.1, 256.4</td>
<td>487 [M+Na]+, 303 [Y0+, M+H−162]</td>
<td>463 [M−H]−, 301 [Y0−H], M−H−162</td>
</tr>
<tr>
<td>8</td>
<td>Quercetin 3-O-glucuronic acid</td>
<td>352.9, 256.4</td>
<td>501 [M+Na]+, 303 [Y0+, M+H−176]</td>
<td>507 [M−H]−, 447 [M−H−162], 301 [Y0−H], M−H−162</td>
</tr>
<tr>
<td>10</td>
<td>Kaempferol 3-O-galactoside</td>
<td>346.3, 265.2</td>
<td>471 [M+Na]+, 287 [Y0+, M+H−162]</td>
<td>447 [M−H]−, 284 [Y0−H], M−H−308</td>
</tr>
<tr>
<td>12</td>
<td>Kaempferol 3-O-glucoside</td>
<td>346.3, 265.1</td>
<td>471 [M+Na]+, 287 [Y0+, M+H−162]</td>
<td>447 [M−H]−, 284 [Y0−H], M−H−308</td>
</tr>
<tr>
<td>15</td>
<td>Kaempferol 3-O-pentose</td>
<td>344.0, 265.0</td>
<td>441 [M+Na]+, 287 [Y0+, M+H−132]</td>
<td>441 [M−H]−, 284 [Y0−H]</td>
</tr>
</tbody>
</table>

* Retention time on the C18 column recorded by DAD.
The absence of ions including Y0−, Z1−, Z2−X1− in NI mode and [Z1+Na]+, [Z1+Na−H2O]+, [B0+Na−H2O]+ in PI mode indicated the interglycosidic linkage type between the two monosaccharides was a 1,6 linkage. Finally, the structure was assigned as quercetin 3-O-rhamnopyranosyl-(1→6)-glucopyranoside (rutin) by co-elution with standard rutin.

Peaks 9 and 11 showed similar mass spectrometric behaviors with peak 6. Based on molecular ions, fragment ions and radical aglycone ions in both NI and PI modes (see Table 3 for details), they were identified as kaempferol 3-O-rutinobioside and isorhamnetin 3-O-rutinoside, respectively. Kaempferol 3-O-rutinobioside was reported in lotus flowers [18], while isorhamnetin 3-O-rutinoside in lotus stamens [32].

Both peaks 7 and 8 produced major fragments at m/z 303 Y0− in PI mode and m/z 301 Y0− in NI mode. Combined with their elution order and UV−vis spectrum, they were identified as quercetin glycosides. The deprotonated molecule ion at m/z 463 [M−H]− and the sodium adduct at m/z 487 [M+Na]+ of peak 7 revealed a hexose linked to the aglycone, while the relatively higher abundance of the radical aglycone at m/z 300 Y0− to the aglycone product ion indicated that the glycoside was located at the 3-position of the aglycone. Peak 7 was finally confirmed as quercetin 3-O-glucuronide (Qu3Gal) by co-chromatography with standard, while peak 10 was identified as quercetin 3-O-galactoside (Qu3Gal) by co-elution with Qu3Gal obtained from a blueberry cultivar ‘Bluecrop’ [33]. Peak 8 was identified as quercetin 3-O-galacturonide considering the UV−vis spectrum, elution order, the disoprotonated molecule at m/z 477 [M−H]− in NI mode and the quasimolecular ion peak at m/z 501 [M+Na]+ in PI mode, and its occurrence in lotus leaf [34]. ESI-MS+ spectrum of peak 7 and 8 in negative mode were illustrated in Fig. 3.

The following three major peaks (peaks 10, 12 and 13) were all identified as kaempferol derivatives on account of the aglycone fragment ions at m/z 287 Y0− in PI mode, the radical aglycone at m/z 284 [Y0−H]− (peaks 10 and 12) or the aglycone ion at m/z 285 Y0− (peak 13) in NI mode, and their UV λmax (Band II) at 265 nm [35]. Peaks 10 and 12 both exhibited predominantly molecular adducts at m/z 471 [M+Na]+ and 447 [M−H]− in PI and NI modes, respectively, which were just the molecular weight of kaempferol plus 162 u. Peak 12 was then straightforward confirmed as kaempferol 3-O-glucuronide by co-chromatography with standard, while peak 10 was assigned as kaempferol 3-O-galactoside based on a previous report by Jung et al. [36]. Peak 13 was identified as kaempferol 3-O-galacturonide which was also detected in lotus stamens [36].

The MS fragmentation data (loss of 162 u) of peak 14 suggested a hexose was the glycosyl substituent. In a similar manner, the predominantly higher amount of the radical aglycone at m/z 344 [Y0−H]− than the aglycone ion at m/z 345 Y0− indicated the 3-
hydroxyl group of syringetin was blocked, namely, there was a substituent at this position. However, we could not differentiate glucose from galactose substituents just from the mass spectrometry. Peak 14 was tentatively identified as syringetin 3-O-glucuronide (B) in lotus petals, obtained with negative ionization. The detailed operating conditions were described in the text.

4. Conclusion

In this study, we established an analytical method that could simultaneously separate a wide range of anthocyanins and flavonols within 40 min. In addition to its high sample throughput, the proposed method is sensitive, reliable, and reproducible for the analysis of lotus petals as the analytical parameters suggest. By combination of powerful tools (HPLC-DAD and HPLC–MS), five anthocyanins and ten flavonols have been characterized, in which two anthocyanins and nine flavonols are reported for the first time in lotus petals.

The present work is indispensable for investigating the composition of anthocyanins and flavonols in hundreds of lotus cultivars. The feature of high sample throughput significantly reduces the workload as well as procedure cost of analyzing flower pigments from different lotus cultivars. With the anthocyanins and flavonols composition data, we could develop a HPLC fingerprinting database of lotus cultivars to solve headachy problems about synonyms and homonyms. Meanwhile, we could select species or cultivars rich in delphinidin, cyanidin, malvidin or quercetin derivatives for functional food materials, such as lotus liquor and lotus tea. It is also a contribution to our understanding of flavonoid biosynthesis, which could obviously help the hybrid breeding of lotus oriented to flower color by providing guidelines on selecting appropriate parents for breeding new cultivars with novel flower colors.

Acknowledgments

The authors gratefully acknowledge Mr. Tianjian Chen for much help and guidance, and his critical review of the manuscript. This research was financially supported by the Knowledge Innovation Program of the Chinese Academy of Sciences, No. KSCX2–YW-N-043.

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