Arsenic extraction and speciation in plants: Method comparison and development

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\textbf{HIGHLIGHTS}

• An optimized extraction method for As speciation in plants based on three different plants and four different methods was developed.
• The optimized method was based on ethanol/water extraction and used 50% less ethanol and 38% less time.
• The optimized method produced satisfactory extraction efficiency (~80% for the roots and >85% for the fronds).
• The optimized method has the potential to be used on other plant samples for As speciation.

\textbf{GRAPHICAL ABSTRACT}

\textbf{ABSTRACT}

We compared four methods to extract arsenic (As) from three different plants containing different As levels for As speciation with the goal of developing a more efficient method, i.e., As-hyperaccumulator \textit{Pteris vittata} at 459–7714 mg kg\textsuperscript{-1}, rice seedling at 53.4–574 mg kg\textsuperscript{-1}, and tobacco leaf at 0.32–0.35 mg kg\textsuperscript{-1}. The four methods included heating with dilute HNO\textsubscript{3}, and sonication with phosphate buffered solution, methanol/water, and ethanol/water, with As being analyzed using high-performance liquid chromatography coupled with inductively-coupled plasma mass spectrometry (HPLC–ICP-MS). Among the four methods, the ethanol/water method produced the most satisfactory extraction efficiency (~80% for the roots and >85% for the fronds) without changing As species based on \textit{P. vittata}. The lower extraction efficiency from \textit{P. vittata} roots was attributed to its dominance by arsenate (82%) while arsenite dominated in the fronds (88%). The ethanol/water method used sample:solution ratio of 1:200 (0.05 g:10 mL) with 50% ethanol and 2 h sonication. Based on different extraction times (0.5–2 h), ethanol concentrations (25–100%) and sample:solution ratios (1:50–1:300), the optimized ethanol/water method used less ethanol (25%) and time (0.5 h for the fronds and 2 h for the roots). Satisfactory extraction was also obtained for tobacco leaf (78–92%) and rice seedlings (70%) using the optimized method, which was better than the other three methods. Based on satisfactory extraction efficiency with little change in As species during extraction from three plants containing different As levels, the optimized method has the potential to be used for As speciation in other plants.

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1. Introduction

Arsenic (As) is a toxic pollutant in the environment, resulting from both natural and anthropogenic sources. Plants growing in As-contaminated soils accumulate As in their biomass. Arsenic in plants causes toxicity such as leaf chlorosis and necrosis, and reduces growth (Abedin et al., 2002; Caille et al., 2005). Although As in plants is mainly present as inorganic forms including arsenite (AsIII) and arsenate (AsV), small amounts of organic species including dimethylarsinic acid (DMA) and monomethylarsonic acid (MMA) have also been found (Jedynak et al., 2009; Bergqvist and Gregier, 2012). Different As species in plants show different toxicity (B’Hymer and Caruso, 2004), so it is important to identify As species in plants to better understand their metabolism.

*Pteris vittata* (PV; Chinese brake fern) is the first-known As hyperaccumulator (Ma et al., 2001). It can accumulate up to 23,000 mg kg$^{-1}$ As in its fronds when growing in an As-contaminated soil containing 1500 mg kg$^{-1}$ As (Ma et al., 2001). Since the plant can effectively accumulate large amounts of As quickly, it has potential for phytoremediation of As-contaminated soils and waters (Cao et al., 2003; Tu et al., 2004). In PV, As is mainly present in inorganic forms, with AsIII dominating in the fronds and AsV in the roots (Ma et al., 2001). Understanding As speciation helps to better examine its uptake, transformation, and detoxification mechanisms in PV. However, the extraction efficiency for As speciation in PV is unsatisfactory, especially for the roots at ~60% (Zhang et al., 2002).

Analysis of As speciation in plants is challenging, as the procedure needs to extract all As from the matrix without altering their species (Burgueras and Burgueras, 1997). Various extraction methods have been developed for As speciation in plants (Amaral et al., 2013). Mild extractants including methanol, water, and methanol/water mixture have been used to extract As by shaking, sonication, or microwave (He et al., 2002; Ruiz-Chancho et al., 2008). For some plants, trifluoroacetic acid (TFA) is more acceptable than methanol. However, the method reduces 20% of AsV to AsIII in the plant (Abedin et al., 2002). Though HCl helps to solubilize As in plants by breaking up the bonds between AsIII and thiol groups (Muñoz et al., 1999), the method suffers from chlorine interference during As analysis using inductively coupled plasma-mass spectrometry (ICP-MS) (Heitkemper et al., 1989).

Many studies investigated As extraction methods in plants, but few focused on As hyperaccumulators. Zhang et al. (2002) developed a methanol/water method to extract As in PV, with recovery of 85–100% in the fronds and ~60% in the roots. The low As recovery in the roots is unsatisfactory so it is important to develop a more efficient method for As extraction in PV. Several extraction methods have been developed using certified reference materials, which are then applied to plant samples without further tests (Bohari et al., 2002; Raber et al., 2012). For example, Heitkemper et al. (2001) obtained 95–105% recovery for NIST standard reference material (SRM) 1568a rice flour based on methanol/water method, but only 24–36% was obtained for enriched long-grain white rice. This might be attributed to the different sample matrices between SRM and plant samples such as different plant species and the amounts of As present in the samples. Therefore, to develop a robust As extraction method for plant samples, the method should be tested using real plant samples in addition to SRM.

To develop a satisfactory As extraction method from plants, we compared four extraction procedures including sonication with phosphate buffered solution, heating with HNO$_3$, and sonication with methanol/water and ethanol/water mixture (Zhang et al., 2002; Su et al., 2008; Sun et al., 2008). The specific objectives of this research were to: (1) compare extraction efficiency of the four methods in extracting As from *P. vittata*; (2) develop an effective method to extract As from *P. vittata* roots by optimizing analysis parameters; and (3) test the developed method in other plant samples including tobacco leaf and rice seedling.

2. Materials and methods

2.1. Plant materials

Three plant materials were used in this experiment including the roots and fronds of As-hyperaccumulator *P. vittata* (PV), tobacco leaf, and rice seedlings. One batch of PV plants was obtained from Xu et al. (2014). The plants grew for 60 d in a soil spiked with 200 mg kg$^{-1}$ AsIII (NaAsO$_2$) or AsV (Na$_2$HAsO$_4$ 7H$_2$O), and a soil with no As. Another batch of PV plants was obtained after growing in 0.2-strength Hoagland nutrient solution containing 0, 1, or 10 mg L$^{-1}$ of AsV for 8 d. Rice seedlings were obtained by growing in a nutrient solution containing 2.5 mg L$^{-1}$ AsV (Na$_2$HAsO$_4$ 7H$_2$O) for two weeks (Ren et al., 2014).

After harvest, all PV and rice seedling plants were washed thoroughly with Milli-Q water, separated into roots and fronds. Both PV and rice plants were freeze-dried for 48 h and ground into fine powder using liquid nitrogen. To compare the difference in sample preparation, part of a fresh PV plant was ground into fine powder using liquid nitrogen. In addition to fresh plants, dried tobacco leaves were also used, which were obtained from cigarettes purchased from a supermarket in Nanjing. The tobacco leaves and freeze-dried PV and rice seedling samples were passed through a 150 μm nylon sieve to obtain uniform size before extraction. For all speciation analysis, freeze-dried PV and rice samples were used unless specified otherwise.

2.2. Comparison of four extraction methods

Four common methods (Zhang et al., 2002; Su et al., 2008; Sun et al., 2008) were compared in extracting As from freeze-dried roots and fronds of PV after growing in a soil spiked with 200 mg kg$^{-1}$ AsV for 60 d.

Sonication with phosphate buffered solution (PBS): 50 mg of ground PV roots or fronds were weighed into a centrifuge tube and 10 mL of PBS (2 mM NaH$_2$PO$_4$ 0.2 mM Na$_2$EDTA, pH 6.0) were added to extract the samples for 1 h under sonication following by 15 min centrifugation at 4000 rpm to retrieve supernatant.

Sonication with HNO$_3$ (HNO$_3$): 5 mL of 1% HNO$_3$ were added into the samples and the mixture was allowed to stand overnight in the dark to minimize As transformation. Then the samples were heated following the procedure using a hot block digestion system: 10 min at 55 °C, 10 min at 75 °C, and 30 min at 9°C. The samples were centrifuged at 4000 rpm for 15 min.

Sonication with methanol/water (methanol): 50 mg of ground PV were ultrasonically extracted with 10 mL 1:1 methanol/water for 2 h. The samples were then centrifuged at 4000 rpm for 15 min, and the supernatant was decanted into a 50 mL centrifuge tube. The procedure was repeated twice and the three extracts were combined. The temperature was set at 25–30 °C during sonication extraction.

Sonication with ethanol/water (ethanol): the same as methanol method except that 1:1 ethanol/water was used.

For all four methods, the extracts were diluted to 50 mL with Milli-Q water and filtered through 0.22 μm filters for As speciation analysis. In addition, As stability during extraction using the four methods was investigated using a PV sample cultivated in a clean soil with low As concentration. AsIII and AsV at 10 mg L$^{-1}$ were spiked into the mixture of the PV plants and extraction solution, which was subjected to the same extraction procedure as the four methods.

2.3. Optimization of ethanol/water extraction method

After finding that the ethanol/water method provided more satisfactory extraction efficiency for PV roots (~80%) and fronds (~90%) than the other three methods as well as the preservation of As species, we...
tried to optimize the extraction efficiency. Different extraction conditions were compared: sonication times (0.5, 1, 1.5 or 2 h), ethanol concentrations (0, 15, 25, 50, 75 or 100%), and ratios of sample mass to solution volume (1:50, 1:100, 1:200 or 1:300). The extraction time was optimized using 1:1 ethanol:water with sample:solution ratio of 1:200 (0.05 g:10 mL). Then the ethanol concentration was optimized with sample:solution ratio of 1:200 under the optimized extraction time, and finally the sample:solution ratio was tested using the optimized extraction time and ethanol concentration. For this experiment, freeze-dried PV plants growing in a soil spiked with 200 mg kg\(^{-1}\) AsIII or AsV for 60 d were used, which contained relatively high As concentrations.

The optimized ethanol method based on PV plants with high As concentrations was then tested using freeze-dried PV plants containing low As concentrations, which were obtained after growing in hydroponics containing 0, 1, and 10 mg L\(^{-1}\) AsV for 8 d. In addition, fresh PV roots and fronds grown in nutrient solution with 10 mg L\(^{-1}\) AsV were extracted using the optimized method and compared to freeze-dried samples. Besides PV, the developed ethanol method was applied to other plant species including tobacco leaf and rice seedling and compared to three other extraction methods (PBS, HNO\(_3\) and methanol). Since tobacco leaf and rice seedlings contain much lower As concentration than PV, the optimized sample:solution ratio of 1:200 was reduced by 10-fold.

2.4. Total As concentration analysis

For total As determination, all samples were digested following USEPA Method 3050B. Briefly, 50 mg plant sample was weighed into 50 mL digestion vials containing 10 mL of 1:1 H\(_2\)O\(_2\):water. The mixture was heated in a Hot Block Digestion System (Environmental Express, USA) at 105°C for 2 h, with ~5 mL digest being left in the vessel. The samples were then removed from the block, cooled to room temperature and two 0.5 mL aliquots of 30% H\(_2\)O\(_2\) were added slowly. After digestion in the block for another 15 min, the samples were cooled and brought to a final volume of 50 mL with Milli-Q water.

The final solution was stored at 4°C prior to measurement using ICP-MS (Perkin Elmer NexION 300X, USA). Since PV is an As hyper-accumulator with high As concentrations in its roots and fronds, digestion samples were diluted using 0.1 M H\(_2\)NO\(_3\) to achieve As concentrations within the calibration curve concentrations of ICP-MS (1−20 μg L\(^{-1}\)). The ICP-MS system was calibrated before each batch of analysis using five point calibration curves (R\(^2\) > 0.999). ICP-MS measurements were performed under the following conditions: sweeps 20, replicates 3, dwell time 100 ms, ICP RF power 1300 W, plasma gas flow rate 17 L min\(^{-1}\), auxiliary gas flow rate 1.2 L min\(^{-1}\), and nebulizer gas flow rate 1.05 L min\(^{-1}\).

For quality control and quality assurance (QA/QC), indium (m/z 115) and germanium (m/z 74) were used as internal standards, which were added to the samples, calibration standards, and blanks to compensate for matrix effects and long-term signal drift produced by matrix components slowly blocking the sampler and skimmer cone orifice. The recoveries of internal standards were within 90–110%. During measurement, standard solution at 1 μg L\(^{-1}\) As was measured every 20 samples to monitor the stability of the ICP-MS. The check recovery was 95–106%.

In addition, blanks and certified reference material for plant samples (CSB-21, Chinese geological reference materials) were used as quality checks, which were within expected values.

2.5. As speciation analysis

Arsenic speciation analysis was carried out using HPLC (Waters 2695, USA) coupled with ICP-MS. An anion-exchange column (PRP-X100, 10 μm, Hamilton, UK) connected with a guard column (Hamilton, UK) was used to separate As species. The mobile phase consisted of 8.0 mM (NH\(_4\))\(_2\)HPO\(_4\) and 8.0 mM NH\(_4\)NO\(_3\), with pH adjusted to 6.2 using H\(_3\)PO\(_4\) and NH\(_4\)OH. It was filtered through a 0.22 μm membrane filter and degassed using an ultrasonic bath before flowing into chromatographic columns. A sample of 50 μL was injected at a flow rate of 1.0 mL/min at room temperature.

AsIII and AsV stock solutions at 1000 mg L\(^{-1}\) were prepared from NaAsO\(_2\) (Sigma-Aldrich, ≥90%) and Na\(_2\)HAsO\(_4\) \(7\)H\(_2\)O (Sigma-Aldrich, ≥98%) with Milli-Q water. Dilute As standard solutions used for speciation analysis were prepared daily using Milli-Q water. All stock solutions were stored at 4°C. Blanks, calibration curves (0, 1, 2, 5, 10, and 20 μg L\(^{-1}\)), and spiked samples were used to assure analysis quality. A standard solution at 10 μg L\(^{-1}\) As was measured every 10 samples to monitor the stability of HPLC–ICP-MS.

3. Results and discussions

Effective As extraction is critical in speciation analysis in plant samples. An acceptable method should extract most of As without changing As species in plants (Amaral et al., 2013). In addition, it should be relatively easy without producing toxic waste.

3.1. Comparison of four extraction methods

PV plants after growing in soil containing 200 mg kg\(^{-1}\) AsV were used to compare As extraction and speciation of four extraction methods. Total As concentrations in the roots and fronds were 586 and 7714 mg kg\(^{-1}\) (Table 1). Arsenic in PV consisted of primarily inorganic species, with 82% of As in the roots as AsV and 85% as AsIII in the fronds (Table 1). This result agreed with a previous study showing that AsV dominates in the roots while AsIII dominates in the fronds (Zhang et al., 2002).

Four extraction methods including PBS, HNO\(_3\), methanol, and ethanol were evaluated. Generally speaking, extraction efficiency of >80% is acceptable but >90% is better. Among the four methods, the PBS method provided the highest extraction efficiency at 98% for the fronds. However, the recovery for the roots was low at 70% (Fig. 1A). In addition, ~10% of AsV in the fronds was reduced to AsIII at high temperature during extraction process. Based on spiked As, 3% of AsIII was oxidized to AsV in the roots and ~14% of AsV was reduced to AsIII in the fronds (Fig. 1B). This was consistent with Huang et al. (2012) who reported that HNO\(_3\) results in ~10% oxidation and ~20% reduction during extraction.

The methanol method has been widely used for As speciation in PV (Singh and Ma, 2006; Mathews et al., 2010). Though As extraction efficiency in the fronds was satisfactory at 80–90%, the efficiency for the roots was low at ~60% (Fig. 1A). This is consistent with Zhang et al. (2002) who reported ~60% for the roots and 85–100% for the fronds. In addition, methanol is toxic during extraction process in addition to producing hazardous waste. Hence, it is important to develop a new method with satisfactory extraction efficiency and less toxic waste.

Since a large amount of As in PV is water soluble (Webb et al., 2003), As can be extracted with water alone or with a mixture of water and organic solvent. Ethanol is non-toxic and powerful to extract plant pigment and can dissolve many compounds in plants. The ethanol method by sonication produced satisfactory extraction efficiency of ~80% for the roots and >90% for the fronds (Fig. 1A), with a small fraction of AsIII being oxidized to AsV (3%) and of AsV to AsIII (~4%) in the roots and fronds (Fig. 1B). Even though the temperature was controlled at 25–30 °C in an ice bath during the extraction process, standing wave patterns create non-uniformity with respect to density, which in turn results in extremely localized high temperatures and pressures (Dietz et al., 2007). Hydrogen peroxide and oxygen radicals are generated due to the radiolysis of the extraction solution induced
by the focused ultrasound energy, leading to redox change and As transformation (Sanz et al., 2005). Our recovery result was in agreement with Yuan et al. (2005) who reported that ethanol/water (1:1, v/v) mixture using microwave-assisted extraction provides better extraction efficiency than methanol/water for rice straw samples, with the extraction efficiency increasing from 66% to 85%. However, a much stricter temperature control has to be used in the microwave-assisted extraction to obtain good extraction efficiency. Due to its high extraction efficiency coupled with its low toxicity, the ethanol method was tested for further improvement.

Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tissue</th>
<th>AsIII (mg kg⁻¹)</th>
<th>AsV (mg kg⁻¹)</th>
<th>AsIII + AsV (mg kg⁻¹)</th>
<th>Total As (mg kg⁻¹)</th>
<th>Extraction efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AsIII</td>
<td>Root</td>
<td>177 ± 9.19³ (57.3%)</td>
<td>133 ± 25.5 (42.6%)</td>
<td>310 ± 22.3</td>
<td>459 ± 21.1</td>
<td>67.5 ± 4.85</td>
</tr>
<tr>
<td></td>
<td>Frond</td>
<td>5506 ± 203 (88.2%)</td>
<td>852 ± 47.5 (13.8%)</td>
<td>6358 ± 435</td>
<td>7346 ± 447</td>
<td>86.9 ± 5.92</td>
</tr>
<tr>
<td>AsV</td>
<td>Root</td>
<td>61.5 ± 11.6 (17.8%)</td>
<td>288 ± 54.7 (82.2%)</td>
<td>350 ± 55.0</td>
<td>586 ± 65.6</td>
<td>59.7 ± 9.38</td>
</tr>
<tr>
<td></td>
<td>Frond</td>
<td>5621 ± 421 (88.7%)</td>
<td>708 ± 129 (11.2%)</td>
<td>6329 ± 355</td>
<td>7714 ± 70.5</td>
<td>81.8 ± 4.60</td>
</tr>
</tbody>
</table>

Control

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tissue</th>
<th>AsIII (mg kg⁻¹)</th>
<th>AsV (mg kg⁻¹)</th>
<th>AsIII + AsV (mg kg⁻¹)</th>
<th>Total As (mg kg⁻¹)</th>
<th>Extraction efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Root</td>
<td>1.89 ± 0.15 (33.8%)</td>
<td>3.73 ± 0.48 (66.2%)</td>
<td>5.63 ± 0.34</td>
<td>10.1 ± 0.57</td>
<td>55.8 ± 3.40</td>
<td></td>
</tr>
<tr>
<td>Frond</td>
<td>24.2 ± 1.85 (73.6%)</td>
<td>9.00 ± 0.53 (26.4%)</td>
<td>33.3 ± 2.37</td>
<td>37.1 ± 3.22</td>
<td>89.7 ± 6.38</td>
<td></td>
</tr>
</tbody>
</table>

³ Sum of AsIII and AsV determined using HPLC-ICP-MS.
⁴ Ratio of AsIII + AsV to total As.
⁵ Total As determined using ICP-MS after digestion.
⁶ Percentage of AsIII or AsV in the sum of AsIII and AsV.

3.2. Optimization of ethanol method

The ethanol method used 1:1 methanol:water (50%), and sample to solution ratio of 50 mg:10 mL with 2 h extraction time. The parameters tested including extraction times (0.5 to 2 h), ethanol concentrations (0, 15, 25, 50, 75, and 100%) and sample to solution ratios (50 mg to 2.5, 5, 10 and 15 mL). Among the parameters, extraction time was the most important. PV plants after growing in soil containing 200 mg kg⁻¹ AsV and AsIII was used for method optimization. The As contents in PV roots and fronds were 459–586 and 7346–7714 mg kg⁻¹ (Table 1).

For AsIII-exposed PV, the extraction efficiency for the roots was 61% after 0.5 h, and it increased to 81% after 2 h (Fig. 2A and B). However, extraction time had little effect on the fronds, which was >90% after 0.5 h. It seemed that As in the roots was more difficult to extract than in the fronds. The difference in As speciation was the possible reason as the fronds were dominated by AsIII at 93% whereas the roots were mainly AsV at 89% (Table 1). Between the two PV samples, As extraction efficiency was higher in PV exposed to AsIII than AsV. This was because more AsIII was present in PV roots exposed to AsIII than AsV (57% vs. 18%), indicating that AsIII in PV was more easily extracted than AsV. This was consistent with Yuan et al. (2005) who reported that AsV extraction is more time-consuming from rice straw than AsIII.

After being taken up by plants, As is distributed in different cell fractions, including cytoplasmic supernatants, cell organelles, and cell walls. Chen et al. (2005) showed that As is mainly in the cytoplasmic supernatant fraction in PV exposed to 7.5 or 15 mg kg⁻¹ AsV for 3 months. However, ~33% of the As accumulates in cell walls in the roots, which is ~2-fold higher than that in the fronds. The cell walls mainly contain protein, lignin, and cellulose, which have a strong ability to chelate heavy metals (Tang et al., 2009). As such, it was possible that As in the cell walls binds more strongly than other fractions, resulting in lower As extraction efficiency and longer time for the roots.

Among the ethanol concentrations tested, 25% ethanol or 1:3 ethanol:water provided the highest extraction efficiency. For AsIII-exposed PV, 1:3 ethanol:water yielded the highest extraction efficiency at 92% for the roots and 103% for the fronds (Fig. 2C and D). In comparison, using water alone produced lower As extraction at 64% for the roots and 77% for the fronds. Water is suitable to extract soluble polar species, which constitute the majority of As in plants (Francesconi and Kuehnel, 2004). The addition of ethanol increases the solubility of non-polar As species in PV (Wolle et al., 2014). However, increasing ethanol concentration beyond 25% decreased the extraction efficiency, with 100% ethanol resulting in 26% extraction efficiency for the roots and 58% for the fronds, which is consistent with another study (B’Hymer and Caruso, 2004). For AsV-exposed plants, similar results were found with a slightly lower recovery.

The impact of sample mass to solution ratio was tested by holding a 0.05 g sample with solution volumes of 2.5, 5, 10 and 15 mL (Fig. 2E and F). However, sample:solution ratio had little effect on the extraction...
Table 2
Application of the optimized ethanol method (25% methanol, 0.5 h and 2 h for roots and fronds, sample/solution ratio of 1:200) to freeze-dried and fresh Pteris vittata grown for 8 d in 0.2-strength Hoagland solution with 0, 1 and 10 mg L\(^{-1}\) of As\(_{\text{V}}\).

<table>
<thead>
<tr>
<th>As exposure concentration (mg L(^{-1}))</th>
<th>Pretreatment</th>
<th>Tissue</th>
<th>AsIII (mg kg(^{-1}))</th>
<th>AsV (mg kg(^{-1}))</th>
<th>AsIII + AsV (mg kg(^{-1}))</th>
<th>Total (mg kg(^{-1}))</th>
<th>Extraction efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Freeze-dried</td>
<td>Root</td>
<td>3.97 ± 0.82</td>
<td>1.02 ± 0.01</td>
<td>5.00 ± 0.70</td>
<td>6.36 ± 0.75</td>
<td>78.6 ± 11.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Frond</td>
<td>49.9 ± 1.20</td>
<td>5.17 ± 1.02</td>
<td>55.1 ± 1.14</td>
<td>63.2 ± 1.06</td>
<td>87.1 ± 1.80</td>
</tr>
<tr>
<td>1</td>
<td>Freeze-dried</td>
<td>Root</td>
<td>0.39 ± 0.06</td>
<td>5.69 ± 0.68</td>
<td>6.08 ± 0.81</td>
<td>7.31 ± 0.74</td>
<td>83.1 ± 11.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Frond</td>
<td>37.7 ± 2.31</td>
<td>44.4 ± 3.76</td>
<td>82.0 ± 2.27</td>
<td>89.1 ± 0.64</td>
<td>92.0 ± 2.54</td>
</tr>
<tr>
<td>10</td>
<td>Freeze-dried</td>
<td>Root</td>
<td>7.42 ± 1.13</td>
<td>71.8 ± 7.18</td>
<td>79.2 ± 8.30</td>
<td>95.7 ± 8.6</td>
<td>82.7 ± 8.67</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Frond</td>
<td>119 ± 9.76</td>
<td>78.5 ± 3.41</td>
<td>198 ± 13.1</td>
<td>232 ± 14.3</td>
<td>85.7 ± 5.64</td>
</tr>
<tr>
<td>10</td>
<td>Fresh</td>
<td>Root</td>
<td>3.74 ± 0.00</td>
<td>14.5 ± 2.14</td>
<td>18.1 ± 2.36</td>
<td>25.1 ± 6.18</td>
<td>72.3 ± 9.40</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Frond</td>
<td>39.7 ± 1.14</td>
<td>36.5 ± 1.11</td>
<td>76.2 ± 0.58</td>
<td>100 ± 5.16</td>
<td>76.2 ± 0.58</td>
</tr>
</tbody>
</table>

Fig. 2. The effect of different extraction times (A and B), ethanol concentrations (C and D) and plant mass/solution volume ratios (E and F) on extraction efficiency of As from freeze-dried roots and fronds of Pteris vittata grown in an AsIII-spiked (A, C and E) and AsV-spiked (B, D and F) soil containing 200 mg kg\(^{-1}\) As for 60 d. The error bars indicate the standard deviation of triplicate analyses.
efficiency. A slight improvement in extraction efficiency was obtained with increasing extractant volume, with 1:200 producing the highest extraction efficiency for both the roots and the fronds (80% and 95%).

During As extraction for speciation, it is important for As species to remain unchanged. Since the predominant As species in PV samples are AsIII and AsV, AsIII or AsV was spiked to PV grown in a clean soil, which contained 37 mg kg\(^{-1}\) As in the fronds and 10 mg kg\(^{-1}\) in the roots. The optimized ethanol method (0.5 h for fronds and 2 h for roots, 25% ethanol, and sample mass to solution ratio of 1:200) was used to extract spiked-As with and without PV tissues. The recovery for AsIII and AsV spike was ~100% in the absence of PV. In the presence of PV, 2% of AsV was reduced to AsIII in the roots and ~4% of AsV was reduced to AsIII in the fronds when a mixture of AsIII and AsV was spiked, indicating little change in As species. In addition, AsIII and AsV was spiked individually. Similar results were obtained, with 3% AsIII being oxidized to AsV and ~4% of AsV was reduced to AsIII in the roots and fronds. Thus the optimized ethanol method, which used less ethanol (reduced from 1:1 to 1:3) and less extraction time for the fronds (reduced from 2 h vs. 0.5 h), produced satisfactory extraction efficiency without changing As species during the extraction.

### 3.3. Application to different PV and other plant species

Satisfactory results were obtained with PV containing high As using the optimized ethanol method, which used 50 mg of sample with 10 mL of 1:3 ethanol:water and sonicated for 0.5 h for fronds and 2 h for roots. To test its suitability to other plants, it was applied to different plant samples, including fresh or freeze-dried PV with low As concentrations grown in hydroponics, tobacco leaves, and rice seedlings. For freeze-dried hydroponic PV, the method produced satisfactory extraction efficiency of ~80% for the roots and ~85% for the fronds (Table 2). However, the extraction efficiency for fresh PV samples was lower at 72%. Analysis of fresh samples was difficult mainly due to their moisture content and sample heterogeneity. On the other hand, freeze-dried plants were easier to be ground into homogeneous samples compared to fresh ones. During freeze-drying, water is removed, causing As compounds to crystallize and the cells to rupture (Jedynak et al., 2010). Bluemlein et al. (2009) recommended that the sample lyophilization process promoted the disintegration of As–phytchelatin complex. Hence, higher extraction efficiency was obtained using freeze-dried plants.

### Table 3

Application of the optimized ethanol method (25% methanol, 0.5 h and 2 h for roots and fronds, sample/solution ration of 1:200) to dry tobacco leaf and freeze-dried rice seedlings.

<table>
<thead>
<tr>
<th>Sample</th>
<th>AsIII (mg kg(^{-1}))</th>
<th>AsV (mg kg(^{-1}))</th>
<th>MMA (mg kg(^{-1}))</th>
<th>Sum (mg kg(^{-1}))</th>
<th>Total (mg kg(^{-1}))</th>
<th>Extraction efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tobacco 1</td>
<td>0.03 ± 0.00</td>
<td>0.13 ± 0.01</td>
<td>0.12 ± 0.01</td>
<td>0.28 ± 0.01</td>
<td>0.35 ± 0.01</td>
<td>80.0 ± 2.85</td>
</tr>
<tr>
<td>Tobacco 2</td>
<td>0.04 ± 0.00</td>
<td>0.14 ± 0.01</td>
<td>0.12 ± 0.00</td>
<td>0.30 ± 0.01</td>
<td>0.32 ± 0.01</td>
<td>93.7 ± 3.12</td>
</tr>
<tr>
<td>Rice root</td>
<td>398 ± 29.4</td>
<td>31.3 ± 4.92</td>
<td>Nd</td>
<td>429 ± 34.2</td>
<td>574 ± 32.3</td>
<td>74.7 ± 5.95</td>
</tr>
<tr>
<td>Rice shoot</td>
<td>32.4 ± 2.67</td>
<td>2.95 ± 0.21</td>
<td>Nd</td>
<td>35.3 ± 2.80</td>
<td>53.4 ± 2.02</td>
<td>66.1 ± 5.24</td>
</tr>
</tbody>
</table>

Nd: not detected.

### Fig. 3

Extraction efficiency of As from freeze-dried rice seedlings (A) and dry tobacco leaf (C); and distribution of spiked AsIII and AsV (1:1) into rice seedlings (B) and tobacco leaves (D) based on the optimized ethanol method (25% methanol, 0.5 h and 2 h for roots and fronds, sample/solution ratio of 1:200) and other three extraction methods. Rice seedlings were obtained by growing in a nutrient solution containing 2.5 mg L\(^{-1}\) AsV for two weeks. Tobacco leaves were purchased from a supermarket in Nanjing, 1 and 2 represent two different brands. The bars indicate the standard error of triplicates.
In addition to PV samples, we applied the optimized ethanol method to other plant species including tobacco leaves and rice seedlings. The As concentration in the tobacco was 0.32–0.35 mg kg\(^{-1}\) whereas rice seedlings contained 57.4 and 53.4 mg kg\(^{-1}\) As in the roots and shoots (Table 3). The optimized ethanol method was compared with the other three methods in term of extraction efficiency and species stability during extraction. The results showed that the ethanol method was not only effective for PV samples but also good for rice seedlings and tobacco leaves (Fig. 3).

For rice seedlings, the PBS method produced the highest extraction efficiency at 80% for the shoots, but the efficiency for the roots was low at 46% (Fig. 3A), with 5% of As\(\text{V}\) being oxidized to As\(\text{III}\) in the roots (Fig. 3B). The extraction efficiency for the roots and shoots was 55% and 71% using HNO\(_3\), with 10–15% of As\(\text{V}\) being reduced to As\(\text{III}\). The extraction efficiencies for the roots and shoots were 74% and 66% using the optimized ethanol method, compared to 56% and 64% with the methanol method. In addition, both methanol and ethanol kept As species stable during extraction. Among the four methods, the optimized ethanol method provided the best As recovery from rice seedling without changing As species. Our results were similar to Yuan et al. (2005) using rice straw and water–ethanol mixture based on micro-wave extraction.

Rice seedlings consisted of ~92% of As\(\text{III}\) and ~8% of As\(\text{V}\) in the current study. Lomax et al. (2012) showed that methylated species are present only in plants growing in media containing MMA and DMA. The rice used in our study was grown in a hydroponic solution without methylated As species, so only inorganic As species were detected. Based on the results from PV, higher extraction efficiency should be obtained from rice seedlings due to the dominance of As\(\text{III}\). However, only 66% and 74% of As were extracted using the optimized ethanol method. The difference in extraction efficiency between PV and rice seedlings was probably because As is stored in PV fronds as uncomplexed As\(\text{III}\) (Lombi et al., 2002), whereas As is stored as As\(\text{III}\)–phytochelatin complexes in rice (Moore et al., 2011).

Unlike PV and rice seedlings, MMA was found in tobacco leaf besides As\(\text{III}\) and As\(\text{V}\), with no DMA being detected (Table 3). The percentage of As extracted varied from 78% to 92% in two brands of cigarettes using the optimized ethanol method with little transformation in As species during extraction (Fig. 3C, D). The HNO\(_3\) method produced the highest extraction efficiency of 94–104% with 3.9–7.7% of As\(\text{V}\) being reduced to As\(\text{III}\). The extraction efficiency of methanol and PBS method was 68.2–86.4 and 39.7–63.8% with 3% of As\(\text{V}\) being reduced to As\(\text{III}\). Since MMA was found in the tobacco leaves, aqueous mixtures of As\(\text{III}\), As\(\text{V}\), DMA and MMA were added to the tobacco powder to check the stability of these species. ~100% DMA and MMA were recovered when the ethanol method was applied. Thus, the optimized ethanol method also produced satisfactory extraction efficiency for tobacco leaves while keeping As species unchanged. In short, the optimized ethanol method was suitable for extraction of As species not only from As-hyperaccumulator PV grown in contaminated soil and hydroponics, but also for other plant species with low As.

4. Conclusions

The optimized ethanol method based on 1:3 ethanol:water mixture with 0.5–2 h sonication was successfully used to perform As speciation in PV roots and fronds with satisfactory extraction efficiency. Extraction >80% of As was obtained for PV roots and >85% for the fronds. In addition, compared with the traditional methanol/water extraction, the optimized method saved time for As speciation in the fronds (0.5 vs. 2 h) with better As recovery in the roots (80 vs. 60%). Replacing toxic methanol with ethanol makes it more environment-friendly. Satisfactory results were obtained when applying the method to other plant species including tobacco leaf (78–92%) and rice seedlings (~70%). In short, the method has the potential to be used in As speciation in plant samples. However, further study is needed to validate its suitability to all plant samples.

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References


