Effects of exogenous addition of cadmium on cadmium speciation in hyperaccumulator of *Sedum alfredii* Hance

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Abstract. Continuous chemical extraction is considered to be one of the most common methods for the extraction of heavy metals speciations in plants. However, the basis for the extraction order of this method is not explained and has been rarely studied. In this paper, we analyzed the speciations of cadmium in plants by a four-step continuous extraction method. The extraction sequence of ethanol and water was studied to study the effect of extraction sequence on the extraction rate of extraction agent. The results showed that cadmium exists mostly in NaCl extraction state and the extraction rate of the two kinds of extraction agents decreased when the order of ethanol and water was changed.

1 Introduction

Sedum alfredii Hance, an ancient lead-zinc mine in Yazhou city, Zhejiang province, is known to be a cadmium hyperaccumulator that can transport heavy metals (HMs) to the ground through its roots. The absorption of HMs by plants and their migration and transformation in plants are hot topics. Chemical continuous extraction is considered to be a common method to study the speciation of HMs in plants. The extracting agents for different forms of HMs are mostly NaCl, ethanol, hydrochloric acid, deionized water, acetic acid, organic solvent, etc [1-3]. And different extracting agents have different extraction rate for HMs. However, in many literatures, the reasons for the selection of extractant have not been explained. In this paper, four extractants, 80% ethanol, deionized water, NaCl and 2% acetic acid, were used to extract speciations of Cd in *Sedum alfredii* Hance. In order to explore the effect of the order of extraction agents on the extraction rate, the extraction order of ethanol and water was changed in this paper.

2 Material and methods

2.1 Plant cultivation

The experimental material was hyperaccumulator *Sedum alfredii* Hance, purchased from Hangzhou Lusheng Institute of Modern Agriculture and Environmental Ecology. The purchased branches were washed with tap water, the roots were removed, and the branches were cut into uniform branches with a height of 3-4cm. The branches were germinated in clear water for 15 days in an incubator, and then cultured in 1/8 Holland nutrient

solution for 6 days and 1/4 Holland nutrient solution for 14 days. The nutrient composition is: 0.1 mM KH_2PO_4 , 0.5 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 mM KCl, 0.7 mM K_2SO_4 , 2 mM $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 10 μM H_3BO_3 , 0.01 μM $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, 0.2 μM $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 1 mM $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 μM $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 100 μM FeEDTA. The environment of the incubator was 26/20°C, photosynthetically active radiation was $500\mu\text{molm}^{-2}\text{s}^{-1}$, illumination intensity was 2500Lux, 70-80% relative humidity, water was changed every three days and ventilation was maintained all day long.

2.2 Cadmium exposure and sample pretreatment

Sedum alfredii Hance was cultured in nutrient solution containing 20mg/L and 100mg/L Cd^{2+} for 9 days, and three parallel samples were set for each treatment. After harvest, the whole plant was divided into three parts: roots, stems and leaves. The roots were soaked in 20mM $\text{Na}_2\text{-EDTA}$ for 15 minutes to remove the Cd^{2+} on the roots surface [4]. Then clean it with deionized water for several times, dry it with absorbent paper, freeze dry it in a freeze-dryer (FD-1A-80) and store it at room temperature for later use. Leaves and stems were washed several times with deionized water, dried with absorbent paper, then freeze-dried in a freeze-dryer and stored at room temperature for later use.

2.3 Extraction of different speciation of cadmium

The freeze-dried samples were ground evenly with a mortar and pestle and extracted by two methods. The first method, which is called the four-step continuous extraction method, refers to the four-step extraction method of Wang et al. The extraction agent and

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extraction speciation are respectively. 80% ethanol: extraction of inorganic salts and amino acid salts based on nitrate and chloride; deionized water: extraction of water-soluble organic acid salts, HMs generation of phosphate, etc.; 1mol/L NaCl: extraction of pectate, HMs bound to protein or adsorbed state, etc.; 2% acetic acid: extraction of water insoluble HMs phosphate, including second generation phosphate, orthophosphate, etc.

The second method uses a two-step extraction method, the first step is extracted with deionized water, and the second step is extracted with 80% ethanol (i.e. switching the order of the first two steps in the four-step extraction method). The specific operation steps are as follows: take about 0.2g of the sample and immerse it in 25ml of the extractant overnight, then extract the precipitate with 25ml of the extractant for 3 times with a time interval of 2 hours, extract it for 4 times in total, and collect 100ml of the supernatant. The supernatant of each extraction is filtered by a filter membrane, and the solids on the filter membrane are flushed to the solid precipitate with extractant. Each extraction step follows the above process.

2.4 Determination of the content of each speciation

100mL of supernatant extracted from ethanol, water and hydrochloric acid was placed on an electric heating plate at 70°C and evaporated to about 2ml. 10mL of supernatant extracted from NaCl and acetic acid was evaporated to about 2ml and then digested with a microwave digestion apparatus ($\text{HNO}_3:\text{H}_2\text{O}_2$, 5:1). After digestion, ethanol and water were extracted to 25ml, and NaCl and acetic acid were extracted to 10mL. The residue was steamed and dried with an electric hot plate and digested under the same conditions, then the volume

was constant to 10mL. The digestion solution was filtered with 0.45 μm membrane and stored at 4°C for later use. Finally, the cadmium content was analyzed by atomic absorption spectroscopy (AA7000).

2.5 Data processing

All data were correlated and statistically analyzed using Excel 2019 and SPSS packets (version#24.0). Data were analyzed by univariate ANOVA, and when the P-value detected by ANOVA F was less than 0.05, the difference between the means was considered significant. Finally, use Orig8.0 drawing software to draw the graph.

3 Results

3.1 Analysis of total speciation and recovery rate

Four extraction agents (80% ethanol, deionized water, NaCl, acetic acid) were used for continuous extraction of the three parts of *Sedum alfredii* Hance roots, stems and leaves, respectively. The extraction results are shown in Table 1. Under the treatment of 20mg/L Cd, the contents of ethanol, water, NaCl, acetic acid and residue in roots were 75.96 μg , 2.52 μg , 0.85 μg , 0.54 μg and 0.01 μg , respectively. The content of each extracted state in the stem was 12.86 μg for ethanol, 80.58 μg for water, 32.90 μg for NaCl, 8.50 μg for acetic acid and 0.02 μg for residue. The extracted contents of leaves were 104.61 μg , 99.41 μg , 173.11 μg , 14.56 μg and 0.07 μg , respectively. It can be seen from the data that the content of Cd in the residue state can be almost ignored by the extraction of the four extractant

Table 1 The content of different speciation in root, stem and leaf of *Sedum alfredii* Hance

Cd treatment (mg/L)	Plant organs	The content of different speciation (μg)					Recycled (μg)	Actual content (μg)
		80% ethanol	Deionized water	NaCl	Acetic acid	Residue		
20	root	75.96 $\pm$ 3.80	2.52 $\pm$ 0.11	0.85 $\pm$ 0.03	0.54 $\pm$ 0.01	0.01 $\pm$ 0.00	79.88 $\pm$ 3.99	69.84 $\pm$ 2.11
	stem	12.86 $\pm$ 0.26	80.58 $\pm$ 3.22	32.90 $\pm$ 0.66	8.50 $\pm$ 0.25	0.02 $\pm$ 0.00	134.85 $\pm$ 5.39	156.58 $\pm$ 6.26
	leaf	104.61 $\pm$ 6.21	99.41 $\pm$ 3.98	173.11 $\pm$ 6.92	14.56 $\pm$ 0.58	0.07 $\pm$ 0.00	391.77 $\pm$ 22.51	374.29 $\pm$ 15.11
100	root	15.23 $\pm$ 0.61	1.90 $\pm$ 0.08	33.95 $\pm$ 1.35	0.75 $\pm$ 0.03	0.03 $\pm$ 0.00	51.85 $\pm$ 1.56	42.25 $\pm$ 1.27
	stem	16.93 $\pm$ 0.51	29.92 $\pm$ 0.59	116.26 $\pm$ 3.49	10.87 $\pm$ 0.11	0.16 $\pm$ 0.01	174.15 $\pm$ 5.22	137.10 $\pm$ 5.48
	leaf	26.56 $\pm$ 1.06	29.33 $\pm$ 1.74	154.65 $\pm$ 6.19	16.01 $\pm$ 0.31	0.24 $\pm$ 0.01	226.77 $\pm$ 9.07	235.02 $\pm$ 12.45

The percentage of the total amount of Cd extracted by four extractants and the actual amount of Cd in the plant was the recovery rate of Cd. The recoveries of roots, stems and leaves were 114.37%, 86.12% and 104.67% under 20mg/L Cd treatment. The recoveries of roots, stems and leaves were 122.72%, 127.02% and 96.49%

under 100mg/L Cd treatment, respectively. The recovery range was between 86.12% and 127.02%, which was similar to the recovery range of HMs extracted by Li through continuous extraction method [5] and met the requirements of speciation analysis

Table 2 The recovery rates of different speciation in root, stem and leaf of *Sedum alfredii* Hance

Cd Treatment (mg/L)	Plant organs	Recovery rate (%)					Recovery rate
		80% ethanol	Deionized water	NaCl	Acetic acid	Residue	
20	root	95.09 $\pm$ 4.35	3.16 $\pm$ 0.11	1.07 $\pm$ 0.02	0.67 $\pm$ 0.01	0.01 $\pm$ 0.00	114.37 $\pm$ 4.57
	stem	9.53 $\pm$ 0.25	59.75 $\pm$ 2.57	24.40 $\pm$ 1.05	6.31 $\pm$ 0.22	0.01 $\pm$ 0.00	86.12 $\pm$ 3.44

100	leaf	26.70±1.12	25.37±1.54	44.19±1.85	3.72±0.16	0.02±0.00	104.67±5.23
	root	29.38±0.73	3.66±0.09	65.47±2.41	1.44±0.04	0.05±0.00	122.72±4.91
	stem	9.72±0.62	17.18±0.87	66.76±4.24	6.24±0.45	0.09±0.00	127.02±2.54
	leaf	11.71±0.45	12.93±0.64	68.19±3.29	7.06±0.27	0.10±0.00	96.49±3.68

3.2 Speciation of Cd under different concentrations of Cd exposure

As shown in Fig.1, the acetic acid extraction state of *Sedum alfredii* Hance under the exposure of 20mg/L Cd showed significant differences with the ethanol extraction state, water extraction state and NaCl extraction state ($p<0.05$). Ethanol, water and NaCl accounted for 31.89%, 30.09% and 34.11% of the total, respectively, and acetic acid accounted for 23.6%. Under the exposure of 100mg/L Cd, the proportion of NaCl extraction was the highest, 67.33%, which showed significant difference with other extraction states ($p<0.05$). There was no significant difference in ethanol, water and acetic acid extracts, which accounted for 12.97%, 13.5% and 6.1% of the total, respectively. It can also be seen from the figure that the extraction rate of NaCl was the highest at the two concentrations, followed by ethanol and water extraction, and the extraction rate was basically the same.

Fig.1 Speciation in *Sedum alfredii* Hance under 20mg/L (A) and 100mg/L (B) Cd exposure

Fraction 1: 80% ethanol extraction speciation; Fraction 2: Deionized water extraction speciation; Fraction 3: 0.1mol/L NaCl extraction speciation; Fraction 4: 2% acetic acid extraction speciation; Fraction 5: residual speciation

3.3 Speciation of Cd in root, stem and leaf

There were obvious differences in the speciation of Cd in the roots, stems and leaves. Different concentrations of Cd also have an effect on the transformation of Cd speciation. This section mainly analyzes the speciation of Cd in roots, stems and leaves and the effects of different concentrations of Cd on speciation.

As shown in Fig.2, Cd speciation in roots were significantly different under exposure of different concentrations of Cd. Under the exposure of 20mg/L Cd, Cd mainly existed in ethanol form (95.09%), 3.61% in water form, and only 1.74% in NaCl and acetic acid form. The forms of Cd in roots mainly existed in ethanol and water extraction states. Under the exposure of 100mg/L Cd, 65.47% of Cd was extracted in the form of NaCl, followed by ethanol, accounting for 29.38%. The proportion of water extraction is relatively small, only 3.66%. Cd in roots mainly exists in the form of NaCl extraction states.

The speciation of Cd in stem was different from that

in roots. As shown in Fig.2, under 20mg/L Cd exposure, 59.75% of Cd in the stem was in the form of water extraction, 24.40% in the form of NaCl extraction, 9.53% in the form of ethanol extraction and 6.31% in the form of acetate extraction. Under the exposure of 100mg/L Cd, 66.76% Cd was extracted in the form of NaCl extraction, 17.18% in the form of water, 9.72% in the form of ethanol and 6.24% in the form of acetic acid.

The speciation of Cd in leaves were not significantly affected by Cd exposure. The leaves of *Sedum alfredii* Hance treated with 20mg/L Cd and 100mg/L Cd were mainly NaCl extraction, followed by ethanol extraction, water extraction and acetic acid extraction. Under the treatment of 20mg/L Cd, the extraction state of NaCl was 44.19%, that of ethanol was 26.70%, that of water was 25.37% and that of acetate was 3.72%. Under the treatment of 100mg/L Cd, 68.19% of the leaves were NaCl extraction state, 12.93% were water extraction state, 11.71% were ethanol extraction state and 7.06% were acetic acid extraction state.

Fig.2 The speciation of cadmium in root of *Sedum alfredii* Hance under 20mg/L (left) and 100mg/L (right) Cd exposure

Fraction 1: 80% ethanol extraction speciation; Fraction 2: Deionized water extraction speciation; Fraction 3: 0.1mol/L NaCl extraction speciation; Fraction 4: 2% acetic acid extraction speciation; Fraction 5: residual speciation

It can be seen from Fig.2 that, under the treatment of 20mg/L and 100mg/L Cd, the higher the position was, the more NaCl extraction state content was in all parts from aboveground part to aboveground part. Under the treatment of 100mg/L Cd, the extraction state of acetic acid also showed the same rule. This may be the reason for the less toxicity of the NaCl and acetic acid extracts.

3.4 The different speciation of Cd were extracted by two-step extraction method

Due to the literature review, we could not find the explicit basis for the extraction methods of different speciation of HMs in plants, nor could we find the basis for the selection of extractant sequence in the continuous extraction method. Therefore, the extraction sequence of ethanol and water is changed in this section, that is, the first step is water extraction, the second step is ethanol extraction, and the change of extraction rate of extraction agent is analyzed after the exchange of the order. In this section, a two-step continuous extraction experiment was

carried out with *Sedum alfredii* Hance under 20mg/L Cd exposure, and compared with the first step ethanol extraction and the second step water extraction in sections 3.2 and 3.3.

The two-step extraction method was used to extract different speciation of Cd in the *Sedum alfredii* Hance. From the point of view of the whole plant, water extracted Cd 80.39 μ g, accounting for 13.38% of the total

Cd. 36.33 μ g Cd was extracted by ethanol, accounting for 6.05% of the total Cd (as shown in Table 3). In the experiment in Section 3.2, ethanol extracted Cd accounted for 31.89% of the total Cd, and water extracted Cd accounted for 30.09% of the total Cd. Therefore, changing the extraction sequence of the extractant will reduce the extraction capacity of the extractant

Table 3 The extraction concentration and extraction rate of two - step extraction method

Cd Treatment (mg/L)	Plant organs	Extraction concentration (μ g)		Extraction rate (%)	
		Deionized water	80% ethanol	Deionized water	80% ethanol
20	root	13.08 $\pm$ 0.52	2.27 $\pm$ 0.13	18.73 $\pm$ 0.56	3.25 $\pm$ 0.16
	stem	15.88 $\pm$ 0.79	9.13 $\pm$ 0.37	10.14 $\pm$ 0.31	5.83 $\pm$ 0.23
	leaf	51.43 $\pm$ 1.54	24.93 $\pm$ 1.25	13.74 $\pm$ 0.69	6.66 $\pm$ 0.33
	plant	80.39 $\pm$ 4.02	36.33 $\pm$ 0.73	13.38 $\pm$ 0.54	6.05 $\pm$ 0.30

As shown in Fig.3, water and ethanol were used to extract Cd from the roots, stems and leaves, and the extraction rate of water was both higher than that of ethanol. Water and ethanol accounted for 18.73% and 3.25% respectively. 10.14% and 5.83% of the stems were extracted by water and ethanol. In the leaves, 13.74% were extracted by water and 6.66% by ethanol.

Compared with the four-step extraction method, the first step was ethanol extraction (extraction rate 95.09%) and the second step was water extraction (3.16%). When Cd was extracted from roots, the extraction rate of ethanol was greatly reduced, while the extraction rate of water was increased. For Cd in stems, ethanol and water accounted for 9.53% and 59.75% by four-step extraction method, and the extraction rate of two-step extraction method was both reduced. For Cd in leaves, the extraction rates of ethanol and water by four-step extraction method were 26.70% and 25.37%, respectively, higher than that of water (13.74%) and ethanol (6.66%) by two-step extraction method. In conclusion, although the speciation of Cd is different in roots, stems and leaves, the extraction sequence of the exchange extractant is not conducive to the extraction of Cd speciation.

Fig.3 The extraction rate of two-step continuous extraction method

4 Discussion

(1) Almost all Cd can be extracted from the *Sedum alfredii* Hance by four successive extraction steps of ethanol, water, NaCl and acetic acid. Xu et al. extracted

Cd in *Siegesbeckia orientalis* L. by a five-step continuous extraction method (ethanol, water, NaCl, acetic acid and hydrochloric acid), and the extraction rate of the first four extracts reached more than 90% [6]. However, Yang et al. [7] used this method to extract different speciation of lead in rice, and found that the extraction form of hydrochloric acid accounted for 28%-52%, followed by acetic acid, NaCl, water and ethanol, which was very low, contrary to the conclusion of this study. This may be the result of the self-regulation mechanism of hyperaccumulator to reduce HMs toxicity.

(2) The toxicity of Cd is directly related to its speciation. In general, ethanol and water-extracted states are more cytotoxic to plants because inorganic salts such as nitrate and chloride are thought to have strong migratory capacity. However, the NaCl and acetic acid extraction showed weak mobility and cytotoxicity. In this paper, the hyperaccumulator *Sedum alfredii* Hance was used as the material, and the exogenous Cd exposure was added to extract Cd from the plants continuously with four extractant. It was found that the extracted state of NaCl accounted for 34.11% of the plants under 20mg/L Cd exposure, and 67.33% of the plants under 100mg/L Cd exposure. The high proportion of NaCl extraction is due to the fact that metallothioneins in the cells of hyperaccumulator can chelate with HMs and form complex with low toxicity after being stressed by Cd. Wei et al. [8] used the five-step continuous extraction method to extract Cd from the hyperaccumulator *Solanum nigrum* L., and found the same rule.

(3) From the underground part to the aboveground part (root $\rightarrow$ stem $\rightarrow$ leaf), the NaCl extraction state increased successively, that is, *Sedum alfredii* Hance transported the less toxic NaCl extraction state upward. We hypothesized that *Sedum alfredii* Hance had selective migration for different speciation of Cd, especially for the less toxic NaCl and acetic acid extraction states. Further study is needed on the migration ability of different speciation.

(4) The extraction rate of two-step extraction method (the first step is water extraction, the second step is ethanol extraction) is lower than that of ethanol extraction and then water extraction. Literature review

found that the extraction rate of water is generally not more than 30%. Xu et al.^[9] extracted different speciation of arsenic in lentinus edodes with water, and the extraction rate was about 25%. Gilon^[10] used water at 60°C to extract selenium from selenium-rich yeast, and the extraction rate was 20%. In this study, Cd was extracted with normal temperature water, and the extraction rate was only 13.38%. Therefore, the order of exchange of extractant reduces its extraction capacity. The extraction yield of ethanol was only 6.05%, which was far lower than that of ethanol as the first order. To sum up, the extraction effect of HMs in plants is better when ethanol is placed in the first extraction sequence and water in the second extraction sequence.

5 Conclusion

(1) The four-step continuous extraction method (80% ethanol, deionized water, 0.1mol/L NaCl, 2% acetic acid) can extract more than 90% Cd from the *Sedum alfredii* Hance. The recoveries ranged from 86.12% to 127.02%, which met the requirements of speciation analysis.

(2) Cd is mainly extracted from NaCl in the *Sedum alfredii* Hance. The Cd extracted in the NaCl extraction state binds to pectate and protein, and has low toxicity and little damage to cells, which is one of the important mechanisms of *Sedum alfredii* Hance against Cd toxicity.

(3) *Sedum alfredii* can transfer the low toxicity form to the aboveground. From the underground part to the aboveground part of the plant, the NaCl extraction states in various organs (roots, stems and leaves) gradually increased, and the migration of different speciation of Cd needs to be further studied.

(4) Changing the extraction sequence of ethanol and water is not conducive to the extraction of HMs in plants. The extraction rate of ethanol decreased from 31.89% to 6.06%, and the extraction rate of water decreased from 30.09% to 13.38%.

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References

1. Wang, W., M. Meng, and L. Li, 2019, Arsenic detoxification in *Eucalyptus*: subcellular distribution, chemical forms, and sulfhydryl substances. *Environ Sci Pollut Res Int.* 26(24): p. 24372-24379.
2. Zhang, W., K. Lin, J. Zhou, W. Zhang, L. Liu, and Q. Zhang, 2014, Cadmium accumulation, sub-cellular distribution and chemical forms in rice seedling in the presence of sulfur. *Environ Toxicol Pharmacol.* 37(1): p. 348-53.
3. Wang, X. and D. Liu, 2017, Integration of cerium chemical forms and subcellular distribution to understand cerium tolerance mechanism in the rice seedlings. *Environ Sci Pollut Res Int.* 24(19): p. 16336-16343.
4. Lai, H.Y., 2015, Subcellular distribution and chemical forms of cadmium in *Impatiens walleriana* in relation to its phytoextraction potential. *Chemosphere.* 138(nov.): p. 370-376.
5. Li, X., B.J. Coles, M.H. Ramsey, and I. Thornton, 1995, Sequential extraction of soils for multielement analysis by ICP-AES. *Chemical Geology.* 124(1-2): p. 109-123.
6. Xu, X., 2018. Mechanisms of uptake and tolerance of hyperaccumulator plant *Siegesbeckia orientalis* L. to cadmium stress. Sichuan Agricultural University.
7. Yang, J., Zha, Yan., Liu, Hong., 1999, The distribution and chemical forms of Cd, Cu, Pb in polluted seeds. *China Environmental Science.* 19(6): p. 500-504.
8. Wei, S., X. Zeng, S. Wang, J. Zhu, D. Ji, Y. Li, and H. Jiao, 2014, Hyperaccumulative property of *Solanum nigrum* L. to Cd explored from cell membrane permeability, subcellular distribution, and chemical form. *Journal of Soils and Sediments.* 14(3): p. 558-566.
9. Xu, J., 2016, Arsenic Speciation Analysis of *Lentinus edodes* by Ultrasonic-Assisted Extraction-High Performance Liquid Chromatography-Inductively Coupled Plasma Mass Spectrometry. *Food science.* 37: p. 216-221.
10. N. Gilon, A.A., M. Astruc and M. Potin-Gautier, 1995, Selenoamino Acid Speciation Using HPLC-ETAAS following an enzymic hydrolysis of selenoprotein. *Applied Organometallic Chemistry.* 9(7): p. 623-628.