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Source / Izvornik: **Poljoprivreda, 2013, 19, 53 - 58**

Journal article, Published version

Rad u časopisu, Objavljena verzija rada (izdavačev PDF)

Permanent link / Trajna poveznica: <https://urn.nsk.hr/urn:nbn:hr:151:584864>

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Download date / Datum preuzimanja: **2024-08-09**



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COMPARISON OF DIFFERENT EXTRACTION METHODS REPRESENTING AVAILABLE AND TOTAL CONCENTRATIONS OF CD, CU, FE, MN AND ZN IN SOIL

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Original scientific paper
Izvorni znanstveni članak

SUMMARY

Various extraction methods are used to predict plant uptake of trace metals. Most commonly it is total concentration that is used for risk assessment and evaluation of trace metal availability. However, recent studies showed that total concentration is a poor indicator of availability while concentrations in soil solution show good correlation with plant uptake. Present study was conducted on agricultural soils with low levels of trace metals where 45 soil samples were collected from different soil types. The main objective was to compare four different extraction methods and examine how total and reactive (EDTA) trace metal concentrations correlate with soil solution concentration (in this study determined by water extraction). The samples were analyzed by four extraction methods: strong acid extraction (ultra-pure HNO₃ extraction and aqua regia), weak acid extraction by EDTA and the most available fraction, fraction in soil solution, were represented by water extraction (weakest extractant). Five elements were investigated (Cd, Cu, Fe, Mn and Zn). Water extraction significantly correlated with EDTA extraction for Cu, Fe and Mn, while total extraction (HNO₃ extraction and aqua regia) correlated significantly with water extraction only for Cu. No correlation between water extraction and total extraction confirmed poor role of total concentration as an indicator of availability. EDTA extraction can be used to represent reactive pool of trace metals in soil but it should be also taken with caution when using it to describe available fraction.

Key-words: availability, extraction, soil, trace metals

INTRODUCTION

Understanding trace metal uptake has been of general concern for food quality, from toxicity point of view as well as deficiency. Metals enter human food chain directly or indirectly through plants. Assessing potential risk requires the knowledge of plant metal concentrations and behavior of trace metals inside the plant (Šimić et al., 2009; Rastija et al., 2009; Eđed, 2012). However, instead of monitoring the plant it is much easier to monitor soil systems, from where the plant is taking up trace metals. Interaction between plant and soil is very complicated and great number of researchers is dealing with the issue of bioavailability, trying to estimate the available fraction by mimicking the plant uptake with extraction methods using neutral salts, weak acids and organic extractants.

Main two approaches trying to explain the bioavailability are *single extraction* and *sequential extraction* procedures. However, sequential extraction has been criticized for its lack of selectivity (Kim and Ferguson, 1991; Peijnenburg et al., 2007). Main reasons for causing uncertainty of sequential extraction are: a) reagents can dissolve other compounds than those predicted, b) metals liberated in earlier steps can be resorbed or redistributed and c) handling before the extraction can change the speciation of metals (Peijnenburg et al., 2007). Tessier and Cambell (1991) agree that there are possible problems with re-adsorption. However, they do

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not consider sequential extraction unsuitable for prediction of trace metal pools in soil. Nevertheless, comparison of single extraction and sequential extraction shows strong deviations between them (Peijnenburg et al., 2007). Due to controversy regarding the sequential extraction, in present study we focus only on single extraction procedures. Single extractions investigated in present study were strong acid digestions by ultra pure HNO_3 and *aqua regia* for the determination of pseudo-total trace metal concentrations, synthetic acid (EDTA) extraction for the determination of reactive metal concentrations and water extraction for the determination of metals in the soil solution.

Latest findings consider weak extraction by water, CaCl_2 or rhizosphere based method (RHIZO), that uses organic acids, as the most appropriate methods correlating with the plant uptake (Feng et al., 2005a; Feng et al., 2005b; Meers et al., 2007), mainly because these extractions express the concentrations of trace metals in soil solution and are able to release some weakly bound trace metals (CaCl_2 and RHIZO method). Plant is basically doing the same. The trace metals in the soil solution are the most available fraction and, in addition to this readily available fraction, plant is capable of influencing the release of some weakly bound trace metals from the rhizosphere by excreting different exudates, mainly in the form of organic acids. However, there is no certainty that these methods will always give us the accurate estimations of bioavailability, as for bioavailability to be accurate it needs to take plant species or plant genotype, metal speciation and soil properties into account (Peijnenburg et al., 2007).

Synthetic chelating agents such as DTPA and EDTA have also been often used to estimate potential availability and mobility of metals (Kovačević et al., 2002; Antunović et al., 2003). However, it has been shown that EDTA and DTPA can act more aggressively than metal chelating under natural circumstances (Meers et al., 2007). In addition, EDTA was originally developed for acidic soils so it shows good correlation when investigating acidic soils (Kovačević et al., 2002) but it seems to correlate poorly for neutral and alkaline soils. On the other hand, DTPA correlates poorly for acidic soils but it can show good correlation for neutral and alkaline soils (Feng et al., 2005a; Feng et al., 2005b).

Total or pseudo-total concentrations of trace metals in the soil, extracted by strong acids, have been by now recognized as poor indicators of toxicity. Nevertheless, they are still being used, in the legislation of many countries, for the determination of trace metal maximum permissible concentrations in soil.

The main objective of present study is to investigate correlations of total (HNO_3 extraction and *aqua regia*) and reactive pool (EDTA extraction) with water extractable fraction, which is used to represent the fraction of trace metals in soil solution. In addition, the total extraction methods will also be analyzed for correlation between them. Our hypothesis is that there will be no

correlation of water extraction with EDTA extraction or total extraction. To test this hypothesis we have conducted extraction of trace metals from 45 soil samples by strong acid (HNO_3 and *aqua regia*), EDTA and ultra-pure H_2O and tested for correlation.

MATERIAL AND METHODS

Forty five soil samples were collected from the unpolluted agricultural area in eastern Croatia. Samples were collected from the depth of 0-25 cm (hereafter called surface soil) from 8 different soils: Stagnosols, Dystric Cambisols, Luvisols, Haplic Gleysols, Chernozems, Eutric Cambisols, Mollic Gleysols and Anthrosols (IUSS Working Group WRB, 2006). From each site, 10 subsamples from surface soil were taken within 5 m distance and then combined into one composite sample of approximately 500 g. Samples were dried and sieved through a 2-mm mesh for the determination of soil pH and trace metal extraction by *aqua regia*, EDTA and water extraction of Cd, Cu, Fe, Mn and Zn. For the determination of soil organic carbon (SOC) and total metal extraction by ultra pure HNO_3 , samples were further ground to finer particle size using agate mortar. The concentration of SOC was determined by a dry combustion method on Leco Carbon Determinator EC12 (Nelson and Sommers, 1982). Soil pH was determined in soil to water solution ratio of 1:2.5 (Mc Lean, 1982). Samples were tested for 4 different single extraction methods: strong acid extraction (ultra pure HNO_3 extraction and *aqua regia* extraction), EDTA extraction and extraction by water (weakest extractant). Ultra-pure HNO_3 extraction and water extraction were conducted at the Norwegian University of Life Sciences and EDTA and *aqua regia* extraction at the J.J. Strossmayer University of Osijek, Faculty of Agriculture in Osijek, Croatia.

- *Aqua Regia extraction* - 0.5 g of air-dried soil was digested with 12 ml of *aqua regia* (1/3 HNO_3 + 2/3 HCl), the extract was filtered into the 100 ml Erlenmeyer flasks and filled with distilled water up to the marker (ISO: 11466). Concentration of trace metals was determined by Inductively Coupled Plasma Optic Emission Spectrometer (ICP-OES).
- *Ultra pure HNO_3 extraction* - Total metal concentration in soil (M_{tot}) was determined after digesting the soil in concentrated ultra pure HNO_3 (1:15 solid:solution ratio) by stepwise heating up to 250°C using a Milestone Ultra clave for 1 hr and 15 min. Trace metal concentrations were determined using a Perkin Elmer Sciex Elan Inductively Coupled Plasma Mass Spectrometer (ICP-MS) for Cd and a Perkin Elmer Optima 5300 DV Inductively Coupled Plasma Optic Emission Spectrometer (ICP-OES) for Cu, Fe, Mn and Zn. Standard reference material (SRM) used was the SRM 2709 (National Institute of Standards & Technology 2003).
- *EDTA extraction* - 10 g of air-dried soil was digested with 20 ml EDTA extract (0,01 M ethylene-diamine-

tetraacetic acid (EDTA) and 1M $(\text{NH}_4)_2\text{CO}_3$, adjusted to pH 8.6) and shaken for 30 minutes. Therefore, soil:solution ratio was 1:2 (Trierweiler and Lindsay, 1969). Extract was filtered and concentrations of trace metals were determined by ICP-OES.

- **Water extraction** – On 4 g of air-dried soil 40 mL ultra pure water (MilliQ H_2O , electric conductivity $< 18.2 \text{ M}\Omega \text{ cm}^{-1}$) was added and shaken in high density polyethylene (HDPE) centrifugation tubes for two days and centrifuged at $1400 \times g$ for 30 min. The suspension was filled in High Density Poly Propylene (HDPP) syringes and filtered through $0.45 \mu\text{m}$ polyethersulfone membrane filters to poly propylene (PP) test tubes. The filters and the test-tubes were provided by VWR International (vwr.com). Blanks of MilliQ H_2O were stored in PP tubes for 48hr. and filtered through the same membrane filters before analysis. Water trace metal concentrations were determined using a Perkin Elmer Sciex Elan Inductively Coupled Plasma Mass Spectrometer (ICP-MS) for Cd and a Perkin Elmer Optima 5300 DV Inductively Coupled Plasma Optic Emission Spectrometer (ICP-OES) for Cu, Fe, Mn and Zn. Standard reference material used for water was the SRM 1643e (National Institute of Standards & Technology, 2004).

Descriptive statistics and simple correlation analysis were performed using Minitab[®] Statistical Software version 15 (2007). Pearson correlation coefficients were determined by correlating different extraction methods.

RESULTS AND DISCUSSION

Soil pH in our samples varied from 4.3 – 8.0 (average 6.8), however 4.3 value was an extreme observation,

Table 2. Trace metal concentrations (n = 45)

Tablica 2. Koncentracije teških metala (n=45)

		Cd	Cu	Fe	Mn	Zn
Aqua regia Extraction (mg kg ⁻¹)	Avg.	0.42	24.4	35363	595	60.6
	SD	0.12	13.4	5315	170	9.86
	Range	0.23-0.97	8.86-96.5	25822-51780	242-928	43.7-86.5
	Skewness	2.45	3.88	0.62	-0.45	0.39
	Kurtosis	10.06	19.50	0.93	-0.34	-0.26
HNO_3 Extraction (mg kg ⁻¹)	Avg.	0.23	25.7	29915	635	80.4
	SD	0.07	13.4	3476	195	13.2
	Range	0.1-0.4	13.3-98.7	24318-40221	238-1038	58.5-119.5
	Skewness	0.24	4.02	0.46	-0.41	0.77
	Kurtosis	0.02	20.34	0.43	-0.56	0.86
EDTA Extraction (mg kg ⁻¹)	Avg.	0.1	6.8	205.2	54	1.4
	SD	0.04	6.4	341	33.9	0.9
	Range	0.015-0.23	1.4-41.4	13.7-1631.9	7.4-131.6	0.3-4.1
	Skewness	0.63	4.15	2.91	0.66	1.41
	Kurtosis	1.97	20.05	8.74	-0.59	1.91
Water extraction (mg kg ⁻¹)	Avg.	0.0006	0.15	20.5	0.89	0.14
	SD	0.001	0.08	30.5	3.2	0.17
	Range	0.00003-0.007	0.05-0.45	0.4-125.3	0.004-20.9	0.08*-0.82
	Skewness	5.35	1.74	1.64	6.02	2.09
	Kurtosis	32.30	3.48	2.19	38.38	5.56

* indicates values that had some observations below detection limit

excluding it we have pH in range 5.2 - 8.0 with more than half of our samples having pH above 7. Soil organic carbon (SOC) was in range: 0.5 – 4.4% (average 1.4%), however two samples are considered outliers for their extremely high values (4.4% and 3.7%). The pH and SOC were positively correlated ($r=0.36$; $p<0.05$). Higher pH has been reported in the northern part of the investigated area (Baranja) (Ivezić et al. 2011).

Table 1. Soil properties (n=45)

Tablica 1. Svojstva tla (n=45)

	Median	Avg.	SD	Min.	Max.	Skewness	Kurtosis
pH	7.3	6.8	1.0	4.3	8.0	-0.65	-0.74
SOC (%)	1.1	1.4	0.8	0.5	4.4	2.12	5.36

Pseudo-total extractions of trace metals resulted with highest concentrations, followed by EDTA extraction and water extraction at the end as the weakest extractant. Ultra pure HNO_3 extraction had highest concentration values for Mn, Cu and Zn while *aqua regia* for Fe and Cd (Table 2). Governmental maximum permissible concentrations (MPC) of Zn and Cd, for agricultural soils, are determined by *aqua regia* method (Official Gazette, 2010). In present study concentrations of Zn and Cd did not exceed MPC. Extraction by EDTA had substantially lower concentrations from pseudo-total concentrations as well as did the water extraction (Table 2). More than half of water extractable Zn was below detection limit, therefore for those samples we used the half of the detection limit value. Calcareous sites with $\text{pH}>7$ were the one having water extractable Zn below detection limit, which indicates strong influence of pH on Zn solubility (Alloway, 1995).

Ultra-pure HNO₃ extraction and *aqua regia* extraction showed good correlation ($p < 0.001$) for all five investigated trace metals (Cd, Cu, Fe, Mn and Zn). Strongest correlations were observed for Cu, Mn and Fe ($r = 0.99$; $r = 0.93$ and $r = 0.90$ respectively) and somewhat weaker for Zn and Cd ($r = 0.69$ and $r = 0.61$ respectively) (Table 3). This is in accordance with previous studies investigating these two extraction methods (Sabiene et al. 2004; Meers et al. 2007). However, for Cd extraction *aqua regia* gave us twice as higher values than HNO₃ extraction (Table 2), therefore when investigating total Cd levels information on the extraction method is important.

EDTA is a strong chelating agent that has been reported to remove organically bound metals and partially metals in oxides and secondary clay minerals (Bermond et al., 1998; Feng et al., 2005a). It is a more aggressive chelating agent than chelation occurring under natural circumstances (Meers et al., 2007; Labanowski et al., 2008). It was originally developed for acidic soils and it correlates well with plant uptake in acidic soils but it is a poor predictor of availability for alkaline and neutral soils (Feng et al., 2005a). In present study EDTA extraction showed good correlation ($p < 0.001$) with pseudo-total extractions for Cd, Cu and Mn ($r = 0.59$; $r = 0.95$ and $r = 0.73$ respectively) with *aqua regia* and ($r = 0.87$; $r = 0.95$ and $r = 0.62$ respectively) with ultra pure HNO₃ (Table 3). Therefore, Fe and Zn were showing no correlation between EDTA and pseudo-total extraction methods.

Water is a weak extractant and in the present study water extraction represents the trace metal concentration in soil solution. Recent studies show that weak extractants such as CaCl₂ and RHIZON method using organic acids are the best indicators of trace metal availability as they take soil solution concentration into account as well as some weakly bound trace metal concentrations (Feng et al., 2005a; Feng et al., 2005b; Meers et al., 2007). Water extractable fraction on the other hand represents only the soil solution concentrations, those are not total concentrations that will be taken up by the plant, still, solution concentrations are in good correlation with the free metal ion (FMI) concentrations in the solution and FMI concentrations are in good correlation with plant uptake (Almås et al., 2005; Lofts et al., 2004; Sauvé et al., 2000). Therefore, information on soil solution concentrations (in our study water extractable fraction) can be used to predict plant uptake. Water extraction showed the best correlation with EDTA, where it was correlated for Cu ($r = 0.60$), Fe ($r = 0.38$) and Mn ($r = 0.47$). With pseudo-total extractions the correlation existed only for Cu ($r = 0.51$ for *aqua regia*) and for Cu and Zn with HNO₃ extraction ($r = 0.53$; $r = -0.30$). Such findings mainly confirm our hypothesis (Cu is an exception) that EDTA and pseudo-total concentrations are not well correlated with concentrations in soil solution. The main reason for lack of correlation is due to the influence of soil properties. Soil proper-

ties, such as pH, play a major role in solubility of trace metals. As mentioned earlier EDTA behaves differently in acid soils than in alkaline soils. Copper was the most interesting element as all of its extractions were correlated among each other (Table 3). Such results suggest that there are not many other factors influencing Cu extractability.

Table 3. Pearsons correlation coefficients (n = 45)

Tablica 3. Pearsonov korelacijski koeficijent (n=45)

	Cd	Cu	Fe	Mn	Zn
HNO ₃ – Aqua regia	0.61***	0.99***	0.90***	0.93***	0.69***
Aqua regia - EDTA	0.59***	0.95***	ns	0.73***	ns
HNO ₃ – EDTA	0.87***	0.95***	ns	0.62***	ns
EDTA – WE	ns	0.60***	0.38*	0.47***	ns
HNO ₃ – WE	ns	0.53***	ns	ns	-0.30*
Aqua regia - WE	ns	0.51***	ns	ns	ns

* and *** indicate significant difference at $p < 0.05$ and $p < 0.001$ respectively, ns - not significant; WE – water extraction

Earlier studies conducted in the same area investigated plant uptake in the relationship with EDTA extraction (Lončarić et al., 2010). The same trace metals as in present study were considered and EDTA showed good correlation only with Fe concentrations in grain. EDTA is an extractant that mimics phytosiderophore release, exudates used by metal deficient plants primarily to obtain Fe (Menzies et al., 2007). We believe that this could be the reason why only Fe correlated with EDTA. In addition, soils from Lončarić et al. (2010) study had a quite high pH (pH was in range 6.95 -8.15) which reduces EDTA ability to predict plant uptake as EDTA method was developed for acidic soils and shows the best correlations with uptake when used on soils with low pH (Feng et al., 2005a; Feng et al., 2005b). In present study we examined 45 soil samples from the same area as in Lončarić et al. (2010) study where we had no information on trace metal concentration in soil solution, which in present study is represented by water extraction. The soil solution is the medium from where plants take up the elements and as such it is a good indicator of availability (Almås et al. 2005; Lofts et al. 2004; Sauvé et al. 2000). Further research should consider investigating correlation between soil solution concentrations of trace metals and plant uptake.

CONCLUSION

Strong acid extraction methods (*aqua regia* and HNO₃ extraction), representing total pool of trace metals in soil, showed good correlation between each other for all of the investigated trace metals. However, they showed no correlation with most readily available pool extracted by water (with exception for Cu) while correlation with available pool (EDTA extracted) exists only for Cd, Cu and Mn. Therefore, total pool has shown itself as a poor indicator of bioavailability. Furthermore, comparison

of these two total extraction methods showed that *aqua regia* extracts have twice as much Cd than HNO₃ extracts. Even if there is a positive correlation between two extraction methods there can be differences in quantity which can lead to misinterpretation of the results.

The most available fraction represented by water extraction showed no correlation with total concentrations and poor correlation with EDTA extraction. No correlation of water extraction with total concentration and poor correlation with EDTA indicate poor role of total extracts and EDTA extracts as indicators of bioavailability. In a nutshell, further research should be conducted to investigate plant uptake in relation to these extraction methods.

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USPOREDBA EKSTRAKCIJSKIH METODA ZA ODREĐIVANJE RASPOLOŽIVIH I UKUPNIH KONCENTRACIJA Cd, Cu, Fe, Mn i Zn U TLU

SAŽETAK

Ukupne koncentracije metala u tragovima u tlu se vrlo često koriste kao indikatori rizika i procjene raspoloživosti metala. Međutim, novija su istraživanja pokazala da je ukupna koncentracija loš pokazatelj raspoloživosti, dok koncentracije u otopini tla pokazuju dobru korelaciju s koncentracijama u biljci. Glavni je cilj istraživanja bio usporediti četiri različite metode ekstrakcije metala u tragovima i korelacije među njima. Istraživanje je provedeno na poljoprivrednim tlima Istočne Hrvatske (Slavonija i Baranja). Prikupljeno je 45 uzoraka tla s oraničnoga sloja (0-25cm). Uzorci su analizirani sljedećim metodama: ekstrakcije jakim kiselinama (HNO₃ ekstrakcija i aqua regia), ekstrakcija slabom kiselinom (EDTA) i najslabija ekstrakcija, koja predstavlja najraspoloživiju frakciju, frakciju u otopini tla, vodotopiva frakcija. Uzorci su ispitani za pet elemenata (Cd, Cu, Fe, Mn i Zn). Vodotopiva je frakcija pokazala najbolju korelaciju s EDTA ekstrakcijom (Cu, Fe i Mn), dok su ukupne ekstrakcije (HNO₃ i aqua regia) korelirale s vodotopivom frakcijom samo za Cu. Nepostojanje korelacije između vodotopive frakcije i metoda za ukupne koncentracije potvrdila je lošu ulogu ukupne koncentracije kao pokazatelj raspoloživosti. EDTA ekstrakcija se može donekle koristiti za predstavljanje dostupne koncentracije teških metala u tlu, ali ju se, također, treba uzeti s oprezom kada se koristiti za opisivanje raspoloživoga dijela.

Ključne riječi: raspoloživost, ekstrakcija, tlo, metali u tragovima

(Received on 5 April 2013; accepted on 13 May 2013 - *Primljeno 05. travnja 2013.; prihvaćeno 13. svibnja 2013.*)