# Recovery of Trace Metal Isotopes in Seawater Samples Using Multifunctional Neem (*Azadirachta indica*) Biosorbent: A Comparison with Monofunctional NOBIAS−Chelate−PA1 Resin

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ABSTRACT: Neem (*Azadirachta indica*) tree leaves (NL) represents a potential alternative to costly conventional materials for recovery of trace metals from seawater. Metal uptake increased in the following order: hard metals (^{44}Ca, ^{24}Mg, and ^{88}Sr) < borderline metals (^{55}Mn, ^{59}Co, ^{61}Ni, ^{63}Cu, and ^{66}Zn), and soft metals (^{208}Pb and ^{111}Cd) based on covalent bond formation with biological ligands. Improved removal of major ions was achieved using NL instead of the monofunctional ($-N(CH_2COOH)_2$) NOBIAS−Chelate−PA1 resin. However, major ion (1.5−500.8 mg/kg) and heavy metal concentrations were much higher (1.0×10^2 to 1.0×10^5 μg/kg) in fresh NL compared to the metal concentrations (0.02−98.2 ng/kg) in seawater. Therefore, before use, the freeze-dried NL was subjected to a rigorous acid cleaning process using HCl, HNO₃, and buffer (NH₄COOCH₃) to remove heavy metals and major ions. Trace metal recovery levels ascertained using inductively coupled plasma-mass spectrometry (ICP-MS) were in the range of 91−115%, with 99.999% of the matrix ions removed. For each trace metal isotope, the procedure blank was <10% of the mean concentration of the seawater, and the precision was 5−8% RSD. The use of reference seawater (NASS-6) was extremely valuable in validating the metal-free condition of the NL and for data quality control. The current adsorption capacities of NL are relatively low; however, biological ligands have excellent potential for rapid separation of trace metals from the seawater matrix.

KEYWORDS: *Acid-cleaned biosorbent, Soft metals, Borderline metals, Trace isotopes, Ion exchange column, regeneration of Neem-biosorbent*
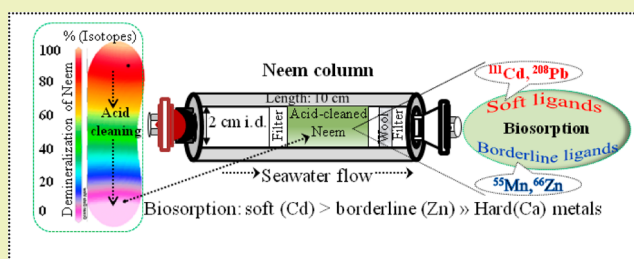
■ INTRODUCTION

The metal uptake ability of biomaterials is well known.[1−5] Biomolecules contain weakly acidic and basic groups capable of chelating macro- and micronutrients critical for growth, including potassium, calcium, sodium, magnesium, manganese, iron, zinc, cadmium, and copper.[6−8] Metal ions may be retained through intracellular accumulation or surface adsorption.[9−11] For example, zinc has important catalytic and structural roles in many biological functions,[12] and it is estimated that 25% of proteins require a metal for function.[13] Metal ion binding to nonviable cells is presumed to occur exclusively through surface adsorption, and under certain conditions, the metal ions are adsorbed in greater quantities than in living biomass.[11] Therefore, biosorption has become an active research topic, with the goal of removing high concentrations of heavy metal ions from aquatic systems using the natural metal sorption capability of biomaterials.

Until recently, biosorbents have been applied as heavy metal scavengers in solutions at high concentration (ppm level), and the initial metal content of the biosorbent was a relatively small factor. However, many heavy metal ions are present in seawater at <1 ppm levels, and a metal-bearing biosorbent may contribute a significant amount to the total measured metal content following the sorption process. Previously published biosorption techniques have not addressed these issues. ICP-MS is capable of multi-element analysis at trace levels (limit of detection: subppt) but suffers from instrumental drift caused by solid deposition at the nebulizer orifice and signal suppression in samples such as seawater that contain high levels of dissolved salts.[14,15] Therefore, control of matrix effects in seawater is essential during ICP-MS determinations. A realistic procedure for trace metal determination in aquatic media using ICP-MS must incorporate preconditioning of the biosorbent for accurate and precise measurements at the ppt level.

The biological properties of Neem (*Azadirachta indica*) tree leaves (NL) have been described elsewhere.[16] NL contains more than 100 bioactive ingredients with versatile metal-binding donor atoms, as well as a native mineral content of 3.4%.[16] According to Pearson,[17] metal cations act as Lewis acids, while organic ligands tend to act as Lewis bases.[18] Complexation of metals with carbohydrates, proteins, and carboxyl groups has been reported.[2,4] Metals may be categorized according to their electron-accepting or -donating behavior under the Lewis theory. For instance, Cd^{2+} and Pb^{2+}

represent a soft and a borderline-to-soft metal, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, and Mn^{2+} are borderline-to-hard metals, and Ca^{2+} and Mg^{2+} are hard metals.

A monofunctional strongly acidic cation-exchange resin (NOBIAS−Chelate−PA1 and carboxymethylated pentaethylenehexamine (CM−PEHA)) is commercially available and has been applied to the separation and adsorption of trace elements.[19,20] The ability to exclusively concentrate trace metals is attractive, although the soft metal recovery was not satisfactory in initial tests.[19,20] Therefore, there is considerable interest in commercial development of novel biosorbents. Numerous biosorption/separation methods have been described for the removal of highly concentrated heavy metals in wastewater,[2−4] but there have been no studies examining the initial metal content of the biosorbent and its magnitude with respect to the amount of metal uptake.

The aim of this paper was to develop a procedure for extracting soft and borderline metal isotopes from seawater using NL, including methods for precleaning, regeneration, and reuse. In practice, the technique would be similar to commercial ion exchange systems. The paper also provides an initial assessment of the utility of biological ligands for precise recovery of trace metals from seawater matrix ions.

■ EXPERIMENTAL SECTION

Reagents and Materials. All reagents used in this work were of analytical grade. Ultrahigh purity HNO_3, HCl, HOAc, Wako Milli-Q2, NH_3, and CH_3OH (Tamapure AA-100, Ultrapur, Kanto Chemical) were used for all operations (cleaning, rinsing, and solution preparation). The MQ water (MQW) was purified using a Milli-Q+ (Millipore) system to a resistivity of 18.2MΩ/cm. All equipment was thoroughly cleaned using an alkaline detergent followed by ultrahigh purity 30% aqua regia, rinsed in water, and dried in a clean room. Sample and reagent bottles (Teflon) were further cleaned in a bath containing 3 M HCl and 3 M HNO_3 at 75 °C for 24 h. A 10 mL eluent of 1 M HNO_3 was placed inside the bottles for 24 h during storage. ICP-MS procedures were performed using a 1 M HNO_3 (TAMAPURE AA-10) eluent containing extremely low levels of trace metals (5−10 ppt). ICP-MS standard solutions of heavy metals and major ions (100 mg L^{-1}, Mn, Co, Ni, Cu, Zn, Cd, Co, Pb, Mo, Ca, Mg, and Sr) were purchased from MERCK, Germany (HC956983, Merck KGaA). The ICP-MS analysis, sample handling, and reagent preparation were carried out in a clean room using trace metal handling techniques.

NASS-6 (Seawater Reference Materials). The accuracy and precision of the column adsorption procedure were evaluated using a seawater reference material (NASS-6) obtained from the National Research Council of Canada (NRC−CNRC).

Seawater Samples. The seawater samples were collected from the East China Sea during the KT-08-19 cruise in 2008. All samples used in the experiments were collected in precleaned 1 L polyethylene bottles. Following sample collection, the bottles were double bagged using clean plastic bags. The water samples were passed through 0.2 μm membrane filters immediately after collection and then acidified to a pH below 1.7−2.0 using 20% HCl (3 mL/L). This work was performed at a clean bench installed on the training ship.

Chemical Demineralization of NL Biosorbent. Fresh NL was collected in June 2007, June 2010, and January 2012 from Andulbaria and neighboring villages in Chuadanga district, Bangladesh. Before use, the material was refrigerated (6 °C) for short (7 days) or long (1 and 5 years) periods. The freeze-dried leaves were pulverized in a glass mortar, sieved through a mesh (particle size range 53−74 μm), and stored in airtight plastic vials in a refrigerator (6 °C).

Then NL was soaked and stirred in 1 M HCl for 4 h and 1 M HNO_3 for an additional 4 h to remove metal ions; the rinsed powder was flushed with water several times followed by treatment with 0.1 M NH_4OAc buffer to establish a pH of approximately 4.5−5. The NL was

then oven dried (50 °C) to a constant weight, and the remaining metals ions in acid-cleaned NL were confirmed. For this, 30 mg of raw and acid-cleaned powdered NL were taken in a digestion apparatus separately, and 0.2 g of conc. H_2SO_4 and 0.4 g conc. HNO_3 were introduced. The mixture was heated slowly on a hot plate, with the addition of 3−4 drops of H_2O_2. This step was repeated until the solution became clear, and then the mixture was dry at about 150 °C. The content was diluted to 40 mL with 1 M HNO_3, and the trace metals and major ions were measured precisely using ICP-MS. The acid-cleaned dried NL (0.5 g) was packed in the column for adsorption. However, high concentrations of acid and strongly alkaline solutions decomposed the NL.

Preparation of Column Set Containing NL and NOBIAS Column. The NL column had an inside diameter of 2.0 cm and a bed height of 10 cm and contained 0.5−1.0 g of NL sandwiched between quartz wool and a 2 mm thick polytetrafluoroethylene (PTFE) tube (Figure 1a). The complete apparatus (Figure 1b) consisted of
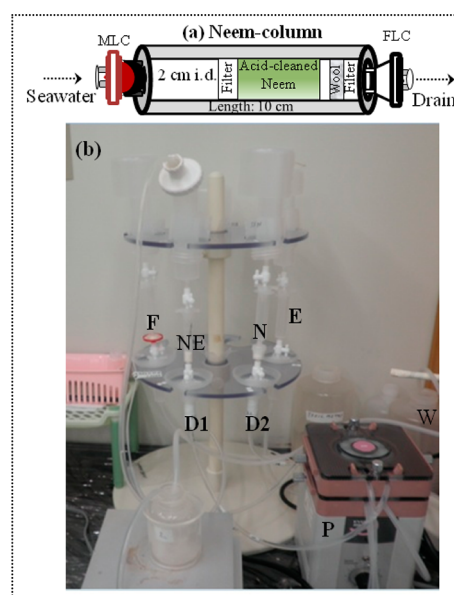

Figure 1. (a) Neem column (MLC, male luer connection; FLC, female luer connection). (b) Schematic diagrams of multi-column preconcentrating and eluting procedure: NE (Neem), N (NOBIAS), F (filtration), E (elution), P (peristaltic pump), D 1 and D2 (drain), and W (column waste).

NOBIAS and NL columns, Teflon bottles, PFA tubing, PTFE joints, PTFE three-way valves, and a peristaltic pump (MP-1100, EYELA). The column set had a two-step round plastic plate, which was fixed in a stand (Figure 1). The system was equipped with four column connections and could process four samples simultaneously during the 30 min processing time.

Adsorption and Desorption Procedure. The NL material was acid cleaned to remove native metals. The NL column was washed with 40 mL of MQW, 100 mL of 1 M HNO_3, and again with 40 mL of MQW. At this point, the column pH was 2.5−2.8. The pH was adjusted to 4.0−5.0 using 0.1 M NH_4OAc buffer. The column was washed to remove major ions using excess 0.1 M NH_4OAc buffer (100 mL) solution.

Prior to initiating the adsorption process, the blank values for the procedure were estimated using 100 mL of MQW as a sample. Solutions of 0.1 M NH_4OAc (5 mL) and 3.6 M NH_4OAc (1.44 g) were added to the 100 mL (pH < 2) of two separate seawater samples. The pH was adjusted using 1 M HCl or 1 M NH_4OH solution to 4−5 for the NL column and 6.0 for the NOBIAS column.

The water sample was passed through the preconditioned columns at a flow rate of 1.0 mL/min using the peristaltic pump. The flow rate was dependent on the weight, surface area, and column thickness.

After the samples were loaded, the columns were rinsed with 40 mL MQW and 40 mL of 0.1 M NH$_4$OAc buffer solution to remove the bulk of the major ions prior to elution. The adsorbed heavy metals were eluted by passing 3 mL of 1 M HNO$_3$ through the column under suction to reduce the risk of contamination from the air. The light green eluate was mixed with a small amount of H$_2$O$_2$ to remove any dissolved organic matter and dried at 160 °C. NOBIAS (Hitachi High Technology)[19] is a commercial resin containing polyaminopolycarboxylic acid groups (PAPC) and was used as a reference against which the efficiency of the NL column could be compared.

Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) Measurements. An ICP-MS (Element II, Thermo Fisher Scientific K.K.) instrument was used for trace metal measurements. The low-resolution MS mode was used for isotope measurements. Samples were pumped into the instrument at 50 μL min^{-1} (Table 1). A ^{103}Rh

Table 1. Operating Conditions for ICP-MS and ICP-AES Instruments

ICP-MS (Element II)	flore vacuum (mbar)	2.31 e^{-004}
	high vacuum (mbar)	2.26 e^{-007}
	argon middle (bar)	2.50
	argon max (bar)	5.16
	samples were pumped into the instruments	50 μL min^{-1}
ICP-AES	**radio frequency output**	1.3 (kW)
	argon gas flow rate	
	plasma gas	15 (L min^{-1}
	auxiliary gas	0.5 (L min^{-1})
	nebulizing gas	0.8 (L min^{-1})
	pump parameter	
	sample flush time	10 (s)
	sample flush flow rate	4.0 (mL min$^-$)
	sample flow rate	1.5 (mL min$^-$)
	read delay time	30 (s)
	reading time auto	minimum 0.01s; maximum 20 s

internal standard (WAKO Pure Chemical Industries Ltd.) was used for normalization of the individual isotopic intensities (cps) to account for variations in instrument sensitivity. Calibration lines for trace metals were prepared using standard solutions of the metals in 1 M HNO$_3$. The curve was linear for each isotope for concentrations of 0.05, 0.1, 1, 2.5, 5, 10, and 50 μg/L, with a correlation coefficient (r^2) of 0.9999. The detection limit was 0.05 μg/L and was calculated using the following equation

$$DL = 3 \times SD_{PB} \times \frac{H_{STD}}{\text{Mean of } H_{STD} - \text{Mean of PB}}$$

in which H_{STD} is the highest standard concentration (10 μg/L), PB is the procedure blank, and SD is the standard deviation.

The metals content in the solution (without preconcentration) was analyzed using a PerkinElmer Optima 3000 DV inductively coupled plasma-atomic emission spectrometer (ICP-AES) equipped with a cross-flow nebulizer and a Scott-type spray chamber or a Hitachi 180-80 polarized Zeeman flame-type atomic absorption spectrometer. Operating conditions for the ICP-MS and ICP-AES are listed in Table 1.

Infrared spectra (IR) of the NL material was recorded using a Horiba FT-710 spectrometer. The metal content of the NL in terms of wt % was measured using a wavelength dispersive X-ray fluorescence (Philips WD-XRF-PW 2404R). All the measurements were performed at the University of Toyama, Japan.

■ RESULTS AND DISCUSSION

Estimation of Naturally Occurring Metals and Nonmetals in Fresh NL. NL naturally contains approximately

3.4% minerals by weight.[16,21−27] The metal and nonmetal content of fresh NL powder (before washing) was determined using WD-XRF (Table 2). Major ion concentrations ranged

Table 2. Determination of Total Metal and Nonmetal Content in Solid NB Using WD-XRF

	Before wash	Acid-cleaned NL	
elements	wt %	1 M HNO$_3$ (without buffer) wt %	1 M HNO$_3$ (with buffer) wt %
major ions			
Na	4.69	nd	nd
Mg	2.18	nd	nd
K	35.85	4.51	1.37
Ca	38.62	2.56	nd
Sr	0.06	nd	nd
trace metals			
Cr	0.02	nd	nd
Mn	0.18	nd	nd
Fe	2.32	11.69	12.82
Co	0.01	nd	nd
Ni	nd	nd	nd
Cu	0.07	0.56	nd
Zn	0.13	nd	nd
Mo	0.02	nd	nd
Cd	nd	nd	nd
Hg	nd	nd	nd
Pb	nd	nd	nd
Ti	0.28	1.65	0.13
nonmetals			
S	8.4	54.02	52.93
Si	2.78	20.55	26.75
Br	0.02	nd	nd
I	nd	nd	nd
Cl	3.62	nd	nd
P	1.60	4.45	nd
total	~100	~100	~100

from 0.06% to 47.0%, with potassium (35.85%) and calcium (46.62%) being present at higher levels than Na (4.69%) or Mg (2.18%). Heavy metal concentrations were Mn 0.177%, Co 0.0090%, Cu 0.0674%, Zn 0.125%, Mo 0.0166%, and Fe 2.32%. The metals Ni, Cd, and Pb were not detected. Other elements such as Si (2.78%), Cl (3.62%), and S (8.4%) were present at relatively high concentrations. Sulfur content was higher in acid-cleaned Neem (NL).

Demineralization. The natural metal content was removed from the NL material before use, and the residual metal content following cleaning was determined using ICP-AES and ICP-MS. The concentrations of Zn (6.4×10^4 μg/kg), Cu (2.2×10^4 μg/kg), Mn (1.0×10^5 μg/kg), and Ni (4.1×10^3 μg/kg) in the fresh NL were higher than the levels in seawater (ng/kg) (Table 3). However, removal of trace metals and matrix ions (^{44}Ca, ^{24}Mg, ^{88}Sr) from metal-bearing NL was 99.99%. The major ion content of the washed NL is presented in Table 3.

HNO$_3$ (in the concentration range of 1−3 mol L^{-1}) or HCl (1 mol L^{-1}) was independently investigated as the acid-cleaning medium, but neither HNO$_3$ nor HCl could remove heavy metals from the NL rapidly and completely (Table 3, cleaning methods 1−9). According to data analysis, HCl was a more effective reagent (Table 3, methods 2 and 5) for the demineralization of metals compared to HNO$_3$ (Table 3, methods 3, 4, 6−9). Finally, 1 M HCl, 1 M HNO$_3$, and 0.1 M

Table 3. Metals Content in Raw and Acid-Cleaned Neem

cleaning methods chemicals	1 before wash	2 1 M HCl/2 h	3 1 M HNO$_3$/2 h	4 1 M HNO$_3$/7 h	5 1 M HCl/24 h	6 1 M HNO$_3$/24 h	7 3 M HNO$_3$/2 h	8 1 M HNO$_3$/2 h	9 1 M HNO$_3$/2 h and buffer	10 1 M HCl/4h, 1 M HNO$_3$/2 h, buffer (0.1 M NH$_4$COOCH$_3$)
Neem wt/acid (g/mL)	NL powder	1g/100 mL	11g/00 mL	1g/100 mL	1g/100 mL	1g/100 mL	1g/100 mL	10.5g/00 mL	1g/100 mL	0.5g/100 mL
concentration	μg/kg	μg/kg	μg/kg	μg/kg	μg/kg	μg/kg	μg/kg	μg/kg	μg/kg	ng/kg
Cd111(LR)	1.0×10^2	Nd	0.10	nd	nd	nd	0.10	nd	nd	0.02
Pb208(LR)	1.7×10^3	0.21	10.60	0.12	0.13	0.12	11.50	0.20	0.17	0.23
Mn55(MR)	1.0×10^5	7.62	206.5	4.85	4.80	5.01	198.20	6.45	5.68	1.67
Co59(MR)	5.1×10^2	0.56	7.70	0.45	0.45	0.46	8.60	0.51	0.49	nd
Ni60(MR)	4.1×10^3	1.23	62.70	0.89	0.81	0.84	62.50	4.73	1.24	1.78
Cu63(MR)	2.2×10^4	3.71	132.6	2.01	2.26	2.04	86.10	1.91	2.54	9.48
Zn66(MR)	6.4×10^4	2.74	84.10	1.57	1.76	1.84	59.50	4.14	2.82	28.47
Mg24 (MR)	190.3×10^3									62.0
Ca44 (MR)	500.8×10^4									508.2
Sr88 (MR)	1.58×10^3									4.05

NH$_4$COOCH$_3$ were chosen for NL cleaning, and efficient cleaning was obtained (Table 3, method 10). Metal removal performance was good for cobalt (Table 3, method 10) and cadmium (Table 3, methods 2, 4, 5, 6, 8, and 9), but the number of samples for zinc was very small.

After cleaning the NL, the total weight loss was 33% ± 0.85% (n = 3) of total fresh NL weight. The NL decomposed during washing with 1 M NaOH and became coagulated in 0.005 M NaOH, possibly due to the fatty acid (1%) like oleic acid, steric acid, palmitic acid, linoleic acid, vitamin C (218 mg/100 g NL), Glutamic acid (73.30 mg/100 g), and polyamino carboxyl acid (7%) content of the NL material.[16]
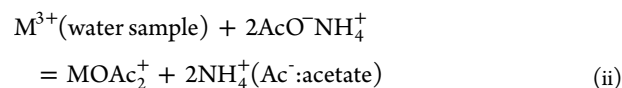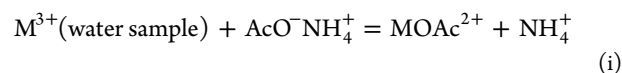
After removing the matrix and assessing system stability, we investigated the behavior of metals toward NL. The extraction efficiency of the NL-packed biocolumn for isotopes was not changed even after several successive cycles of preconcentration/elution procedure, indicating good stability of the developed continuous preconcentration system with NL as sorbent.

Functional Group Characterization of Fresh and Acid-Cleaned NL Using IR. The functional groups present in fresh and freeze-dried NL were identified using IR spectroscopy. The important characteristic absorption bands included −OH, >C═S, −CH(NH$_2$)COOH and −COOH, −NH$_2$ ≡CH, >C═N, ≡C−C≡, ≡C−N < , and ≡C−O−, >C═O, −C−O, −C−H stretching, −O−CH$_3$, −C═C−, −CO−NH−, and −C−O−C− stretching (Table 4).

The functional groups were classified according to the Pearson concept into three types of Lewis bases:[17] hard (−OH, RO−, R$_2$O, and RNH$_2$), borderline (CH(NH$_2$)COO−, ≡C−N < , and RCOO−), or soft (>C═S and S═O) (Table 4).[28]

IR spectra were acquired at three stages to assess the functional group stability: within 72 h after harvesting of the leaves from tree, after long-term refrigerated storage (1 or 5 years), and after initial cleaning and metal removal. There were no major changes in the IR spectrum, suggesting that NL may be reused and that desorption was feasible, reducing the need for disposal of toxic residues common with other biosorbents.

Adsorption Mechanism and Sorption Capacity of NL. Metal ions (M^{x+}) interact with ammonium acetate buffer to produce stable metal−acetate complexes before ion exchange (eq i, ii)

$$M^{3+}(\text{water sample}) + AcO^-NH_4^+ = MOAc^{2+} + NH_4^+ \tag{i}$$

$$M^{3+}(\text{water sample}) + 2AcO^-NH_4^+ = MOAc_2^+ + 2NH_4^+(Ac^-\text{:acetate}) \tag{ii}$$

Carboxylic acid anions including acetate are important ligands for a number of metals.[40] Soft and borderline cations exhibit a higher affinity for NL than hard cations, and their binding mechanism most likely includes a combination of ion exchange and chelation reactions. Cation exchange on the NL surface is an equilibrium process. Metals (M^{x+}) are most likely to bind to polar functional groups (−COOH, −CHO, and −OH, etc.) through the mechanism (eq iii)
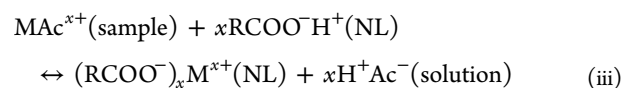
$$MAc^{x+}(\text{sample}) + xRCOO^-H^+(NL) \leftrightarrow (RCOO^-)_x M^{x+}(NL) + xH^+Ac^-(\text{solution}) \tag{iii}$$

Table 4. Position of Characteristics Adsorptions and Classes of Functional Groups in Neem

NL contents[16]	percentage (%)[16]	this work band position (cm^{-1})	this work (functional groups)	ligand atoms	ligand types (Lewis bases)	metals uptake (Lewis acids)
carbohydrates	22.9%	1237.11−3625.52	R−O−H	oxygen	hard	hard metals: Ca^{2+}, Mg^{2+}, Mn^{2+}
		3297	≡CH			
		1656	≡C−C≡			
		3399	−NH$_2$	nitrogen	hard	
proteins	7.1%	1656	>C=N	sulfur		soft metals: Cd^{2+} Pb^{2+}
		1099.23−1318.11	>C=S	nitrogen	soft	
		3399	−CH(NH$_2$)COOH		soft borderline	
tyrosine	31.50 mg/100g	1319.11−1733.69	−CH(NH$_2$)COOH	nitrogen	borderline	borderline: ^{59}Co, ^{61}Ni, ^{63}Cu, ^{66}Zn
lipids (fats)	1.0%	1319.11−1733.69	R−COOR, −COOH	oxygen	borderline	
vitamin C	218 mg/100g NL	1319.11−1733.69	−COOH	oxygen	borderline	
glutamic acid	73.30 mg/100 g	1319.11−1733.69	−CH(NH$_2$)COOH	nitrogen	borderline	
alanine	6.40 mg/100 g	1319.11−1733.69	−CH(NH$_2$)COOH	nitrogen	borderline	
minerals	3.4%					

[5,41] in which M^{x+} represents a cation and R represents a polar portion of NL interacting with M^{x+} through an ion-exchange mechanism.

Following sorption of trace metals by NL, several functional groups that were initially present in IR spectra disappeared (e.g., −CH(NH$_2$)COOH, >C=S, S=O, −COOH, amides, and −OH). The most important factor in the functional group behavior is the nature of the donor atom (for example, electronegativity S ~ N < O), and the most stable interactions are generalized as hard−hard (ionogenic) or soft−soft (covalent) Lewis acid−base interactions (Table 4).[42,43]

In this study, hard metals may interact through electrostatic mechanisms with oxygen-containing basic functional groups, and the soft metal ions Cd and Pb formed stable bonds with sulfur containing ligands (>C=S and S=O), while borderline metals interact with soft and borderline (CH(NH$_2$)COO−, RCOO$^-$) nucleophilic ligands.[17,18] Therefore, Cd and Pb recovery was higher due to the soft functional groups in NL compared to the monofunctional (−N(CH$_2$COOH)$_2$) NO-BIAS resin.

Adsorption capacity measurements were carried out using initial soft (Cd), borderline (Zn), and hard (Ca) metal concentrations of 5 mM in 0.1 M NH$_4$OAc adjusted to pH 5.0 (pH 7 for Ca) using 1 M HCl or aqueous ammonia. The dried and cleaned NL (0.5 g) was suspended in 100 mL of the solution and stirred at room temperature. The amount of each element remaining in the solution nearly reached a constant after stirring for at least 3 h. The metals concentration in the water samples was determined using ICP-AES. The adsorption capacity was estimated, and the metal uptake values increased in the order of major ions (Ca: 0.00001 ± 0.52 mmol/g) < borderline metals (Zn: 0.04 ± 0.12 mmol/g) < soft metals (Cd: 0.06 ± 0.052 mmol/g) (n = 3).

NL contains 7.1% protein in the form of amino acids and sulfur compounds.[16] According to Table 2, sulfur (soft ligand) content was higher in acid-cleaned NL. Aminocarboxylic acids (−CH(NH$_2$)COOH) in proteins provide imperfect selectivity for metals, and metal affinities tend to follow a universal order of preference, which for essential divalent metals is the Irving−Williams series: Mg^{2+} and Ca^{2+} (weakest binding) < Mn^{2+} < Co^{2+} < Ni^{2+} < Cu^{2+} < Zn^{2+}< Cd^{2+}).[29,30] Divalent Cd^{2+} is highly competitive and is expected to bind tightly to molecules containing sulfur and nitrogen ligands.[8] Metal classifications and ligand affinities have been described in previous studies.[31−33] A variety of sites available on the external and internal surfaces of NL are capable of strongly binding soft and borderline acid cations.[24] In addition to metal sorption performance, biosorbent applications require functional group stability to prevent decomposition during use or regeneration.

pH Optimization. The pH$_{pzc}$ (pH$_{point\ zero\ charge}$: pH at which the surface charge is zero) of NL is 4.5.[34] Maximum sorption occurred when the initial pH was greater than pH$_{pzc}$, which facilitates electrostatic attraction between the negatively charged surface of NL and metal ions (4.5 < pH < 6). The sorption process is a function of (1) the metal ions present in solution, (2) the metal charge, and (3) the functional groups present on the NL surface at a given initial pH. At pH 4.5−6, more negatively charged surface species become available, facilitating greater metal uptake. At lower pH (pH < 4.5), adsorption decreases as the surface charge of NL becomes positive, causing binding site competition between the metal ions and the protons present in the solution. At higher pH (pH > 6), metal adsorption decreases due to precipitation of hydroxyl species onto the adsorbent.

Recovery of Trace Metals after Removal of Matrix Ions Using Acetate Buffer. Before elution (desorption), the column was washed with acetate buffer to remove major ions. Because of size and steric effects, some monovalent cations such as the NH$_4^+$ in the buffer can apparently displace specific adsorbed ions. Pickering[35] reported that NH$_4^+$ released Ca from CaCO$_3$ suspensions, and that NH$_4^+$ may promote the liberation of major ions from interlayer exchange sites.[28] Metal desorption increased with increasing acid concentration (0.05 M > 1 M). The tendency of metals to bind to soft ligands or organic substrates (which are usually soft) is greatest for electronically soft and borderline metals, typically in the order Cd^{2+} > Zn^{2+} > Ca^{2+}.[28] The greater the covalent index value (soft metals > borderline metals > major ions) of a metal ion the greater its potential to form covalent bonds with biological ligands (covalent index is defined as Xm^{2}r, where Xm is the electronegativity and r is the ionic radius).[36]

The active ingredients in NL and NOBIAS resin are hydrophilic, unlike most chelating and ion-exchange resins.[37] The hydrophilic character increases the accessibility of the functional groups to the metal ions, improving the efficiency of metal adsorption and desorption.[26]

Buffer Effect. The acetate buffer used in the NL column process increased the metal adsorption capacity from 50% to 95% as well as reducing the required contact time (flow rate was increased to 0.5 mL/min). One possible explanation is the

Table 5. Procedure Blank and Detection Limit (Neem and NOBIAS Column)

elements	measured isotopes	n	procedure blank (NL) ng/kg ave SD	detection limit NL (ng/kg)	procedure blank NOBIAS ng/kg[19] ave SD	detection limit NOBIAS (ng/kg)[19]	mean concn. in the open ocean (ng/kg)[44]	% [(procedure blank/mean concn. in the open ocean) × 100] NL	NOBIAS
Mn	55	4	1.54 ± 0.210	0.638	0.06 ± 0.07	0.2	20	7.7	0.3
Co	59	4	0.061 ± 0.05	0.16	0.002 ± 0.005	0.014	1	6.1	0.2
Ni	61	4	7.16 ± 0.05	0.17			500	1.43	
Cu	63	4	8.79 ± 1.4	4.12	0.3 ± 0.1	0.32	200	4.4	0.15
Zn	66	4	29.01 ± 1.45	4.34	4.6 ± 1.3	3.9	300	9.67	2.2
Pb	208	4	0.219 ± 0.14	0.45	0.32 ± 0.07	0.21	2	4.45	16
Cd	111	4	3.67 ± 0.01	0.03	0.002 ± 0.003	0.009	70	5.24	2.9×10^{-3}

Table 6. Recovery Experiment from Surface Seawater (KT-08-19) Using Neem Biosorbent

elements	isotope	n	KT-08-19 surface (NL) ng/kg ave ± SD	metal addition ng/kg added	found ng/kg ave ± SD	recovery of trace metals %[a] ave ± SD	removal of major ions %[a]
Mn	55	4	56 ± 0.025	540.4	614.29 ± 9	103 ± 4	
Co	59	4	3.4 ± 0.029	2.14	5.4 ± 4.02	98 ± 1	
Ni	61	4	69.5 ± 0.679	68.56	133.91 ± 8	97 ± 1	
Cu	63	4	98.2 ± 0.04	88.45	188.5 ± 1	101 ± 7	
Zn	66	4	23.6 ± 0.17	138.8	168.8 ± 6.5	104 ± 2	
Pb	208	4	1.62 ± 0.049	10.45	12.1 ± 6	102 ± 5	
Cd	111	4	0.019 ± 0.003	0.036	0.054 ± 0.01	99% ± 1	
Mg	24	4	53.4×10^{4} ± 0.09				99.999
Ca	44	4	20.8×10^{4} ± 0.01				99.999
Sr	88	4	3.4×10^{2} ± 0.06				99.999

[a]Percentage against seawater concentration.

Table 7. Comparative Results on Certified Reference Materials of Seawater NASS-6 Using NOBIAS Resin and Neem Leaves[a]

elements	measured isotopes	n	NASS-6 certified values μg/kg	NL μg/kg	recovery% (NL)	RNL (reused NL) μg/kg	recovery% (RNL)	NOBIAS (NASS-6) μg/kg	recovery % (NOBIAS)	precision % RSD[b] (NL)
Mn	55	3	0.53 ± 0.05	0.54 ± 0.094	102	0.48	90	0.5 ± 0.0001	94	0.43
Co	59	3	0.02 ±	0.018 ± 0.001	90	0.0168	84	0.0175 ± 0.012	97	2.36
Ni	61	3	0.30 ± 0.025	0.25 ± 0.01	83	0.233	78	0.3 ± 0.001	100	3.03
Cu	63	3	0.25 ± 0.025	0.23 ± 0.015	92	0.231	90	0.21 ± 0.000	84	1.34
Zn	66	3	0.26 ± 0.020	0.30 ± 0.083	115	0.28	108	0.28 ± 0.001	108	0.47
Cd	208	3	0.03 ± 0.0019	0.032 ± 0.012	105	0.031	102	0.025 ± 0.0001	83	1.32
Pb	111	3	0.006 ± 0.002	0.0065 ± 0.001	106	0.0059	99	0.004 ± 0.0006	67	1.13

[a]All concentrations are in μg/km (n = 3). [b]Precision is calculated as the percent relative standard deviation (% RSD) based on the analysis of NASS-6 by Neem.

interaction of the acetate buffer ($AcO^{-}NH_4^{+}$) with heavy trace metals (M^{x+}). At sufficiently high concentrations, the acetate ion (AcO^{-}) can combine with trace metals (M^{+}) to create $MOAc^{(x+)-1}$ complexes.[38,39] The stability constants of complexes between AcO^{-} and hard major ions (Mg or Ca) and between soft and borderline metals and AcO^{-} reach maximum values at different pH levels. Therefore, major ions may be easily separated from heavy metals by controlling the pH using acetate buffer.[28]

Procedure Blank and Detection Limit. Procedure blanks were prepared for the rinse solution (MQW, 1 M HNO_3, 0.1 M NH_4COOCH_3), eluent (1 M HNO_3), conditioning buffer solution (0.05 M CH_3COONH_4), biosorbent, and ICP-MS system. The blank samples were treated in a manner similar to the seawater samples before loading into the column. The ICP-MS/HNO_3 blank was obtained by introducing pure HNO_3 solution after every five samples. The detection limits of the

procedure (defined as 3 times the standard deviations of the procedure blanks) are listed in Table 5. Detection limits (DLs) for the trace metals in the procedure blank (0.5g NL powder/100 mL water) were 6.38 ng kg^{-1} for Mn, 0.42 ng kg^{-1} for Co, 0.17 ng kg^{-1} for Ni, 4.12 ng kg^{-1} for Cu, 4.34 ng kg^{-1} for Zn, 0.03 ng kg^{-1} for Cd, and 0.45 ng kg^{-1} for Pb (Table 5). In this study, the standard deviations (SD) values for NOBIAS were less than 10% of the previously reported values.[19] Some metal concentrations (e.g., Zn and Cd) following NL extraction were below the detection limit for NOBIAS or the procedural blank for Pb was higher than 15% of the mean concentration in the open ocean.[19]

The procedure blank values for trace metals of NL were less than 5%, except Zn (9%), of the average concentrations of the seawater samples (Table 5). The standard deviation of the blank was reported to be ng/kg. For Mn, Co, Ni, Zn, Cu, and Cd, the blanks of NOBIAS in the eluate were less than the

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detection limits for ICPMS, but significant blanks were observed for Pb (~16%).[19]

Recovery. Seawater collected from the East China Sea was used for recovery experiments (Table 6). The concentrations determined for the various isotopes were in agreement for most of the metals, while the value for Zn^{66} was greater than in the procedure blank. The RSD was 3−8%. All of the target metals were recovered from seawater. The recovery efficiency was greater than 90%. The concentrations for Zn in surface water were close to or lower than the detection limits, and Cd was not detected (NOBIAS).[19] The trace metal adsorption ability of NL was 10−12 times higher when a buffer was used. At low pH, metal recovery was reduced because of competition between H^+ and metal ions for basic functional groups in NL.[45] The soft and borderline metals binding are stronger (to form chelation complexes) than calcium and magnesium binding (by ion exchange). Removal of matrix ions (^{44}Ca, ^{24}Mg, ^{88}Sr) from seawater samples was 99.999% (Table 6). The SD of recovery of soft to borderline metals for NL was less than 7% (Table 6), whereas the SD of recovery of soft metals (Cd and Pb) was 9−11% for NOBIAS−Chelate−PA1.[19]

Analysis of Reference Materials Using NL-Biosorbent. The accuracy, validity, and precision of the NL-biosorbent, reused NL (RNL), and NOBIAS evaluation process were assessed using the standard seawater sample NASS-6 (salinity 33.5 g/L) for eight metal isotopes (^{55}Mn, ^{59}Co, ^{61}Ni, ^{63}Cu, ^{66}Zn, ^{208}Pb, and ^{111}Cd) (Table 7). The adsorption capacities of the NL for soft metals (Cd and Pb) were larger than that of NOBIAS−Chelate−PA1 (Table 7). The analytical results obtained using NOBIAS were in excellent agreement with the reported consensus values compared to NL. The relative standard deviations (% RSD) for the analyzed metal concentrations were lower than 5% for NL. The target metals Co, Cu, Mn, Zn, Ni, Cd, and Pb were quantitatively recovered using 1 M HNO_3. The relatively high recovery of Zn^{2+} (Table 7, found) may be due to contamination from precleaned NL. The recovery order for NL, according to the metal-binding capacities after the NASS-6 tests, was Pb^{2+} > Cd^{2+} > Ni^{2+} (Table 7). This recovery difference was due to soft metals (Pb^{2+} and Cd^{2+}) having a higher affinity to the NL due their ability to bind ligands by coordinative, covalent, and ionic interactions, while borderline metals (e.g., Ni^{2+}) bind ligands mostly by ionic interactions. The precision % (RSD) for NL was less than 5%.

Regeneration was then evaluated by analyses of certified materials, NASS-6 coastal seawater using RNL. The concentration for Zn was higher in NL compared to RNL for high content of Zn in fresh and cleaned NL. The soft metals (Cd and Pb) recovery was higher for reused NL than that of borderline metals (Mn, Ni, Co, and Cu). This demonstrates the reuse of the NL and its suitability for successive analyses of soft metals and stability of functional groups.

CONCLUSIONS

These experiments indicate that the hard and soft ligand principle may be applied to predict the recovery of soft and borderline trace metals and to markedly improve the separation of hard major ions in seawater samples using the NL biosorbent. The initial metal content of NL was completely removed using an acid-cleaning process. The procedure blank was below 7% of the mean concentration of water collected from the open ocean, except for an excess initial Zn content in NL of 9.67%. The method accuracy was assessed by analyzing certified natural filtered seawater (NASS-6). Aminocarboxyl groups in NL were the dominant species responsible for biosorption, especially in the case of borderline metals (^{55}Mn, ^{59}Co, ^{61}Ni, ^{63}Cu, and ^{66}Zn). Groups containing −CN and sulfur such as amido and thiol functionalities were involved in the adsorption of the soft metals tested, particularly ^{208}Pb and ^{111}Cd; thus, recovery levels were higher compared to the monofunctional (aminocarboxylic) NOBIAS resin. NL was chemically stable throughout the analyses. No significant change in peak distribution was observed in IR spectra after chemical treatment and long-term preservation. The column apparatus was successfully applied to simultaneously compare various ion-exchange materials. These preliminary results warrant further investigation using other biosorbents for multi-trace metal recovery from seawater.

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Notes
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