Comparison of a rhizosphere-based method with other one-step extraction methods for assessing the bioavailability of soil metals to wheat

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Received 24 June 2004; received in revised form 17 October 2004; accepted 22 November 2004

Abstract

There is no method recognized as a universal approach for evaluation of bioavailability of heavy metals in soil. Based on the simulation of the rhizosphere soil conditions and integration of the combined effects of root–soil interactions as a whole, a rhizosphere-based method has been proposed. Wet fresh rhizosphere soil was extracted by low-molecular-weight organic acids (LMWOAs) to fractionate metal fractions of soil pools, which were then correlated with the metal contents of wheat roots and shoots. The rhizosphere-based method was compared with other one-step extraction methods using DTPA, EDTA, CaCl_2 , and NaNO_3 as extractants and the first step of the Community Bureau of Reference (BCR) method. Simple correlation and stepwise multiple regression analysis were used for the comparison. Simple correlation indicated that the extractable Cu, Zn, Cr, and Cd of soils by the rhizosphere-based method were significantly correlated with the metal contents of wheat roots. For DTPA, BCR1 and EDTA methods there was a relatively poor correlation between the extractable Cu, Zn and Cd of soil and metal contents of wheat roots. Stepwise multiple regression analysis revealed that the equation of the rhizosphere-based method was the simplest one, and no soil properties variables needed to be added. In contrast, the equations of other one-step extraction methods were more complicated, and soil properties variables needed to be entered. The most distinct feature of the rhizosphere-based method was that the recommended method was suitable for acidic, neutral and near alkaline soils. However, the DTPA and EDTA extraction methods were suitable for calcareous soils only-or-only for acidic soils. The CaCl_2 , and NaNO_3 extraction methods were only suitable for exchangeable metals. In short, the rhizosphere-based method was the most robust approach for evaluation of bioavailability of heavy metals in soils to wheat.

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Keywords: Rhizosphere; Low-molecular-weight organic acids; Bioavailability; Heavy metals in soil; Wheat

1. Introduction

Many efforts have been made to estimate bioavailability of metals in soils to plants. The most important approaches include the free ion activity model (FIAM) (Morel, 1983; McLaughlin et al., 1997; Hudson, 1998;

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Errécalde and Campbell, 2000), the correlation between the labile metals in soil pools determined by single or sequential extraction methods and their accumulation in plants (Haq et al., 1980; Novozamsky et al., 1993; Shan et al., 2003), the isotope dilution exchange method (Collins et al., 2003) and the diffusive gradients in thin films (DGT) (Zhang et al., 1998, 2001).

Linear correlations between various indicators of metal bioavailability and the metal content in plant parts are often used for identifying the best-suited method of assessment for soil testing procedures. Both single and sequential extraction approaches are used for fractionation of metals in soil pools. Ethylenediamine tetraacetic acid (EDTA) (0.005–1 M) acts as a chelating agent, and EDTA can form stable chelates with many metal ions (Haq et al., 1980; Hammer and Keller, 2002; Chaignon et al., 2003). Diethylenetriaminepentaacetic acid (DTPA) (0.005 M) (Haq et al., 1980; Norvell, 1984; Chaignon et al., 2003) is most suitable for calcareous soils, as it is buffered at a pH 7.3 and therefore prevents CaCO_3 from dissolution and release of occluded metals. The DTPA extraction method is chosen to minimize dissolution of carbonates, although uncertainty concerning the dissolution of iron and aluminum compounds remains (Novozamsky et al., 1993). Sodium nitrate and ammonium nitrate are also widely used for single extraction (Whitten and Ritchie, 1991). The use of CaCl_2 is also recommended due to the same salt concentration as the average Ca^{2+} concentration in soil solution and ability of Ca^{2+} to extract other adsorbed cations in soils (Novozamsky et al., 1993). The Community Bureau of Reference (BCR, now Standards, Measurements and Testing Programme of the European Committee) launched a project to coordinate measurements of the extractable trace metal contents in soil and sediments (Quevauviller et al., 1994). More recently, this method has been applied to estimate bioavailability, but no consistent data have been obtained (Qian et al., 1996; Sauvé et al., 1996; Shan et al., 2003). Gleyzes et al. (2002) comprehensively reviewed the single and sequential fractionation methods. Low-molecular-weight organic acids (LMWOAs) are important plant exudates. It should also be stressed that parts of LMWOAs normally found in the rhizosphere are microbial metabolites. It is for instance often considered that acetic and lactic acids are more likely originating from rhizosphere bacteria (Jones, 1998). LMWOAs play an important role in coping with nutrient deficiencies, metal tolerance and plant-microbe interactions operating at the root-soil interface (López-Bucio et al., 2000). In our previous study, LMWOAs were used to predict the bioavailability of heavy metals and rare earth elements (REEs) in soil pools to plants (Shan et al., 2003). In order to further understand whether the LMWOAs extraction method can be more widely used for bioavailability, we studied the effects of LMWOAs on release of REEs and heavy

metals from soils (Shan et al., 2002; Qin et al., 2004). The results indicated that metals could be easily released from soils in the presence of LMWOAs. In addition, soil and hydroponic culture experiments also verified that LMWOAs enhanced the uptake of lanthanum by wheat roots (Wang et al., 2004) and barley roots (Han et al., 2004). LMWOAs also increased the accumulation of light REEs in perennial fern (Wang et al., 2004). All these results demonstrated that organic acids were involved in soil-root interactions, which rationalize the rhizosphere-based method for assessing the bioavailability of metals in soil to plants.

Although a good progress has been made in bioavailability methods, no universally applicable approach has been recognized. The derived data from the correlation methods are inconsistent and make interpretations difficult (Sauvé et al., 1996). It was ascribed to the poor simulation of the conditions of field-grown plants, while the combined-effects of soil-root interactions have not been fully integrated in the current methods of bioavailability.

It has been progressively understood that many factors influence the bioavailability, including soil properties and plant species. There is no simple cause-effect relationship between the metal bound with a particular geochemical phase and metal uptake by plants. It is especially true that the rhizosphere-plant interactions influence the metal bioavailability, and therefore the development of new methods for evaluation of bioavailability should be based on the rhizosphere-plant interactions. The principles of new bioavailability methods must simulate the real field conditions as close as possible, and also simulate the combined effects of rhizosphere-root interactions as a whole. The key parameters of new bioavailability method are the uses of fresh rhizosphere soil and LMWOAs as extractant. Hereafter, this method is referred to as rhizosphere-based extraction method.

To our knowledge a systematic comparison between different extraction methods for assessing the bioavailability of soil metals is lacking. Therefore, the aim of the present study was to compare the rhizosphere-based method with other one-step extraction methods using DTPA, EDTA, CaCl_2 , NaNO_3 as extractants and the first step of the Community Bureau of Reference (BCR) method. Simple correlation and stepwise multiple regression analysis were used for comparison.

2. Materials and methods

2.1. Soil sample

Sixteen A_p horizon cultivated soil samples were collected from different provinces of China, which represented a spectrum of typical Chinese soils (Gong,

1999) with various physical and chemical properties. The soil samples were air dried, homogenized and ground to pass through a 1 mm nylon fiber sieve, and stored for subsequent analysis. Organic matter (OM) was determined by the Walkley–Black procedure (Nelson and Sommers, 1982). Soil pH was measured at soil to 0.01 M CaCl₂ solution of 1:1 (w/v). Cation-exchange capacity (CEC) was determined by the BaCl₂ method

(Hendershot and Duquette, 1986). Amorphous iron oxides (amorphous-Fe) were determined by ammonium-oxalate extraction method (Blakemore et al., 1987). Crystalline iron oxides (crystal-Fe) were determined by the oxalate-ascorbic acid extraction method of Shuman (Shuman, 1982). The selected soil properties are presented in Table 1. The total concentrations of metals in soils are given in Table 2.

Table 1
Selected properties of soils

Site	Taxonomy	pH	CEC ^a cmol kg ⁻¹	OM% ^b	Fe		MnO ₂ g kg ⁻¹
					Amor% ^c	Cry% ^d	
Heilongjiang	Isohumisols	7.34	20.2	6.86	0.73	18.3	4.02
Jilin	Isohumisols	5.65	14.0	2.56	0.70	26.6	6.58
Gansu	Isohumisols	6.55	41.2	0.96	0.26	21.9	3.27
Neimenggu	Luvisols	6.32	56.8	3.81	0.26	13.1	3.09
Shanxi	Luvisols	7.29	21.9	4.12	0.27	19.4	2.21
Beijing	Cambisols	6.91	15.7	1.35	0.37	15.5	4.09
Shandong	Luvisols	6.75	18.0	0.98	0.47	16.7	4.74
Jiangsu	Luvisols	6.37	22.3	0.68	0.47	16.1	3.62
Anhui	Luvisols	5.16	25.0	2.09	0.66	18.0	3.54
Hubei	Luvisols	6.67	22.8	0.92	1.04	32.9	5.25
Fujian	Ferralsols	6.09	5.1	1.81	0.54	13.2	3.53
Jiangxi	Ferrisols	4.70	14.2	0.80	0.29	24.4	1.90
Yunnan	Ferrisols	6.64	15.5	2.23	0.97	29.9	5.25
Chengdu	Cambisols	6.50	42.6	1.03	0.54	22.0	4.82
Guizhou	Cambisols	6.30	51.3	6.76	1.04	32.9	6.37
Xinjiang	Aridisols	7.44	74.0	0.90	0.26	14.1	4.14

^a CEC = cation exchange capacity.

^b OM = organic matter.

^c Amor-Fe = amorphous iron oxide.

^d Cry-Fe = crystalline iron oxide.

Table 2
Total concentrations of metals in soils (mg kg⁻¹)

Site	Metal						Soil quality ^a
	Cr	Ni	Cu	Zn	Cd	Pb	
Heilongjiang	52.8	43.3	83.8	215.3	0.306	39.8	I
Jilin	76.1	37.7	80.7	150.3	0.595	27.3	II
Gansu	44.3	61.4	41.7	38.5	0.200	45.1	II
Neimenggu	32.7	48.9	43.6	156.3	0.462	21.9	II
Shanxi	25.3	42.0	31.9	67.2	0.221	29.7	I
Beijing	33.6	30.9	37.5	105.5	0.230	24.3	I
Shandong	32.2	27.3	60.9	172.4	0.335	51.2	I
Jiangsu	53.8	31.6	51.4	163.9	0.487	25.7	II
Anhui	54.2	23.1	57.2	180.6	0.401	42.9	I
Hubei	53.8	33.8	62.3	254.5	0.419	22.4	II
Fujian	4.2	10.0	96.7	509.5	0.282	86.6	III
Jiangxi	45.3	20.8	48.5	270.0	0.217	36.7	II
Yunnan	36.2	27.4	116.3	138.6	0.875	70.4	II
Chengdu	49.5	43.8	141.7	213.7	0.292	32.5	II
Guizhou	145.1	69.1	83.1	409.3	0.738	51.2	III
Xinjiang	42.8	95.4	45.7	97.5	0.333	35.8	III

^a Classification of soil quality based on GB15618-1995 (China). Grade I: natural background level; Grade II: lightly polluted; Grade III: mildly polluted.

2.2. Pot experiments and sample preparation

Soil samples (0.50 kg) were fertilized with 0.4 g/kg each of N, P, K as KH_2PO_4 and $(\text{NH}_4)_2\text{CO}_3$ and KNO_3 in solution, homogenized and incubated for 1 week before planting. Winter wheat (*Triticum aestivum* L.) seeds were soaked in distilled water for 24 h and then placed on moist filter paper to germinate. Seedlings were sown in each pot and thinned to 15 seedlings per pot after 2 weeks. Water loss was compensated for by daily addition of deionized water. Six weeks after planting, seedlings were harvested. Wheat shoots and roots were first rinsed thoroughly with tap water and then with de-ionized water. Roots were cut off. Roots and shoots were dried in an oven at 65°C for 2 days to constant weight for determination of metals.

Rhizosphere soil (adhering to the root system) was obtained by gently shaking off the loosely bound soil first and then was separated from the roots by a more vigorous shaking or by hands. Rhizosphere soil was subjected to metal fractionation immediately or placed in polyethylene bags, and stored in a refrigerator at -18°C for further metal fractionation.

2.3. Extraction procedure

Moist rhizosphere soils were used for all extraction procedures in order to avoid the speciation change during drying process. The extraction procedures using 0.05 M EDTA, 0.005 M DTPA, 0.01 M CaCl_2 , 0.1 M NaNO_3 , and the first step of a three-step sequential extraction procedure recommended by the Community Bureau of Reference (BCR), now Standards, Measurements and Testing Programme of European Community, were performed. The detailed operations were identical to the references (Table 3), with the exception that the moist rhizosphere soils were used.

The rhizosphere-based extraction protocol was performed as below: 2.00 g soil was mixed with 20 ml of acetic, lactic, citric, malic, and formic acids (two drops of toluene was added as a biocide) in 50 ml pre-cleaned plastic centrifuge tube. The total concentration of these organic acids was 10 mM, and their molar concentration ratio was 4:2:1:1:1. The soil suspension was shaken by an end-over-end shaker for 16 h, and centrifuged at $3000 \times g$ for 10 min. An aliquot of 5 ml supernatant was removed immediately by a transfer pipette to 10 ml volumetric flask and diluted to volume with 2% HNO_3 . The extracts obtained were used for the determination of heavy metals. The soil moisture contents were measured by drying soil at $105 \pm 2^\circ\text{C}$ for 6 h to constant weight. All extraction procedures were carried out in triplicate, including the blanks.

2.4. Determination of heavy metals

Total concentrations of heavy metals in soil and in plants were determined by inductively coupled plasma mass spectrometry (ICP-MS, PQ3, Fisons Instruments, UK) after digestion of soil and plant samples in 3 ml of $\text{HNO}_3/\text{HClO}_4/\text{HF}$ (1 + 1 + 1) mixture in PTFE vessel under high-pressure conditions (Zhang and Shan, 1997). The concentrations of heavy metals in the extracted soil solutions were determined after appropriate dilution with 2% HNO_3 .

2.5. Reagents and glassware

All reagents used were of analytical reagent grade or better. All glassware and plastic containers were previously soaked into 1:2 (v/v) HNO_3 overnight and rinsed thoroughly with deionized distilled water before use. High purity of water ($18 \text{ M}\Omega \text{ cm}$) was prepared by double distillation of deionized water in a fused quartz still (Westdeutsche Quarzschmelze, Geesthacht, Germany).

Table 3
Extraction procedures used in the study

Extraction method	Procedure	Refs.
EDTA	2.0 g soil in 20 ml 0.05 M EDTA adjusted by ammonia solution to pH 7.0, shaking for 1 h	Wear and Evans (1968)
DTPA	10.0 g soil in 20 ml 0.005 M DTPA + 0.01 M TEA + 0.01 M CaCl_2 adjusted to pH 7.3, shaking for 2 h	Lindsay and Norvell (1978)
CaCl_2	2.0 g soil in 20 ml 0.01 M CaCl_2 , shaking for 3 h	Novozamsky et al. (1993)
NaNO_3	8.0 g soil in 20 ml 0.1 M NaNO_3 , shaking for 2 h	Gupta and Aten (1993)
BCR1 ^a	1.0 g soil in 20 ml 0.11 M acetic acid, shaking for 16 h	Ure et al. (1993)
RHIZO ^b	2.0 g soil in 20 ml LMWOAs, total concentration of acetic, lactic, citric, malic, and formic acids was 0.01 M, their molar ratio was 4:2:1:1:1 (c/c), shaking for 16 h	This work

^a BCR1 = the first step of European Community Bureau of Reference.

^b RHIZO = rhizosphere-based method.

2.6. Statistical analysis

Statistical analyses were performed using SPSS 11.5 for Windows (SPSS Inc., USA). Pearson correlation coefficients were determined by correlating metal contents in roots and shoots with extractable heavy metals from soils by various extraction methods.

Forward stepwise multivariate regression was used to derive best-fit models of metal concentrations in plants to soil properties and extractable metals in soils. The variance inflation factor (VIF), the condition indices and the regression coefficient variance-decomposition matrix were also assessed in order to evaluate the collinearity between independent variables. Collinearity becomes an issue when values of VIF exceed 10.0 and condition index is over 30.

3. Results and discussion

3.1. Optimization of rhizosphere-based extraction procedure

In our separate study (Li et al., 2003) the concentrations of acetic, lactic, malic and formic acids in rhizosphere soil and plant roots were determined, which ranged from below the detection limits of capillary zone electrophoresis to several mM. Other studies have also reported that the most abundant monocarboxylic acids (such as formic, acetic and propionic acids) were up to 1 mM in soil solutions (Jones, 1998; Strobel, 2001). The concentration of organic acids in roots cell vacuoles was usually ranged from 5 to 50 mM (Ryan et al., 2001). In order to study the effect of LMWOAs concentrations on extractable heavy metals from soils a series of experiments were performed. The total concentrations of acetic, lactic, citric, malic, and formic acids varied from 0.4 to 100 mM, and their molar ratio of 4:2:1:1:1 was chosen. Sixteen moist rhizosphere soils collected from different provinces of China after wheat planting was used for the optimization of LMWOAs extraction. All extractions were repeated in triplicate. The pH of the soil solution after extraction was monitored. No attempts, however, were made to maintain the pH during LMWOAs extraction for different soils. The errors of the determinations of heavy metals were less than 5% ($n = 3$) for each soil.

The relationship between the LMWOAs concentrations and extractable metals from soils were quite similar for all soils. Here taking Heilongjiang soil as an example, and the results are shown in Fig. 1. Generally, the extracted metals increased with increasing LMWOAs concentrations. Over the total concentration range of 1–10 mM LMWOAs the extractable metals increased very slowly. After that limit the extractable metals increased rapidly with further increasing LMWOAs con-

Fig. 1. The extractable metals from Heilongjiang soil as a function of the concentration of LMWOAs (acetic:lactic: citric:malic:formic acids at molar ratio = 4:2:1:1:1). (■): Cr; (●): Ni; (▲): Cu; (▼): Zn; (◇): Cd; (+): Pb.

centrations. And it seems that no equilibrium was reached at the concentration range of LMWOAs tested. The reason for this was probably that higher concentration of LMWOAs might attack soil minerals, resulting in the release of heavy metals associated with various geochemical phases of soil. Considering a concentration gradient of LMWOAs from root surface to soil solution, and a short-term extraction for simulation of long-term reaction in the rhizosphere the total concentration of 10 mM LMWOAs was chosen as an extractant in the remainder of experiments.

The effect of extraction time on the extractable metals and soil solution pH was also studied. The results demonstrated that the extractable metals reached equilibrium after extraction time of 4 h. The pH of extracted soil solutions remained unchanged after extraction time of 12 h. In the remainder of this study an extraction time of 16 h was chosen.

3.2. Comparison of extractable metals using various extraction methods

In this study, the rhizosphere-based extraction method was compared with other one step extraction methods using DTPA, EDTA, CaCl_2 , and NaNO_3 as extractants and the first step of BCR (BCR1) method. The range and mean of the extractable metals with these methods are shown in Table 4. The comparison of the extractable metal concentrations was performed using *T* test with 95% confidence intervals. Generally, the extractable metals followed the descending order $\text{EDTA} \approx \text{BCR1} > \text{DTPA} > \text{rhizosphere-based method} \approx \text{CaCl}_2 > \text{NaNO}_3$. Chelating agents of EDTA and DTPA and high concentration of acetic acid (BCR1) extracted more metals from soils than CaCl_2 and NaNO_3 . The extractable metals with the rhizosphere-based method were in between.

Table 4

Ranges and means of extractable heavy metals of rhizosphere soils ($\mu\text{g/kg}$) ($n = 16$)

Element	RHIZO ^a		DTPA		BCR1 ^b		EDTA		CaCl ₂		NaNO ₃	
	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean
Cr	32.0–224	67.4	8.13–66.4	31.9	55.1–1666	477	123–756	383	0.11–15.1	4.32	4.24–22.3	9.06
Ni	45.4–900	205	54.8–916	331	178–6168	2115	164–5044	1249	9.92–499	75.1	10.6–77.9	32.8
Cu	36.0–1215	186	127–5417	1211	2.50–1642	579	53.6–7996	3389	2.42–1595	182.5	5.95–95.8	32.5
Zn	25.2–1110	273	248–9350	1666	158–28606	4775	409–18269	3653	8.47–5935	558	1.19–182	36.8
Cd	17.2–44.9	26.8	19.6–634	94.0	4.70–1913	290	61.7–829	162	17.4–95.5	35.6	0.61–105	22.8
Pb	0.405–88.7	16.2	378–4681	1409	29.7–1019	297	799–9221	3724	0.56–1558	236	1.37–34.5	8.82

^a RHIZO = rhizosphere-based method.^b BCR1 = the first step of European Community Bureau of Reference.

The reasons for this extraction order are easily understood. EDTA is a strong chelating reagent and has been reported to remove both the organically bound metals and metals occluded in oxides and secondary clay minerals in part (Payá-Pérez et al., 1993). EDTA is a non-specific extractant, and it can extract labile and non-labile fractions (Bermond et al., 1998). A higher concentration of 0.11 M acetic acid was used in the first step of the BCR method. Acetic acid could extract organic matter bound metals in part (Payá-Pérez et al., 1993), and acetic acid was able to release most of metals associated with calcium carbonate, and minerals (kaolinite, potassium feldspar and ferrihydrite) (Whalley and Grant, 1994). DTPA was originally developed to identify near-neutral and calcareous soils with insufficient transition metals (Lindsay and Norvell, 1978). Iron and manganese oxides are potentially extracted from acidic soils by DTPA (O'Connor, 1988). As far as the soil background electrolyte solutions were concerned, easily exchangeable metals could be extracted with NaNO₃ and CaCl₂ (McBride et al., 2003). Lower con-

centrations of metals were extracted with NaNO₃ than with CaCl₂, because the monovalent cations exert weak competition with the adsorption sites on organic matter (Novozamsky et al., 1993). For rhizosphere-based method, complexation of metals by organic acids plays an important role in controlling metal solubility (Cieřliński et al., 1998; Naidu and Harter, 1998; Shan et al., 2002; Qin et al., 2004). Metals extracted by the rhizosphere-based method correspond to the mobile fractions in soil solution (Naidu and Harter, 1998), which are responsible for the short-term available pool (Cieřliński et al., 1998).

3.3. Comparison of the rhizosphere-based method with other extraction methods for bioavailability using simple correlation analysis

The relationship between the extractable metals in soil solutions and metal accumulation in wheat roots and shoots are summarized in Table 5. Generally, the correlation coefficients between extractable metals in soil

Table 5

Linear correlation coefficients (r) between metal concentrations in shoots and roots and extracted metals from soils by different extraction methods ($n = 16$)

	Element	RHIZO ^a	DTPA	BCR1 ^b	EDTA	CaCl ₂	NaNO ₃
Shoot	Cr	0.306	−0.266	0.063	0.273	0.170	−0.265
	Ni	0.197	0.259	−0.002	0.192	0.787***	0.386
	Cu	0.823***	0.418	0.429*	0.352	0.006	0.795***
	Zn	0.690**	0.496*	0.573*	0.500*	0.412	0.030
	Cd	0.792***	0.548*	0.498*	0.533*	0.134	−0.241
	Pb	0.438*	0.290	0.417	−0.068	0.448*	0.144
Root	Cr	0.519*	0.033	0.257	−0.216	0.540*	0.229
	Ni	−0.235	−0.265	0.415	0.107	−0.113	−0.158
	Cu	0.731***	0.524*	0.541*	0.381	−0.093	0.676**
	Zn	0.733***	0.512*	0.590*	0.747***	0.762***	0.273
	Cd	0.796***	0.738***	0.724**	0.765***	0.225	−0.232
	Pb	0.037	0.099	0.043	0.106	−0.013	0.333

*, **, and *** = the probability level of $p < 0.1$, $p < 0.01$, and $p < 0.001$, respectively.^a RHIZO = rhizosphere-based method.^b BCR1 = the first step of European Community Bureau of Reference.

pools and metals in roots are better than that in shoots (Table 5). This was in agreement with the criticism by Chaignon et al. (2003) that metals in shoots are not a good indicator of metal bioavailability. Thus, the following discussion was limited to the relationship between metals in roots and in soil pools.

When comparing the correlation coefficients obtained by the rhizosphere-based method with that obtained by DTPA, BCR1, EDTA, CaCl_2 , and NaNO_3 extraction methods, the rhizosphere-based method was preferred to evaluate metal bioavailability. For the rhizosphere-based method a good correlation was obtained between the extractable Cr, Cu, Zn and Cd in soil pools and their contents of wheat roots. The correlation coefficients were 0.519*, 0.731***, 0.733*** and 0.796*** for Cr, Cu, Zn and Cd, respectively. When DTPA, BCR1 and EDTA extraction methods were used, there was a good correlation between the extractable Cu, Zn and Cd and their contents of wheat roots, but the correlation coefficients were lower, especially for Cu and Zn. For the CaCl_2 extraction method only extractable Cr and Zn were correlated with those metals in roots ($r = 0.540^*$ to 0.762^{***}). NaNO_3 extraction method was only good for assessing the bioavailability of Cu ($r = 0.676^{***}$). These results were consistent with Hammer and Keller (2002), who suggested that plant appeared to take up heavy metals from other pools than the NaNO_3 -extractable one.

3.4. Superiority of the rhizosphere-based method over other extraction ones to different types of soils

As it has already been pointed out before that the DTPA extraction method is most suitable for calcareous soils, the EDTA extraction method was originally developed for acidic soils, the NaNO_3 and CaCl_2 extraction methods are suitable for easily exchangeable heavy metals (Hammer and Keller, 2002). In order to compare the suitability of the rhizosphere-based method for different types of soils the 16 soils were divided into two groups based on soil pH. Soils of $\text{pH} \leq 6.5$ were referred to as acidic soils, and soils of $\text{pH} > 6.5$ as neutral and near alkaline soils. Generally, the extractable metals from the acidic soils were higher than that from the neutral and near alkaline soils, and the metal contents in wheat roots grown in the acidic soils were also higher than that in the neutral and near alkaline soils (data not shown).

A simple correlation analysis was performed between the extractable metals by different extraction methods from the acidic, neutral and near alkaline soils, and metal contents of wheat roots, separately. The comparison results are shown in Table 6. The extractable Cr, Cu, Zn, and Cd from both types of soils by the rhizosphere-based method were significantly correlated with their contents of wheat roots. These results suggested that

Table 6
Linear correlation coefficients (r) between metal concentrations in roots and extracted metals from the acidic soils ($\text{pH} \leq 6.5$) and from the neutral and near alkaline soils ($\text{pH} > 6.5$) by different extraction methods ($n = 8$)

Element	RHIZO ^a		DTPA		BCR1 ^b		EDTA		CaCl_2		NaNO_3	
	Acidic	Neutral and near alkaline	Acidic	Neutral and near alkaline	Acidic	Neutral and near alkaline	Acidic	Neutral and near alkaline	Acidic	Neutral and near alkaline	Acidic	Neutral and near alkaline
Cr	0.614*	0.657*	-0.202	0.302	0.600	-0.185	-0.398	0.154	0.624*	0.380	0.214	0.123
Ni	-0.042	-0.423	-0.464	-0.032	0.079	0.696*	0.095	0.079	-0.267	-0.177	-0.270	-0.037
Cu	0.775**	0.886***	0.301	0.839**	0.506	0.653*	0.688*	0.181	0.676*	-0.224	0.384	0.840***
Zn	0.805**	0.691*	0.458	0.499	0.539	0.680*	0.831**	-0.092	0.887***	-0.453	0.250	-0.271
Cd	0.816*	0.907***	0.278	0.971***	0.995***	0.152	0.979***	0.477	-0.255	-0.196	-0.178	-0.840***
Pb	-0.107	0.619*	-0.152	0.923***	0.276	-0.151	0.378	-0.268	0.258	-0.276	0.144	0.465

*, **, and *** = the probability level of $p < 0.1$, $p < 0.01$, and $p < 0.001$, respectively.

^a RHIZO = rhizosphere-based method.

^b BCR1 = the first step of European Community Bureau of Reference.

the extraction behavior of the rhizosphere-based method was similar for both types of soils, suggesting that the rhizosphere-based method simulated the soil conditions and root–soil interactions well. When the rhizosphere-based method was used the extractable Pb from acidic soils was poorly correlated with Pb content of wheat roots because the concentration of organic acid used in the rhizosphere-based method was too low to extract as much Pb as plant needed from acidic soils. This might be so because Pb was organic matter-bound, iron and manganese oxides-bound in soil, which is largely unavailable to plants (Blaylock et al., 1997; Pitchel et al., 1999). However, a good correlation was observed between the extractable Pb from the neutral and near alkaline soils by the rhizosphere-based method and Pb content of wheat roots because organic acids increased the release of Pb from the neutral and near alkaline soils (Blaylock et al., 1997).

There was a good correlation between the DTPA extractable Cu, Cd and Pb from the neutral and near alkaline soils and the metal contents of wheat roots, but poor correlation for the acidic soils. DTPA is a strong extractant for the acidic soils (O'Connor, 1988; Hammer and Keller, 2002). DTPA could extract much more divalent cations from the acidic soils due to high concentration of CaCl_2 used and high pH of extractant solution (Hammer and Keller, 2002). Miner et al. (1997) also obtained poor correlation between plant metal concentrations and the DTPA extractable metals from acidic soils. Therefore, the DTPA extraction method is unsuitable for assessing the bioavailability of heavy metals to plants for acidic soils.

It was interesting to find that the extractable Ni, Zn, and Cu by BCR1 showed a reasonably good correlation for the neutral and near alkaline soils and poor correlations for the acidic soils. In contrast, a good correlation was observed between the BCR1 extractable Cd and Cd content of wheat roots for acidic soils. There were no clear explanations for this phenomenon.

The EDTA extractable Cu, Zn, and Cd had a good correlation for the acidic soils, but not for the neutral and near alkaline soils. This was easily understood because the EDTA method was originally developed for acidic soils.

The CaCl_2 extractable Cr, Cu, and Zn had a good correlation with their contents of wheat roots for the acidic soils, but poor correlation for the neutral and near alkaline soils. The NaNO_3 extractable Cu and Cd showed positive and negative correlation for the neutral and near alkaline soils, respectively. In high pH soils ($\text{pH} > 6.5$), Cd desorption was increased by ligand rather than by proton such as soil electrolyte (NO_3^- , Cl^-) (Naidu and Harter, 1998). The NaNO_3 extractable Cd was low at pH value > 5.5 (Naidu and Harter, 1998), which supported our results that the NaNO_3 extractable Cd correlated negatively with root Cd.

Based on the above comparison one can draw a conclusion that the extractable Cu, Zn, Cr, and Cd of soils by the rhizosphere-based method were significantly correlated with the metal contents of wheat roots. For DTPA, BCR1 and EDTA methods there were relatively poor correlations between the extractable Cu, Zn and Cd of soils and metal contents of wheat roots. However, it must be pointed out that the most distinct feature of the rhizosphere-based method was that the recommended method was suitable for both the acidic soils, the neutral and near alkaline soils. The DTPA, EDTA, CaCl_2 and NaNO_3 methods were suffered from their limited practical applicability. The DTPA and EDTA extraction methods were only suitable for calcareous or only for acidic soils, respectively. The CaCl_2 and NaNO_3 extraction methods were only suitable for exchangeable metals.

3.5. Comparison of the rhizosphere-based extraction method with other extraction methods for bioavailability using stepwise multiple regression analyses

Availability of metals to plants is affected by soil properties including soil pH, OM, Fe–Mn oxides, CEC, and plant species (Haq et al., 1980; Li et al., 1998). Ideally, the chemical extraction methods used to predict the bioavailability of metals in soil should represent the soil–root interactions in the rhizosphere. In order to test whether the different extraction methods meet these requirements, a stepwise multiple regression analysis was performed.

It should be pointed out that the simple correlation analysis indicated that the correlations of Pb and Ni concentrations in roots with extractable Pb and Ni by all extraction methods were insignificant (Table 5). The stepwise multiple regression analysis revealed the same results. Therefore, no discussion was given in detail afterwards for Pb and Ni.

The stepwise multiple regression analysis for Cr, Zn, Cu, and Cd concentrations in roots as a function of extracted metals in soil solution fractions, soil pH, OM, CEC, amorphous-Fe and crystal-Fe and MnO_2 are presented in the order that they were added to the equations (Table 7). For simplicity, the coefficients in the equations were omitted, whereas the negative sign of variables was maintained. The correlation coefficients, r , allow an evaluation of the overall prediction of bioavailability. Generally, the inclusion of soil properties variables improved the correlation coefficients to different extent for different extraction methods. It could be concluded that the equation of the rhizosphere-based method was the simplest one and no other soil properties variables needed to be added. In contrast, the DTPA, BCR1, EDTA, CaCl_2 and NaNO_3 extraction methods required the addition of various soil properties variables for the different metals in the equations.

Table 7

Stepwise multiple regression equations for metals of roots as function of extractable metals by different extraction methods and soil properties

Method	Regression Step	Cd _{root}		Cr _{root}		Cu _{root}		Zn _{root}	
		Variables	<i>r</i>	Variables	<i>r</i>	Variables	<i>r</i>	Variables	<i>r</i>
RHIZO ^a	1	Cd	0.796***	Cr	0.519*	Cu	0.731***	Zn	0.733***
	2							(-)Zn × CEC	0.843***
DTPA	1	Cd × Amor-Fe	0.801***	Cry-Fe	0.541*	Cu	0.524*	Zn	0.512*
	2	(-)Cd × CEC	0.875***			(-)Cu × OM	0.715**	(-)Zn × CEC	0.825***
	3	(-)pH	0.899***			(-)Cry-Fe	0.815**		
BCR1 ^b	1	Cd × Amor-Fe	0.800***	Cr × Cry-Fe	0.477	Cu	0.541*	Zn	0.590*
	2	(-)Cd × OM	0.889***	(-)Cr × pH	0.697*	(-)Cu × CEC	0.629*	(-)Zn × CEC	0.868***
	3	(-) Cd × pH	0.937***	(-)MnO ₂	0.783**				
EDTA	1	Cd × Amor-Fe	0.807***	Cry-Fe	0.541*	ns		Zn	0.747***
	2	Cry-Fe	0.854***	(-)Cr × MnO ₂	0.682*			(-)Zn × OM	0.819***
	3	(-)pH	0.883***						
CaCl ₂	1	Cry-Fe	0.682**	Cr × Cry-Fe	0.615**	ns		Zn	0.762***
	2	(-)Cd × Cry-Fe	0.749**	(-)Cr MnO ₂	0.715**			(-)Zn × CEC	0.861***
NaNO ₃	1	Cry-Fe	0.682**	Cry-Fe	0.541*	Cu	0.676**	ns	

*, **, and *** = the probability level of $p < 0.1$, $p < 0.01$, and $p < 0.001$, respectively. ns = no significance.

^a RHIZO = rhizosphere-based method.

^b BCR1 = the first step of European Community Bureau of Reference.

Here we take the regression equations for Cd in roots as example to discuss the parameters in the equation. The extractable Cd by various extraction methods and soil properties variables are listed in Table 7. The equation of Cd of the rhizosphere-based extraction method was the simplest one with r of 0.796***. However, the regression equations developed for root Cd with the DTPA, BCR1, EDTA, CaCl₂, and NaNO₃ extractable Cd required additional soil properties variables to be entered. For DTPA the first variable was Cd × amorphous-Fe, the second variable Cd × CEC and the third variable soil pH, which resulted in correlation coefficients of 0.801***, 0.875*** and 0.899***, respectively. According to the data of Table 7 the equation of the DTPA extractable Cd is as following:

$$\text{Cd in wheat root} = 1365 + 8.38 \text{ Cd} \times \text{amorphous-Fe} - 0.12 \text{ Cd} \times \text{CEC} - 49 \text{ pH}$$

The addition of the second variable CEC led to negative coefficient. It means that extractable Cd from soils of high cation exchange capacity was less available to roots, and this effect was more pronounced if the extractable Cd was high. This observation was in agreement with the findings of Buchter et al. (1989), higher CEC soils retained greater quantities of the cation species than low CEC soils did (Elzinga et al., 1999).

For equation of the extractable Cd by BCR1 method, the first variable was Cd × amorphous-Fe, the second and third variables entered were Cd × OM and Cd × pH interactions. The negative coefficients suggested that the

extractable Cd was less available to roots when the soil OM and soil pH were high. It is understood that organic matter bound metals are less available to plants (Del Castillo et al., 1993). For EDTA extraction method the first, second, and third variables were Cd × amorphous-Fe, crystal-Fe, and soil pH. These variables resulted in EDTA extraction method with correlation coefficients of 0.807***, 0.854*** and 0.883***, respectively. For NaNO₃ extraction method the first variable was crystal-Fe. For CaCl₂ extraction method the first and the second variables were crystal-Fe and Cd × crystal-Fe extractable Cd, which resulted in r of 0.682** and 0.749**, respectively.

Miner et al. (1997) also reported that soil properties needed to be included in the best fit to multiple regression models to improve the relationship between plant metal concentrations and soil-extractable metals for EDTA and DTPA extraction methods. In the present study, the equations of the rhizosphere-based method were the simplest ones for bioavailability.

4. Conclusion

Based on the aforementioned systematic comparison between the rhizosphere-based method and the DTPA, BCR1, EDTA, CaCl₂ and NaNO₃ extraction methods one can conclude that the rhizosphere-based method was the most robust one and it was able to properly predict the bioavailability of Cr, Cu, Zn, and Cd in soils to

plants, but it failed at assessing the bioavailability of two out of the six studied metals, i.e. Ni and Pb. The DTPA and BCR1 extraction methods were good for assessing the bioavailability of Cu, Zn and Cd although the correlation coefficients of Cu and Zn were relatively lower. While the EDTA extraction method was also good for assessing the bioavailability of Zn and Cd, and the CaCl_2 extraction method was able to assess the bioavailability of Cr and Zn. The most important feature of the rhizosphere-based method was that the suggested method was suitable for acidic, neutral and near alkaline soils. In contrast, the DTPA and EDTA extraction methods were suitable only for calcareous and only for acidic soils, respectively. The CaCl_2 and NaNO_3 extraction methods were only suitable for exchangeable metals. The results obtained ascertain that the rhizosphere-based extraction method simulates the rhizosphere conditions and takes into account the effect of soil–root interactions as a whole, at least to some extent. Thus, more widespread application of the rhizosphere-based extraction method for bioavailability of heavy metals in soils to plants is promising. Of course, none of the methods was tested against field-grown plants.

Acknowledgment

The study was supported by the National Natural Science Foundation of China (grant Nos. 20177030, 20237010).

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