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Chelation of Molybdenum in *Medicago sativa* (Alfalfa) Grown on Reclaimed Mine Tailings

D. R. STEINKE,*,† W. MAJAK,† T. S. SORENSEN,‡ AND M. PARVEZ‡

Agriculture and Agri-Food Canada, 3015 Ord Road, Kamloops, B.C., Canada V2B 8A9, and Chemistry Department, University of Calgary, Calgary, Alberta, Canada T2N 1N4

Extraction and analytical procedures were developed from 1999 to 2005 for the chemical investigation of molybdenum (Mo) in aerial portions of alfalfa (*Medicago sativa* L.) grown on a reclaimed mine tailings site at the Highland Valley Copper Mine in British Columbia, Canada. The purification procedures were guided by colorimetric analyses specific for Mo. The Mo levels in freeze-dried plant samples exceeded 100 ppm, which is more than 20 times the maximum level recommended for livestock feed. In contrast to previous work, which detected the inorganic molybdate anion (MoO_4^{2-}) in alfalfa from the site, the present study identified the major pool of Mo as a chelate bound to malic acid in five sources of plant material. However, the inorganic form of Mo was characterized in aqueous tailings samples, but once imbibed by vegetation, the anion was chelated to the α-hydroxy organic acid. Synthetic chelates were synthesized to differentiate the Mo-malate complex from the Mo-citrate by ^{95}Mo NMR. Crystal structure of the synthetic Mo-malate determined that the Mo was bound to two malato ligands as $Na_2[MoO_2(malate)_2]\cdot 5H_2O$, which confirmed the structure of the isolates deduced by ^{95}Mo NMR. The chelation of Mo at the site may well explain the apparent lack of long-term clinical effects in cattle grazing the site.

KEYWORDS: ***Medicago sativa*; molybdenum; chelation; malic acid; mine tailings**

INTRODUCTION

Reclamation of mine tailings is a primary concern for the Highland Valley Copper mine (HVC), one of the largest open pit mines in the world, located near Kamloops, British Columbia, Canada. Deposits of molybdenum (Mo) have been mined with a recovery of approximately 50% Mo over the past 25 years, resulting in high levels of residual Mo in the tailings ponds on the site. These areas have been revegetated with domestic species and fertilized to return the land to sustainable agricultural use. Studies have shown however that residual Mo in the tailings is imbibed by vegetation and can accumulate to extremely high levels depending on the plant species and site (*1, 2*). *Medicago sativa* L. (alfalfa), a major forage at the Highmont site at HVC, accumulated 95−460 ppm Mo on a dry matter basis with no evidence of significant decreases in Mo since 1999, the year of establishment of plant cover at Highmont (*2, 3*). Animal health guidelines recommend a maximum level of 5 ppm Mo in feed for beef cattle (*4*). Vegetation has adapted and sequestered the high levels of Mo, since plant cover and vigor has remained fairly constant without plant yellowing, a major symptom of Mo phytotoxicity (*5*).

Earlier studies by Surridge et al. (*6*) identified the Mo species in alfalfa at Highmont as the molybdate anion (MoO_4^{2-}). This was not anticipated, as plants under heavy metal stress are known to sequester metals by forming organo-metallic complexes (*7*). Furthermore, the widespread occurrence of the molybdate anion would not explain the apparent lack of acute or chronic animal toxicity at Highmont (*3*). Excess dietary molybdate is known to be toxic to cattle (*8*). It was suspected that experimental elevation of pH levels (>8) may have dissociated the organo-metallic complexes in the original isolate from alfalfa (*6*).

The end land use plan for the mine includes sustainable cattle grazing on reclaimed areas, and animal trials were conducted at HVC for 7 years (1998−2004) to determine the long-term impact on cattle exposed to forage extremely high in Mo (*9*). Early, clinical signs were observed in some grazing cattle that may have indicated some degree of Mo toxicity, but widespread chronic morbidity or mortality were not evident as would be expected if excess inorganic molybdate was the only species of Mo available in the forage. In fact, liveweight gains in cows and calves were acceptable throughout the study (*3*). A mode of detoxification of molybdate by the plant cover was conjectured. Accordingly, chemical speciation of Mo in alfalfa from Highmont was re-examined. The purification scheme presented by Surridge et al. (*6*) was modified to permit structure elucidation at slightly acidic pH to minimize the potential dissociation effects of alkaline pH. The structure of the Mo configuration in alfalfa should provide insight into the mode of

* To whom correspondence should be addressed. Phone: (250) 554-5208. Fax: (250) 554-5229. E-mail: steinked@agr.gc.ca.
† Agriculture and Agri-Food Canada.
‡ University of Calgary.

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Table 1. Plant Material for Isolation of Mo Chelates and Yields of Mo from Alfalfa Samples at Highmont

alfalfa isolate	collection date	amount extracted (g DM)	yield (mg Mo)
1	August 17, 1999	75	5.1
2	August 3, 2000	30	3.4
3	August 2, 2001[a]	75	1.2
4[b]	August 13, 2003[a]	50[c]	1.3
5	August 17, 2005	90[c]	7.1

[a] Extraction with H_2O. [b] Oven dried at 40 °C for 48 h. [c] Additional adsorption of impurities on CMC.

biochemical detoxification and could explain the attenuation of clinical signs in grazing livestock.

MATERIALS AND METHODS

Water, Tailings, and Plant Material. Seepage water and tailings samples, the waste product from the milling process, were obtained from Highmont during 2002 to characterize the species of Mo in the environment that could be imbibed by the plant cover. Aqueous extracts of tailings and seepage water were clarified on Whatman #1 filter paper and directly analyzed by ^{95}Mo nuclear magnetic resonance (NMR) without further processing. Composite aerial portions of alfalfa were collected from Highmont from 1999 to 2005 (**Table 1**), transported on ice and frozen. The plant samples were freeze-dried and ground to pass through a 1 mm sieve using a Wiley Mill prior to extraction.

Molybdenum and Organic Acid Analyses. Total Mo values for all phases of the extraction and purification procedures were determined spectrophotometrically using the colorimetric dithiol procedure (*10*). Transmittance readings were recorded at room temperature at 675 nm. The quantitative colorimetric method for total Mo determination required sample ashing prior to analysis (*6*). A qualitative spot test for Mo was derived from the dithiol procedure, which was preceded by acidification, as described by Surridge et al. (*6*). The intensity of the green chromophore estimated the relative amount of Mo present in the chromatographic fractions. The detection limit was approximately 1 ppm Mo. The colorimetric reagent, stored at 4 °C, could also be used directly as a spray reagent on TLC.

Aliquots of the crude extracts from the plants at Highmont were initially examined for the presence of α-hydroxy organic acids, specifically malate and citrate. Aqueous extracts were initially acidified and continuously extracted with diethyl ether (24 h) to yield the pool of free organic acids, which were chromatographed on cellulose TLC (Aldrich, Z 12 283-1) with pentanol/formic acid/water (48.8:48.8:2.4) as the developing solvent system and detection with the bromocresol green spray reagent (*11*).

Purification of Mo Chelate. The following is the extraction protocol for 75 g of plant material adapted from the methods of Surridge et al. (*6*). It was initially washed on a Büchner funnel with 0.75 L of hexane for defatting and with 0.75 L of ethanol to precipitate the proteins. Five liters of 10 mM $(NH_4)_2CO_3$ was percolated as the extraction solvent, which was removed by freeze drying. Aliquots of the two washes, the aqueous extract, and plant residue were assayed for Mo distribution. The major pool of Mo was in the aqueous phase. Distilled water, without $(NH_4)_2CO_3$, was also used as the extraction solvent on two occasions (**Table 1**).

The crude extract was concentrated, redissolved in 0.1 L of solvent, and transferred into 20 cm of 3500 g/mol molecular weight cutoff dialysis tubing (Fisher) that was equilibrated in 10 mM $(NH_4)_2CO_3$. The extract was dialyzed for 4 days with constant stirring at 4 °C and daily changes of $(NH_4)_2CO_3$. Aliquots of the dialyzed and undialyzed phases were concentrated and assayed for Mo. The major pool of Mo was in the external dialysis media, confirming the low molecular weight of the Mo species. Both samples extracted with distilled water (**Table 1**) were also dialyzed against water.

Diethylaminoethyl cellulose (DEAE, Whatman, DE-32, microgranular form, 100 g) was precycled with 0.5 N HCl, washed with water until neutral, and then with 0.5 N NaOH and water again. The resin was converted to the acetate form by treatment with 10% acetic acid

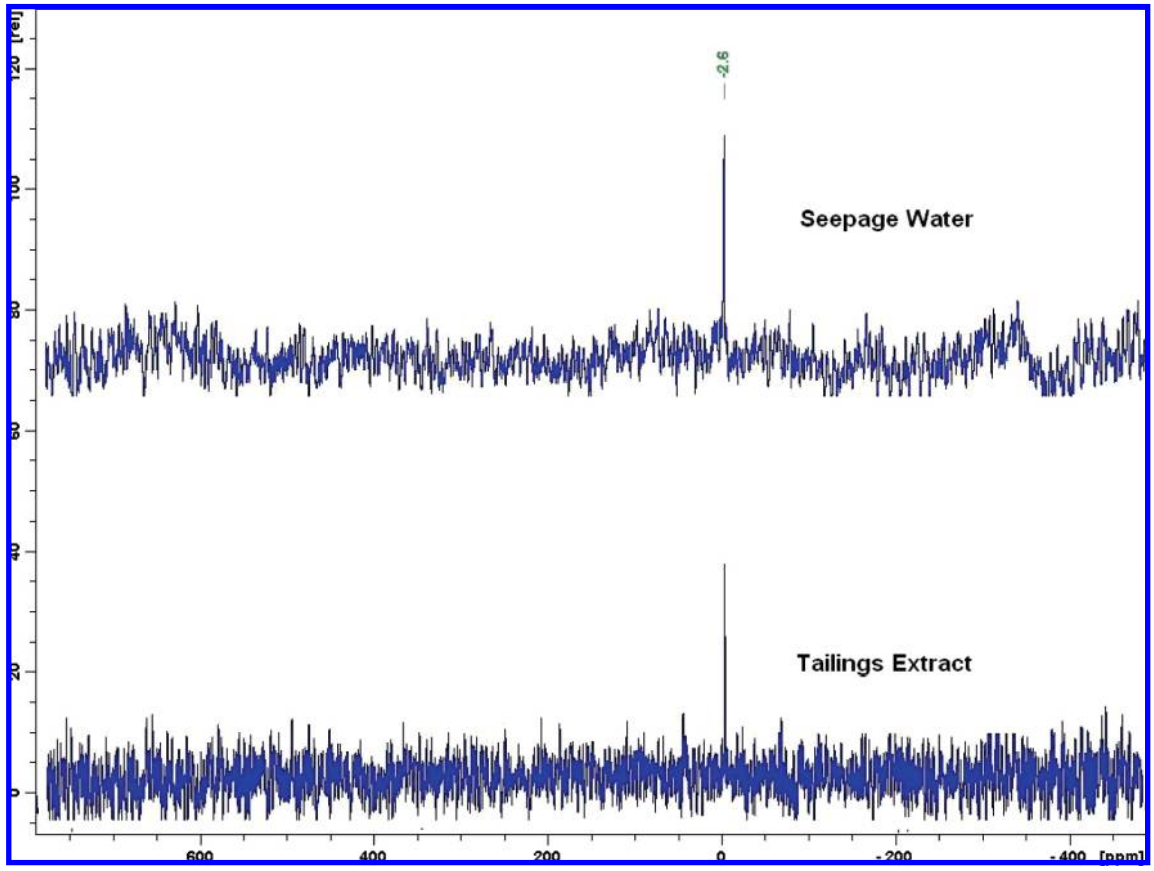

Figure 1. ^{95}Mo NMR chemical shifts confirming the presence of the molybdate anion (−2.6 ppm) in seepage water (66 398 scans) and tailings extract (38 690 scans).

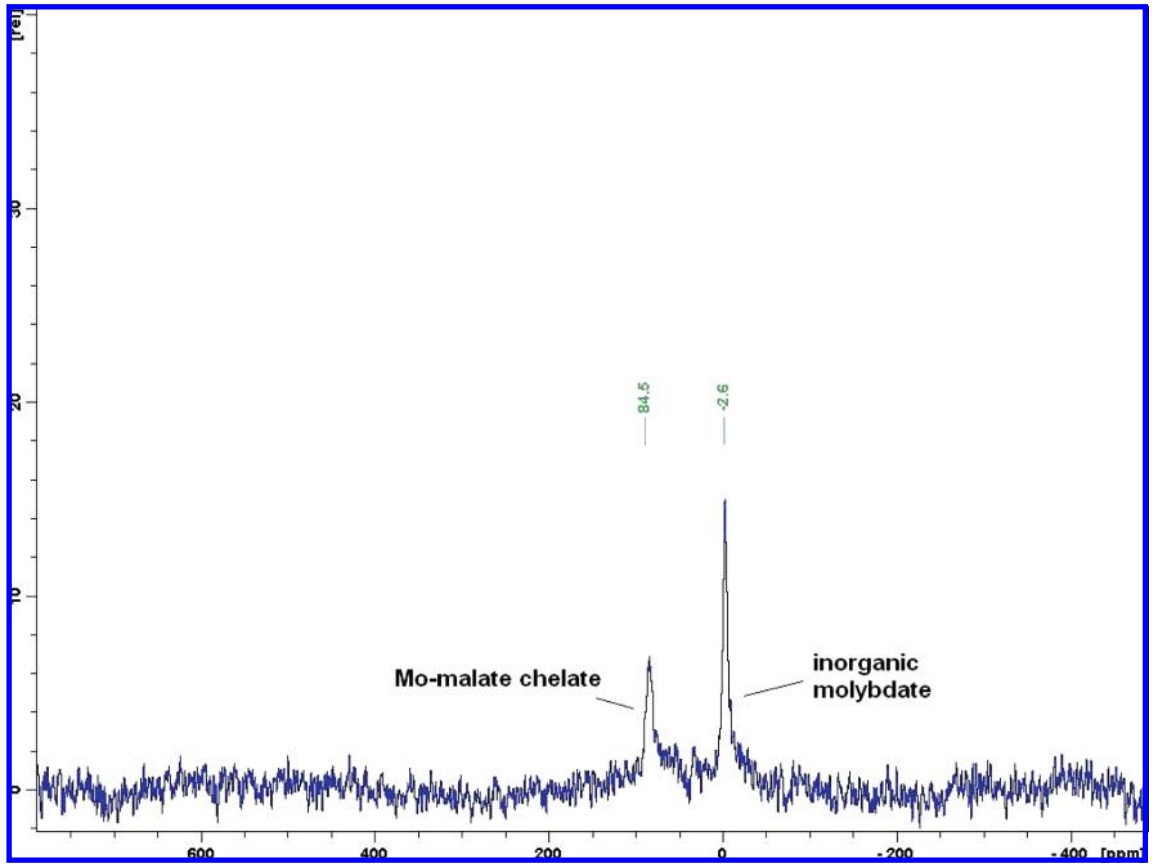

Figure 2. ^{95}Mo NMR chemical shifts in a mixture of the molybdate anion (−2.6 ppm) and the synthetic molybdenum-malate chelate (84.5 ppm, 9166 scans).

and rinsed with water to remove excess acid. A DEAE slurry in water was combined with the dialysis media and suspended in 100 mL of water with continuous stirring for 30 min. The mixture was filtered on a Büchner funnel and washed with water. The gel was treated with 6 × 1 L of 0.5 M NaCl to elute the Mo species, and the eluant was concentrated.

Desalting of the eluant was achieved with Sephadex G-10 (Sigma), which was equilibrated in water overnight and decanted into a column (37.5 cm × 4.5 cm). The DEAE fraction was suspended in water (150 mL), and 25 mL aliquots were chromatographed in water. The fractions rich in Mo were pooled and concentrated. Carboxymethyl cellulose (CMC, Whatman, CM-32, microgranular form, 100 g) could be used as a further cleanup after Sephadex (**Table 1**). The CMC was precycled with 0.5 N HCl and washed with water until neutral. A CMC slurry in water was combined with the Sephadex fractions dissolved in 100 mL of water with continuous stirring for 30 min. The resin was washed with 3 × 1 L of distilled water, and the eluant was concentrated to dryness. Colored components remained on the gel.

Prior to HPLC, fines in the eluant were progressively removed on Whatman #1 filter paper and then on 0.8, 0.45, and 0.2 μm porosity filters (Millipore) under vacuum. The filtrate, in 6 mL of water, was injected in 500 μL increments. Preparative HPLC of the Mo containing fraction was adapted from Surridge et al. (*6*) using a PRP X-100 (250 mm × 21.5 mm) column connected to a Varian 9012 pump equipped with a Varian 2550 variable λ detector and a model 704 Varian fraction collector. The mobile phase consisted of 100 mM NH_4OAc/CH_3CN, 98:2 (A) and distilled water (B) with the following stepwise elution: 100% B for 2 min; 100% A for 140 min; 100% B for 10 min at a flow rate of 8.0 mL/min with detection at 235 nm. The Mo-positive fractions, eluted with (A), were pooled and concentrated.

Spectroscopic Studies. The optimized instrument calibrations for ^{95}Mo NMR spectroscopy were described by Surridge et al. (*6*). It was feasible to obtain these spectra with isolates that were not pure because of the specificity of the Mo signal. ^{95}Mo NMR spectra were obtained at 26.07 MHz with a Bruker AM-400 spectrometer in 99.9% D_2O solutions with 0.1 M Na_2MoO_4 as an external standard set to 0 ppm. The chemical shifts are reported in parts per million, and the line widths at half-height ($\nu_{1/2}$) are in hertz. Sample pH was adjusted with 0.1 N NaOD and measured directly in 5 mm NMR tubes with a Corning 140 pH meter equipped with a capillary probe. Signals were not discerned by ^{1}H and ^{13}C NMR spectroscopy owing to the presence of impurities in the isolates.

Preparation of $Na_2[MoO_2(malate)_2]\cdot 5H_2O$. The synthetic chelate was prepared according to the procedure for the cesium salt (*12*) with the following modifications. The sodium salt was prepared by combining 3.60 g of molybdic acid (H_2MoO_4), 2.12 g of Na_2CO_3, and 5.36 g of L-malic acid, each dissolved in 20 mL of distilled water, and gently heating the mixture until all cloudiness was gone. Slow evaporation at 4 °C yielded fine crystals suitable for X-ray analysis. Yield: 8.0 g. ^{95}Mo NMR: 85.4 ppm ($\upsilon_{1/2}$ = 113 Hz). Migration on cellulose TLC was nil. The citrate chelate of Mo was prepared by the same procedure, but the product was amorphous (^{95}Mo NMR: 71.2 ppm, $\upsilon_{1/2}$ = 113 Hz).

X-ray Structure Determination. A colorless prismatic crystal of $C_8H_{18}MoNa_2O_{17}$ was coated with Paratone 8277 oil (Exxon) and mounted on a glass fiber. All measurements were made on a Nonius KappaCCD diffractometer with graphite monochromated Mo Kα radiation. The data were collected at 173 K using the ω and φ scans to a maximum θ value of 30.0°. The data were corrected for Lorentz and polarization effects and for absorption using the multiscan method.

The structure was solved by the direct methods and expanded using Fourier techniques. The non-hydrogen atoms were refined anisotropically. Hydrogen atoms were included at geometrically idealized positions and were not refined. The final cycle of full-matrix least-squares refinement using SHELXL97 converged with unweighted and weighted agreement factors, R = 0.032 and R_w = 0.075 (all data), respectively, and goodness of fit, S = 1.02. The weighting scheme was based on counting statistics, and the final difference Fourier map was essentially featureless.

Crystal Data. $C_8H_{18}MoNa_2O_{17}$, formula weight = 528.14, monoclinic, $P2_1$, a = 10.198(2), b = 7.798(2), c = 11.889(3) Å, β =

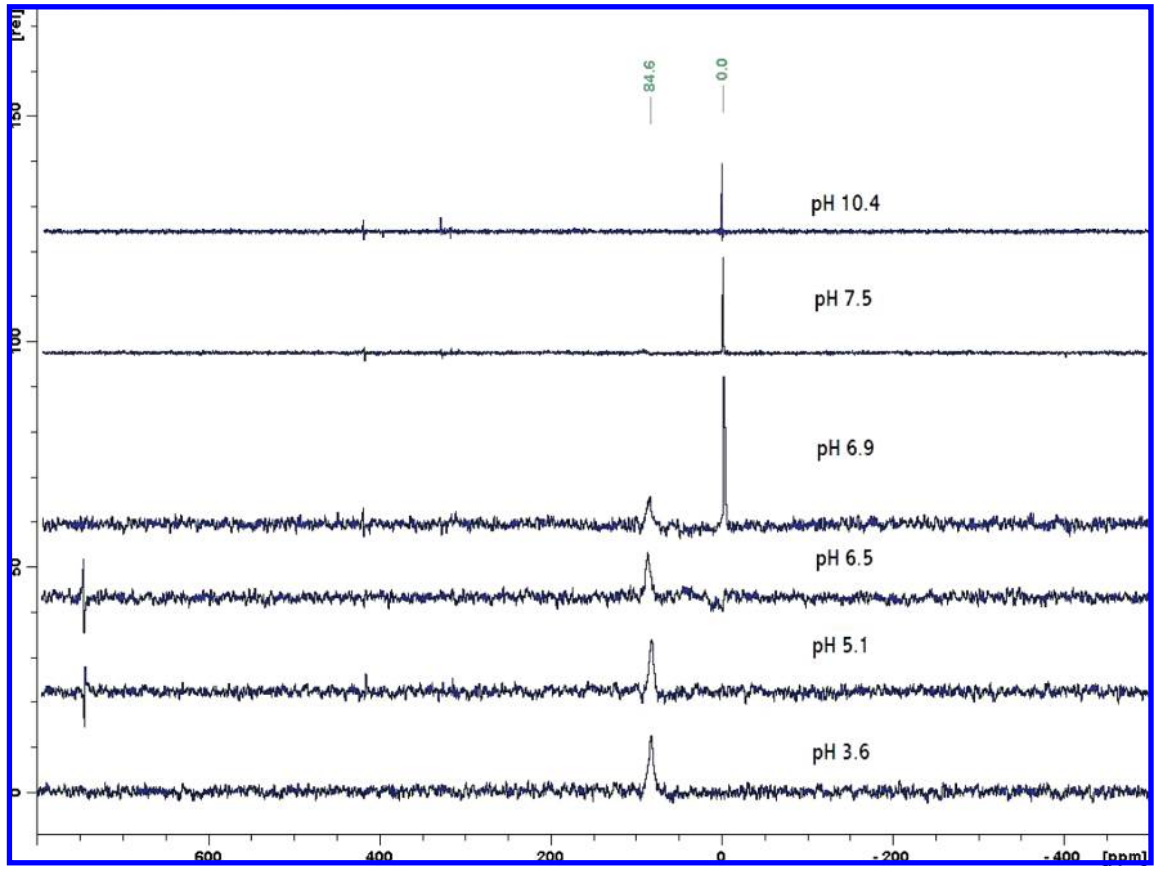

Figure 3. ^{95}Mo NMR spectral shifts showing the dissociation of the synthetic molybdenum-malate chelate as a function of pH (30 000 scans).

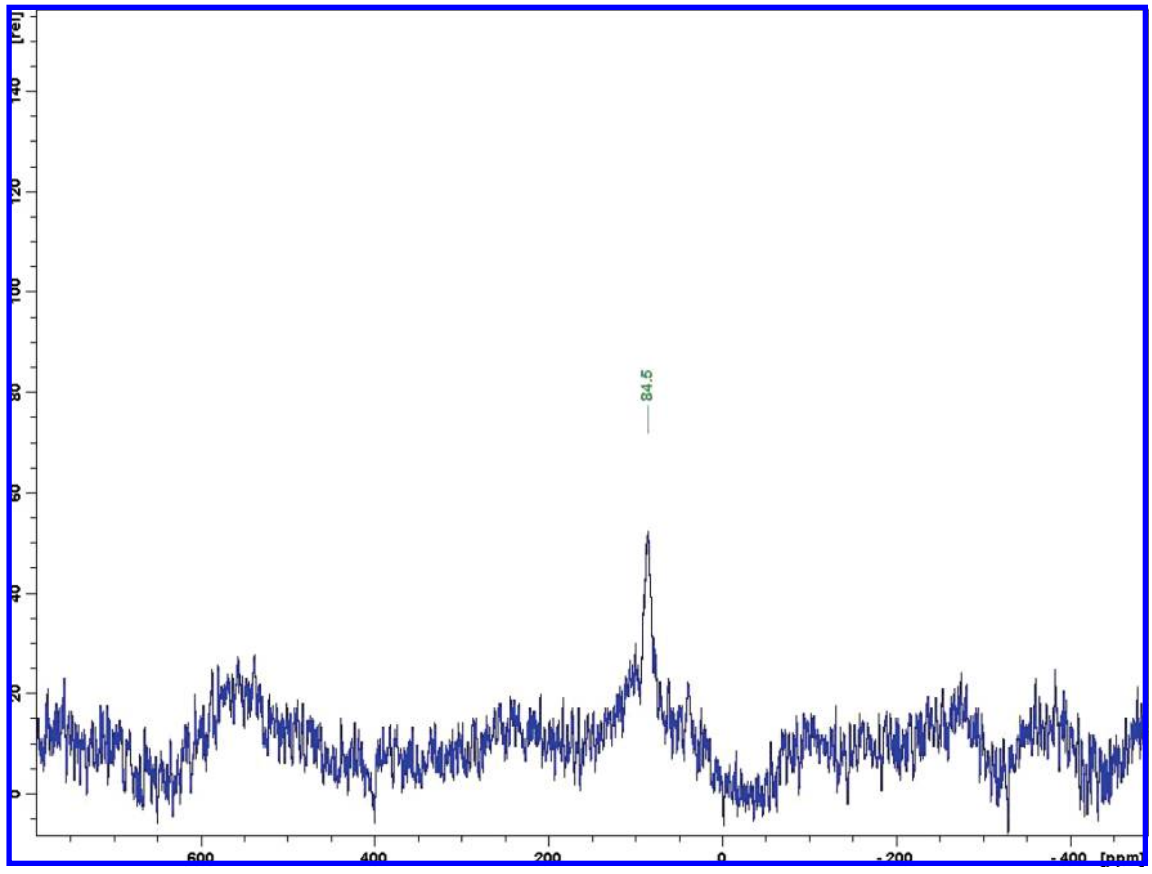

Figure 4. ^{95}Mo NMR spectrum of molybdenum-malate chelate (isolate 2) in alfalfa (754 733 scans).

103.567(10)°, $V = 919.1(4)$ Å^3, $Z = 2$, $D_{calcd} = 1.908$ mg/m^3, Mo Kα radiation ($\lambda = 0.71073$ Å), $\mu = 0.846$ mm^{-1}, $F(000) = 532$, crystal size $= 0.14 \times 0.12 \times 0.06$ mm^3, θ range for data collection $=$ 1.76−30.01°. Independent reflections = 5105 [R(int) = 0.00], maximum

and minimum transmission = 0.951 and 0.891. Absolute structure parameter = −0.03(3) and largest diff. peak and hole = 0.453 and −0.674 e·Å^{-3}.

RESULTS AND DISCUSSION

Crystal Structure of $Na_2[MoO_2(malate)_2]\cdot 5H_2O$. The asymmetric unit contains a central *cis*-oxohydrated MoO_2 core wherein Mo is coordinated to two malato ligands that are further bonded to Na atoms; three water molecules are coordinated to Na atoms, and two water molecules are present in the lattice, as water of hydration. The structure of the Na salt is consistent with that of the cesium salt complex of malate, as reported by Knobler et al. (*12*).

Speciation of Mo in Seepage Water and Tailings. Molybdenum species were determined in seepage water and aqueous extracts of tailings to characterize the form of Mo that could be imbibed by the plant cover at the site. Levels of Mo in aqueous media at the site were estimated to be 7.5 ppm ($n = 3$, SD = 2.5), in agreement with previous estimates of 8.7 ppm Mo (*3*). Direct ^{95}Mo NMR analysis of the tailings and water samples demonstrated that the species of Mo available to the plants from the environment was the inorganic molybdate anion (**Figure 1**), which has a chemical shift near 0 ppm (**Figure 2**). Soil pH ranged from 7.7 to 8.4 while the pH of the seepage water was 8.0. The pH of both matrices was sufficient to maintain the integrity of the Mo anion (MoO_4^{2-}) before absorption by plant root systems. Polymerization of MoO_4^{2-} can occur at pH < 6 (*13*).

Spectroscopic Studies on the Synthetic Mo Chelate. ^{95}Mo NMR spectroscopy readily distinguished the chemical shift of inorganic MoO_4^{2-} from the synthetic Mo-malate chelate (**Figure 2**). As well, the signal for the Mo-citrate chelate (70.1 ppm) was also well resolved in the system (spectral data not shown). The NMR chemical shifts of the synthetic Mo-malate chelate were examined as a function of pH (**Figure 3**). The Mo-malate chelate was stable in acid pH but reverted to the inorganic molybdate anion at pH > 7. It should be noted that the vacuole in plant cells, the storage organelle for heavy metals (*7*), is considered to be slightly acidic (*14*), which would maintain the integrity of the Mo chelate. Previously, the identification of molybdate from alfalfa was reported on the basis of ^{95}Mo NMR identification at pH 9 (*6*), which could have resulted in the dissociation of the chelate, as demonstrated in **Figure 3**.

Isolation of the Molybdenum-Malate Chelate from Alfalfa. Extracts from the plants at Highmont were initially examined by TLC for the presence of α-hydroxy organic acids, specifically malate and citrate. Their abundance in plant tissue was qualitatively confirmed with the specific spray reagent and by cochromatography with authentic standards on TLC. The total levels of Mo in alfalfa ranged from 201 to 433 ppm Mo (*2, 3*).

The Mo chelate, $[MoO_2(malate)_2]^{2-}$, was isolated four times from samples of fresh alfalfa and once from oven-dried alfalfa to simulate hay (**Table 1**). The ^{95}Mo NMR spectrum (**Figure 4**) shown for isolate 2 is in complete agreement with the NMR signal derived from the synthetic chelate (**Figure 2**) as were the signals from the other isolates. The $(NH_4)_2CO_3$ solvent was used to extract the plant material because the yields of Mo with water were lower (**Table 1**). However, alfalfa samples that were extracted with water and dialyzed in water, pH 6, (**Table 1**) yielded the same Mo end product as the $(NH_4)_2CO_3$ extraction at pH 8, which indicated that the slight alkalinity at the initial crude extraction phase was not causing dissociation. As well, oven drying the alfalfa to simulate hay did not appear to disrupt Mo chelate formation, which suggested its relative stability to moderate heat. Yields of Mo from the isolates ranged from 1.2 to 7.1 mg (**Table 1**), in agreement with that reported earlier by Surridge et al. (*6*).

The inorganic molybdate anion was not detected in plant tissue in our studies nor was it lost in the extraction protocol because every soluble fraction was assayed for Mo as was the insoluble residue. The latter may contain a bound, unavailable form of Mo (*6*). The earlier study detected the NMR signal of the anion only after adjusting the pH of the isolate to alkalinity. At pH 5.6, Surridge et al. (*6*) did not detect any signal, which is perplexing. The impact of pH on the isolation of the Mo chelate is critical in this study and a slight elevation in pH of purified samples to alkaline conditions could alter the end products of isolation. The number of attempted isolations of the Mo anion from alfalfa was not reported in the earlier work, and therefore, configuration of the Mo species was apparently not corroborated in that report. In contrast, our isolates were obtained from five sources of plant material over a period of 6 years (**Table 1**), and the results were consistent. The earlier authors also concluded that the occurrence of the Mo anion did not explain the apparent absence of long-term clinical signs in grazing livestock at Highmont. In contrast, we conclude that Mo detoxification was achieved through chelate formation in plant tissue, which would reduce direct intake of inorganic MoO_4^{2-} by grazing livestock at Highmont. The chelate could be stable in the rumen environment and could possibly be excreted intact from the gastrointestinal tract (*2*).

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