UNIVERSITY OF CALGARY

Purification and Characterization of Mo species from Medicago sativa grown on Mine Tailings

by

Blair Surridge

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ABSTRACT

Extraction, purification, and identification procedures were developed for the chemical investigation of Mo in freeze-dried and ground aerial portions of alfalfa (*Medicago sativa* L.) collected in August 1996 from the Highmont site at Highland Valley Copper (HVC). The purification procedures were guided by Mo ICP and dithiol analyses. They included developing an efficient aqueous extraction protocol then sample cleanup by partitioning against n-butanol and filtration through celite. Further purification by anion exchange chromatography using DE-32 and desalting by chromatography over Sephadex G-10. Final purification of the Mo containing fraction was carried out using preparative anion exchange HPLC. Molybdenum was found to be present in its purified form as the MoO_4^{2-} anion based primarily on multinuclear {^{1}H, ^{13}C, ^{95}Mo} NMR studies.

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TO MY WIFE, CHRISTINE

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CHAPTER 1. INTRODUCTION

1.1 Highland Valley Copper (HVC) and Reclamation

Highland Valley Copper[1] is located in south central British Columbia approximately one hour travelling distance from Kamloops (75 km). Highland Valley Copper is a partnership between Cominco Ltd. (50%), Rio Algom (33.6%), Teck Corporation (11.4%) and Highmont Mining Company (5%). Approximately 215,000 tons of rocks are mined daily with the ore averaging 0.42% Cu and 0.008 % Mo . After crushing, approximately 133, 000 tons of ore are processed daily producing 1,255 tons of concentrate containing 40% copper (Cu). Recovery for the mining process ranges between 87 and 93% for Cu. The copper concentrate is sold to smelters in Canada, Japan, and other Pacific-rim buyers. The mine also produces molybdenum (Mo) concentrate, yielding approximately 2000 tons of Mo annually in the concentrate. Process recovery for Mo averages 55 %. Generally the higher the metal content the more efficient the extraction process. Being one of the worlds largest open pit copper mines, reclamation of disturbed lands, 5,900 hectares (see Figure 1.1), costs over 2 M annually. It is the residual mineral content from the extraction process, which is of concern for reclamation. The planned primary uses of the reclaimed land site are: tree farming in cooperation with the provincial forestry service, grazing of cattle and wild life, irrigation and hay production on flat lands, and recreational land for public use.

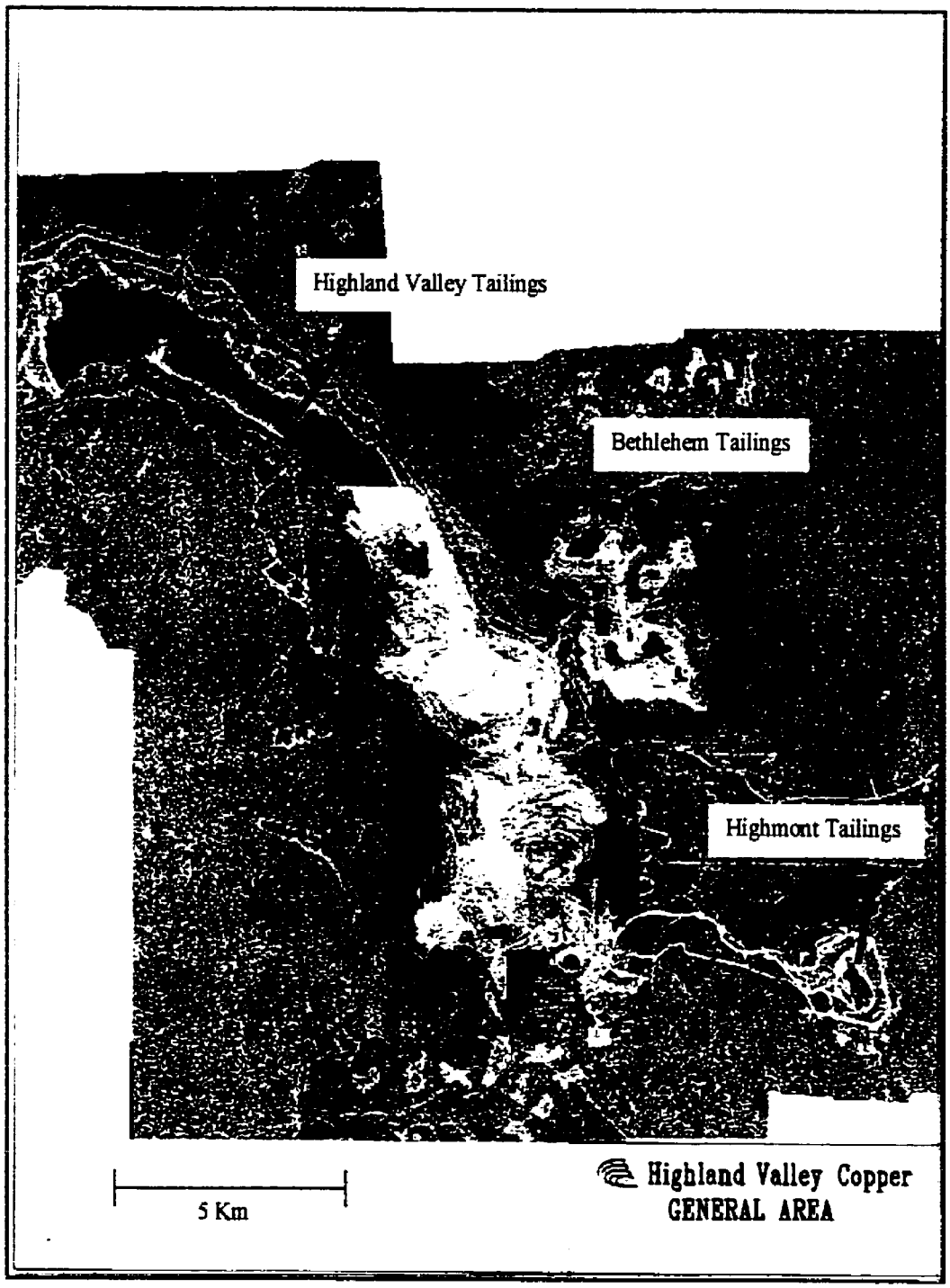


Figure 1.1 Map of Highland Valley Copper Mine

The HVC reclamation program[1] largely consists of establishing and testing of various plant species, planting schedules and frequencies, and fertilizer selections and applications on diverse site conditions at HVC. However, the established[2] vegetation (see Figure 1.2) on the various sites contains[3] elevated levels of residual Mo and Cu (see Figure 1.3 for typical Cu and Mo values on the Highmont site-Refer

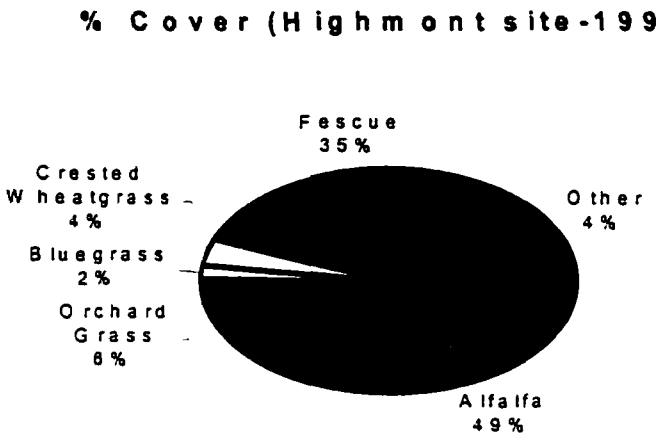


Figure 1.2 Plant speciation found on Highmont site

to Figure 1.1). Close monitoring of the Cu and Mo levels has established the Highmont site as a primary concern, since the Mo levels are the highest in the area. In grazing animals elevated Mo and S levels are important dietary factors that can reduce absorption or availability of Cu. Current theory[4] suggests Mo can interact with Cu in ruminant systems through the formation of thiomolybdates ($MoO_{4-n}S_n^{2-}$) to form unabsorbable Cu thiomolybdate complexes (Cu-TM), thus leading to secondary Cu deficiency. This topic will be discussed later in more detail. The 1984 National Research Council guidelines recommend a maximum level of 5-6 ppm Mo in feed for beef cattle in recognition of Mo as an antagonist of dietary Cu.

It has also been established[5] that a Cu: Mo ratio of 2:1 should be maintained to avoid a Cu deficiency in grazing ruminants.

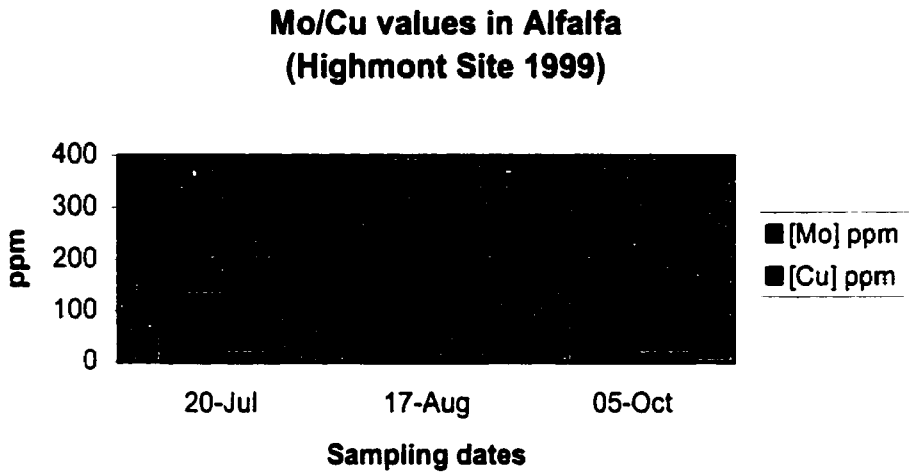


Figure 1.3. Mo Levels in Forages

To address these concerns more recent reclamation studies have included grazing trials to determine the effects of elevated levels of Mo on animal health and performance. Studies were initially carried out in a three year grazing study at the Bethlehem tailings pond[6], where Mo concentrations in alfalfa feed are moderately elevated (21 ppm to 44 ppm), and more recently at Highmont tailings pond[3] (125 ppm to 455 ppm). The Bethlehem site averaged Cu: Mo ratios of 43:100 compared to 12 : 100 to 5 : 100 for Highmont site alfalfa, which clearly falls below the recommended minimum of 2:1. Field studies[6] at Bethlehem indicated no clinical signs of molybdenosis or other adverse effects on animal performance, even though the Mo levels are up to one hundred times higher than the guideline figure. The results of the grazing studies, which will be discussed in more detail later, prompted a chemical investigation of the form of the Mo as it occurs in alfalfa (*Medicago sativa* L.) and will be the focus of this thesis report.

Alfalfa, as a legume, is a known accumulator of molybdenum[7] and is one of the more predominant plant species at the Highmont site (Figure 1.2) and therefore

was selected for chemical investigation. The Highland tailings pond (Figure 1.1) represents the largest area at HVC for reclamation, therefore research conducted at the smaller sites should yield management strategies for future reclamation of mining sites with high Mo-containing forage.

A possible explanation for the observed lack of Mo toxicity in grazing livestock was hypothesized to be the result of an organometallic Mo plant metabolite, which survives the digestive processes and alters the biochemical behavior of Mo in ruminants. A discussion of Molybdenosis is required at this point to provide an understanding of Mo toxicity in ruminants and to review current theories and work in this area. Also, Mo is involved in several primary metabolic functions in plants and can take on several chemical forms[8]. General aspects in this area will be reviewed as well.

1.2 Molybdenosis a Cu Deficiency

1.2.1 Biochemical Aspects

Molybdenosis[9], which has also been termed teart, is a form of Mo toxicity producing symptoms similar to copper-deficiency or hypocupraemia and is therefore termed a secondary copper deficiency. Clinically, molybdenosis is characterized[9] by severe diarrhea, weight loss, joint abnormalities, anemia, osteoporosis, hair discoloration, reduced sexual activity, and death. Ruminants are considered to be the most sensitive to this disorder as the mechanism of action has been shown to involve a three way Mo-Cu-S interaction[10]. It was proposed that Cu, Mo, and S combine in the rumen to form an unabsorbable complex, possibly copper-tetrathiomolybdate ($CuMoS_4$), thus depleting tissues of Cu. It should be noted that the difference between ruminants and non-ruminants is due to the anaerobic reducing conditions, which are required for the formation of thiomolybdates (TM's). A more recent review[11] of the Mo-Cu-S interaction describes both qualitative and quantitative evidence for the formation of TM's, which has

been found to be a key chemical species in the Mo-Cu antagonism. Price[12] reported that approximately 70% of administered $^{99}MoO_4^{2-}$ was converted to tri and tetrathiomolybdates, which were bound to the solid phase of rumen contents. The current TM theory[11] is nutritionally valid on the basis of studies involving the influence of Mo and S on the Cu status in the animal, but an exact cause and effect relationship remains to be established.

As mentioned above, Mo entering the ruminant system as the MoO_4^{2-} anion is subseptable to displacement reactions with S^{2-} leading to thiomolybdates ($MoO_{4-n}S_n^{2-}$), as shown below in Figure 1.4. The levels of S^{2-} and MoO_4^{2-} present are very important and will control where the equilibrium will lie and what the predominant species will be in the rumen.

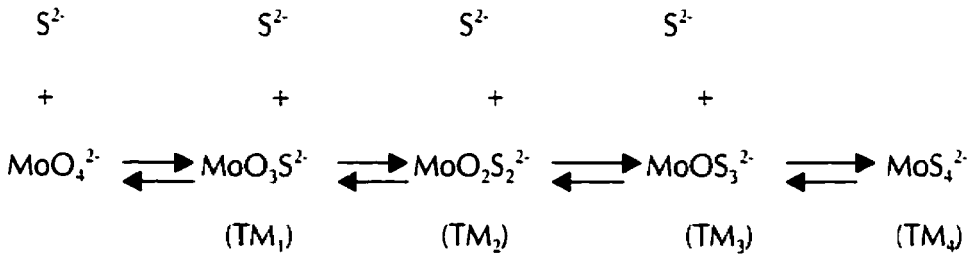


Figure 1.4 Possible TM species

Sulfur's (S) participation as sulfide (S^{2-}) in ruminants can occur[10] from the reduction of both inorganic and organic sulfur compounds to the S^{2-} anion by anaerobic rumen bacteria.

Treating Mo-induced disorders[11] in grazing ruminants usually involves removing animals from feed in acute cases. Chronically affected animals with characteristic hair, fleece, bone abnormalities, and impaired growth will respond well to dietary Cu supplements, oral Cu boluses, or Cu injections. Other strategies[11], that are aimed at the prevention of molybdenosis, attempt to restrict the uptake of Mo in pastures. Keeping soil pH low will decrease Mo uptake, but more successful is sulfate fertilization, which antagonizes Mo uptake in plants. Cereals

take up less Mo than grasses, therefore forage type also plays a role in the management of Mo rich soils.

Treating disorders of excess Cu should also be mentioned, as the Cu x S x Mo interaction has been successfully exploited in the treatment and prevention of Cu poisoning in sheep[11]and humans[13]. In humans, ammonium tetrathiomolybdate is an experimental chelating agent for conditions such as Wilson's disease that cause elevated concentrations of Cu.

Little data at this time is available on human molybdenum related toxicity. High concentrations of molybdenum may irritate the upper respiratory tract as shown by studies on the biochemical abnormalities in workers exposed to Mo dust in a molybdenite roasting plant[14]. However, lack of control groups and adequate follow up limited the establishment of causation.

1.2.2 Bioinorganic Aspects

Inorganic chemists have also studied Cu-Mo systems in search of explanations to the Mo-Cu antagonism. Early biological studies were concerned with administration of Cu, Mo, and S compounds to ruminant and monogastric animals and then speculating on the mode of interaction on the basis of the concentration of these elements in various tissues. Attempts to understand the chemistry have only been recently[15] addressed. A review of synthetic and structural aspects of the Mo-Cu-S system follows.

In ruminants, evidence for the formation of MoS_4^{2-} *in vivo* has been made by identification of the characteristic absorption spectra in the UV/visible region[16] (λ_1- 467 nm and λ_2- 318 nm for synthetic complexes[17]). In non-ruminants the accumulation of S^{2-}, which is required for the formation of TM's, has not been found[18]. The observation that ruminants respond to preformed MoS_4^{2-} in the same way as they do to dietary salts of MoO_4^{2-} + SO_4^{2-} further supports the idea of a MoS_4^{2-} metabolite in the rumen.

Synthetic studies[15] with Mo and tungsten (W) have shown Cu^{2+} to form several complexes with S-containing ligands, where the oxidation state of copper is retained. It has also been shown[19] that various thiometallates of the type $MO_{4-n}S_n^{2-}$ (where M = Mo, W), react with cupric ion through a reduction to the cuprous state. The same study also reports that the reaction proceeds with quantitative formation of a insoluble polymeric solid containing a 1.5:1 Cu:Mo stoichiometric ratio. The measurements were made using elemental and X-ray powder diffraction analysis. It should also be noted that a less facile pathway[19] involves the direct reaction with S^{2-}, precipitating CuS. The reduction of Cu^{2+} to Cu^{+} in the above mentioned processes are biologically relevant as it is believed[20] that the metabolic functions involving copper require the Cu^{+} state. Non-aqueous chemistry of Cu-Mo-S systems has also been studied[21,22] in an attempt to block the polymerization reaction, however this chemistry will not be discussed further.

1.2.3 Grazing Trials at HVC

As was mentioned earlier, monitoring of mineral levels in forage grown on mine tailings has caused concern for the intended land use after mine closure. In response, a three-year grazing study[5] was conducted at the Bethlehem site over a twelve-week period per year. The primary objective was to determine if cattle could safely graze the forage on the reclaimed site. Other objectives included feeding high Mo forage preserved as hay to calves to determine if high Mo forage affected carcass meat quality. In addition, a one-year water quality study was carried out where animals were given mine seepage water with fresh forage over the twelve-week period. No adverse effects on animal health and performance were observed throughout the grazing trial. Throughout each grazing period weight, blood, milk, liver, and various forage samples were analyzed for selected minerals and found to be within the normal range.

Extreme Mo forage levels at HVC are found at the Highmont tailings site (130-330 ppm in alfalfa). Following the above study[5] a grazing trial was initiated at the Highmont site. The primary objective remained the same. Results[2] to date again have indicated no long-term adverse effects on animal health and performance, but there were some short-term abnormalities, which will be the topic of further study.

1.3 Mo Metabolism in Plants

Molybdenum is considered a micronutrient in plants[8] and is required at levels lower than any other known micronutrient. For example, the average concentration in normal growing alfalfa was found on average to be 0.23 mg/kg DM (dry matter) [23]. Physiologically Mo is required at levels less than 1 ppm in the DM. Mo deficiency in plants can result from the following soil conditions[24]: i) low total Mo; ii) where Mo is sequestered by oxyhydroxides; iii) in extensively weathered soils; iv) soils with pH values below 6; and v) in well drained sandy soils. On the other hand, molybdenum toxicity in plants is rare, but has been induced under extreme experimental conditions[25].

The physiological function of Mo in plants is known[8] to involve three enzyme systems, which catalyze various redox reactions by altering the oxidation state of Mo. The enzymes include: (i) nitrate reductase (NR), which catalyses the first step in nitrate assimilation; (ii) xanthine dehydrogenase (XDH), which involves purine catabolism; (iii) aldehyde oxidase (AO), which is involved in various oxidative processes (for a recent review see ref. 26). Molybdenum becomes biologically active when it binds the unique dinucleotide cofactor, pterin or molybdopterin, to give the molybdenum cofactor called Moco (see Figure 1.5.)

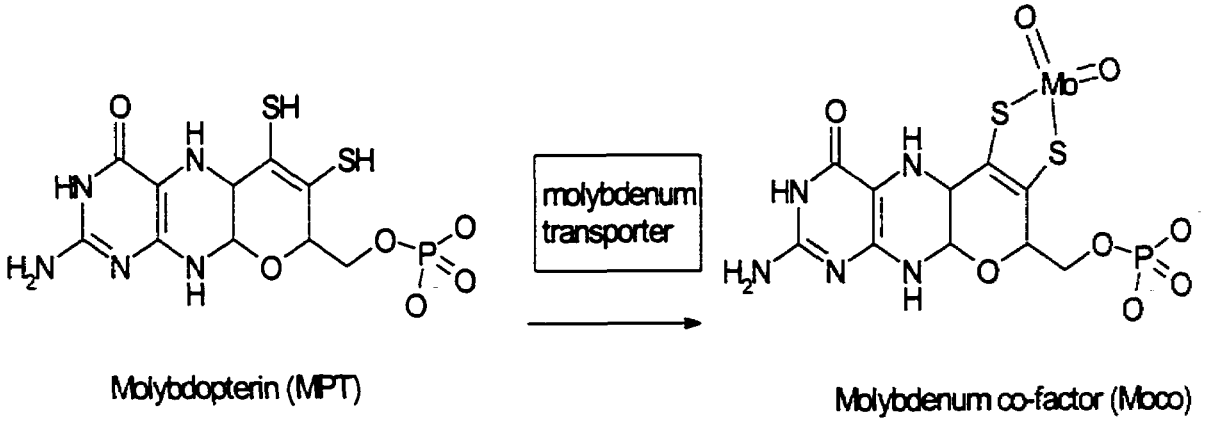
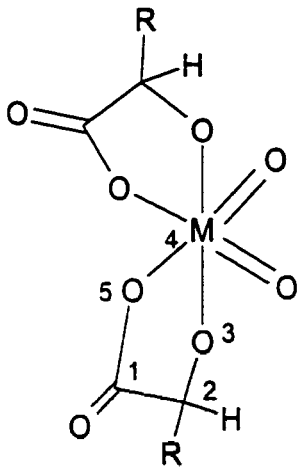


Figure 1.5 Molybdenum Co-factor (Moco)

Molybdenum-containing enzymes are very large and complex and it is notable that they have multiple units and other cofactors besides Mo. There are several plant species that are host to bacteria capable of nitrogen fixation. This plant-microbe symbiosis involves a Fe-Mo co-factor, that activates the enzyme nitrogenase for the reduction of N_2 to NH_3. In this case Mo plays a structural role[27] in the complex. Investigation of the function and role of Mo in plants is currently focusing on Mo-cofactor biosynthesis, where the emphasis is on characterizing the structure and function of regulatory enzymes.

1.4 Bioinorganic complexes of Molybdenum

The coordination chemistry of Mo(VI) in aqueous solutions has been studied in a variety of systems. The ligands have biological relevance and have included organic acids[28a,b,c], carbohydrates[29 a,b,c,d], and catecholates[30]. The ligands all have



M = Mo, W, etc.
R = H (glycolic acid)
= various side chains

Figure 1.6 General structure for 1:2 metal : ligand polyhydroxyl complex

vicinal hydroxyl functionality enabling the formation of stable five membered chelate rings (Figure 1.6). These studies also report Tungsten (VI) forming similar chelates.

In the above mentioned studies, the techniques which have been used to study the structure and properties of these complexes have ranged from the more classic potentiometry, conductimetry, polarimetry, and spectrophometry to the now more common high field multinuclear NMR spectroscopy (i.e. ^{1}H, ^{13}C, ^{95}Mo, and ^{183}

W, 1D and 2D). The latter allows for more complete characterization, as the dominant species in solution depends on pH, concentration and molar ratios. Characterization has included[28b] determining the number, stoichiometry, geometry, and stability of the various complexes. Perhaps most interesting is the use of a Jobs plot[29c]. This technique is based on proton signal intensity, enabling the concentration of the various species to be determined and consequentially, determination of the stoichiometries of the predominant species and their formation constants. Clearly, NMR has played a leading role in investigating the metal complexes. It should be noted that the spin $^5/_2$ ^{95}Mo nucleus is quadrupolar and in some cases[31] gives rise to broad signals, whereas spin ½ ^{183} W produces sharp signals and is therefore often studied in place of Mo.

The above material serves as a quick overview and is not meant to be comprehensive, i.e. this Chapter considers molybdenum issues that are relevant to reclamation at HVC. Specifically, it may be more accurate to say the focus is on the chemistry of Mo as it relates to ruminants consuming forage grown on mine tailings. The aim of this study was to provide a fundamental understanding of this chemistry as an aid to developing management strategies for reclamation programs at HVC and other mines.

1.5 Objectives and Research Plan

After finding no long-term signs of Mo-related toxicity in livestock grazing high Mo forage it was suspected that the form of Mo in the forage was not the free MoO_4^{2-} anion. It was speculated that under the growing conditions on the reclamation area, Mo was complexed or sequestered by an organic acid or sugar, thereby changing the biological activity of Mo in the animal. In addition, a study[32] of the effects of high Mo forage on animal reproduction found no adverse effects and speculation was made as to the chemical form of Mo in the forage. It is recognized that other possible explanations may exist for the observed lack of toxicity. Accordingly, a chemical investigation of the form of the Mo in alfalfa

grown on the Highmont tailing site was carried out and will be described. The research objectives were as follows: 1) to develop a purification protocol adequate for the structural characterization of a potentially labile Mo species 2) to develop a purification protocol, which recovers the Mo as quantitatively as possible, and 3) to characterize the isolated Mo species through spectroscopic analysis.

CHAPTER 2. RESULTS AND DISCUSSION

2.1 Initial extraction protocol

Extraction studies were carried out on freeze-dried and ground aerial portions of alfalfa (*Medicago sativa* L.) collected in August 1996 from the Highmont site at HVC. There is a paucity of information on the extraction and purification of metal-containing secondary metabolites from plants. However, phytochemical studies on several Ni-accumulating plant species report[33] room temperature aqueous extraction of freeze-dried leaf material, which yielded 50 –70 % of the total Ni. There was no mention of other attempted extractions. In the present study, all extraction procedures were also carried out at room temperature unless otherwise stated. Solvents of increasing polarities were tested for efficiency of Mo extraction from plant material (Table 2.1).

Table 2.1 Solvent screening for extraction of Mo, from 1g of alfalfa

Solvent	Volume (mL)	mg Mo	[Mo] (mg/kg PM)
hexanes	100	0.001	1.0
ethanol	100	0.001	1.4
methanol	100	0.026	25.8
H2O (1)	50	0.358	358
H2O (2)	50	0.002	2.3
extracted plant material (residue)		0.069	69.2
total Mo		0.458	457

Extracts were directly subsampled for Mo analysis by ICP. Details of Mo ICP analysis is described in the experimental section. The combined water extracts

contained 79 % of the total Mo in the plant material (PM). The methanol extract contained 6 % of the total Mo. It should be noted that the total amount of Mo in the PM was found to vary between approximately 430 ppm and 550 ppm. This inconsistency was partly attributed to a inhomogenious sample of the ground plant sample.

The Mo species appeared to be polar and very water-soluble. Non-polar solvents such as hexanes and ethanol were unsuitable for use as extracting solvents for the Mo. However, it was thought that defatting the PM with a lipophilic solvent might aid in the subsequent aqueous extraction, allowing for increased wetting and solublization of polar constituents. It was anticipated this could be achieved by prewashing the PM with hexanes. The hexanes wash was followed by washing with ethanol (EtOH) as an intermediate solvent. Prewashing with EtOH may remove some water-soluble interferences, providing a cleaner water extract in the subsequent extraction step. The above experiment was repeated and the aqueous extraction was fractionated in order to determine the minimum volume that was required for extraction of the Mo (Table 2.2). Consistent with the previous experiment, only trace amounts of Mo were found in the hexanes and EtOH washes. The major portion of the Mo was extracted in the initial 40 mL of H_2O and negligible amount of Mo was extracted after 100 mL. A total of 395 ppm Mo was found in the combined H_2O extracts representing 72% of the total Mo. The amount of Mo remaining in the residue was 147 ppm Mo. Upon repeating this experiment this value was found to vary between 100 –150 ppm Mo.

Table 2.2 Solvent extraction sequence for 1 g of alfalfa

Solvent	Volume (mL)	[Mo] (mg/kg PM)
Hexanes (1)	75	1.0
Hexanes (2)	75	0.2
Ethanol (1)	75	1.0
Ethanol (2)	75	1.2
H2O (1)	40	350
H2O (2)	40	33.4
H20 (3)	40	7.8
H20 (4)	40	3.7
PM	—	147
total Mo		546

Attempts were made to access the non-extractable Mo remaining in the residue. An ultrasonic disruption experiment was carried out to determine if the non-extractable Mo was located within plant cell fragments and therefore not accessed by the extracting solvent. One gram of plant material was suspended in 50 mL of H_2O and sonicated for 150 minutes. The suspension was filtered on a Buchner funnel and washed with 50 mL of H_2O. A recovery of 68.7 % Mo was found after Mo-ICP analysis of the plant residue and H_2O extract. Two similar experiments were conducted separately to see if acid or base would access the insoluble Mo. Neither 6 N HCl nor 5 % NH_4OH produced a significant increase in the yield of soluble Mo. More successful was a maceration of the alfalfa during aqueous extraction (see Figure 2.1). After prewashing with hexanes and EtOH the alfalfa was suspended in H_2O and ground in a mortar and pestle with sand. Further

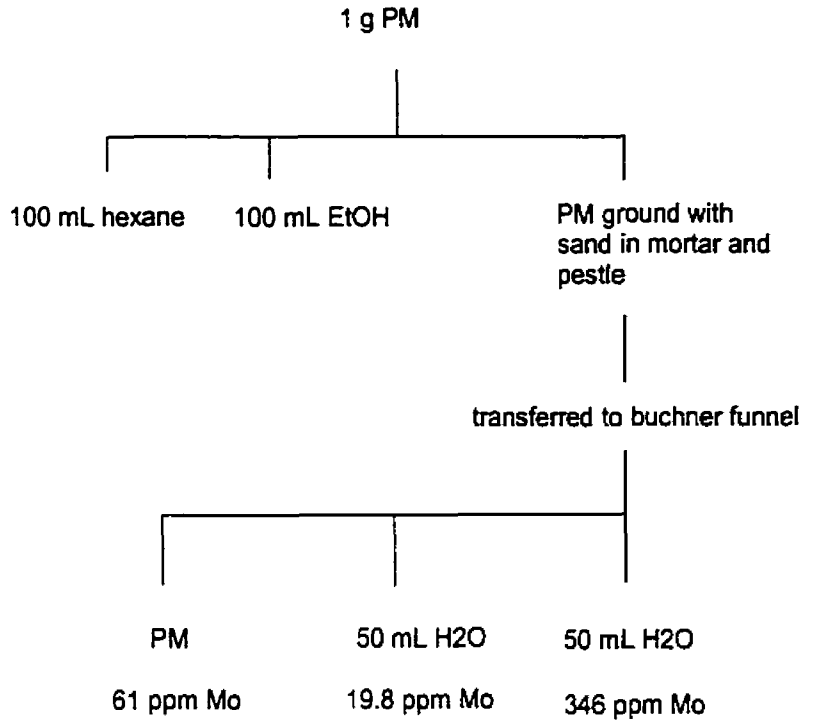


Figure 2.1 Modified extraction protocol for Mo in alfalfa

extraction was then carried out on a Buchner funnel. Mo contents were then determined by ICP. The results obtained were an improvement, but it was concluded that attempts to further optimize the extraction protocol did not yield significant increases in the amount of Mo extracted. It appears that a portion of the Mo is bound and cannot be recovered from the plant sample using the extraction conditions mentioned above.

2.2 Determination of Physical properties for purification

To begin, it should be noted that other investigators studying the effects of high Mo forage in hay on reproduction in cattle reported[32] no signs of molybdenosis, and it was suggested that cattle were more sensitive to molybdenum in fresh vegetation than in hay. It was speculated that Mo might be complexed by a protein, which forms in hay. Accordingly, studies were carried out to determine the molecular weight range of the Mo species as best as possible. This information was then used as a protocol for purification on the basis of molecular weight. The ionic properties were also examined to better understand the nature of the Mo species as it occurs in the plant. Knowledge of the general physical properties of an unknown chemical species are required before purification can be attempted.

2.2.1 Molecular weight determinations

Molecular weight experiments were initially carried out using Sephadex G-10 as a gel filtration media[34]. Sephadex is the registered trademark of dextran gels used for chromatography. G-10 refers to the degree of cross-linking and thus the swelling properties of the gel in H_2O. The molecular weight fractionation range for G-10 is < 700 g/mol, therefore steric effects lead to complete exclusion of molecules > 700 g/mol, while smaller substances will be fractionated and retarded by the gel. Separation within the fractionation range proceeds with increased retention of lower molecular weight species. For a description of the general aspects used in gel filtration refer to the experimental section. In this study 1 g of PM was extracted directly with 100 mL of distilled H_2O and concentrated *in vacuo* at 40°. The residue was redissolved in 2 mL of H_2O and applied to a 1.5 x 18 cm Sephadex G-10 column. The void and exclusion volumes were predetermined with Blue Dextran 2000 (molecular weight of one million) and elution was carried out with H_2O. Repeated experiments found the Mo species occurring in the first fraction immediately following the exclusion volume. Therefore, it seemed likely

that the Mo species has a molecular weight of < 700 g/mol. This was further verified using the modified extraction procedure shown in Figure 2.1. The H_2O extract (0.363 mg Mo, 237.37 mg residue) was concentrated to dryness and redissolved into 2 mL of H_2O and applied to a 47.5 x 1.5 cm Sephadex G-10 column. Elution was carried out with H_20 and fractions were collected as shown in Table 2.3 and analyzed for Mo by ICP. A total of nine fractions were collected. An elution curve was generated from the

Table 2.3 Sephadex G-10 purification of Mo species from 1g alfalfa

Fraction	Fraction volume (mL)	Elution volume (mL)	µg Mo	[Mo] (ug/mL)
Void volume	31	31	0.6	0.0
Exclusion volume	6.1	37.1	6.7	1.1
1	4.6	41.7	59.5	12.9
2	5	46.7	71.5	14.3
3	5.5	52.2	33.4	6.6
4	3.3	55.5	4.4	1.3
5	5.7	61.2	6.5	1.1
6	8	69.2	8.3	1.0
7	5.3	74.5	5.0	0.9
8	4.5	79	4.0	0.9
9	26	105	10.2	0.4

data in Table 2.3 and this is shown in Figure 2.2. It was important to determine the elution profile of the Mo species on G-10 so as to be certain of the molecular weight range.

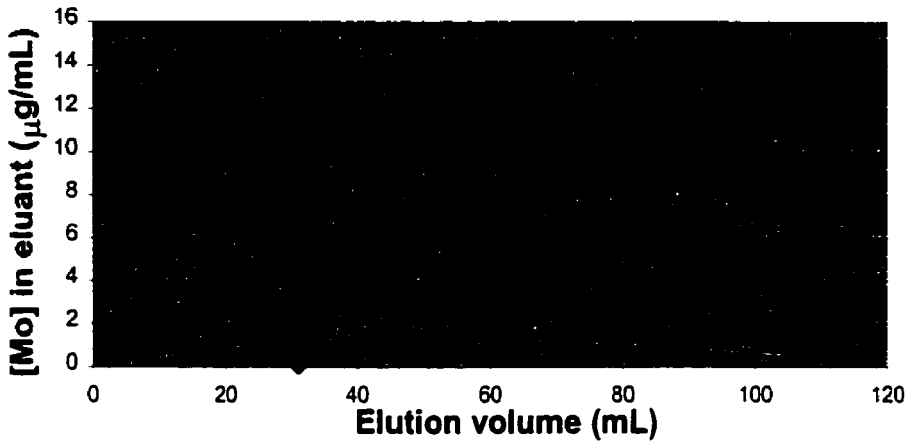


Figure 2.2 Sephadex G-10 elution profile

From the Figure 2.2 profile, it is also appears that the Mo-species chromatographed as a single entity on Sephadex G-10.

Complementary evidence was required to verify the molecular weight range. The above experiment assumes the Mo species to be stable to the extraction and concentration processes prior to the molecular weight determination step. To avoid the concentration process, dialysis seemed a suitable substitute for gel filtration. It is possible to use a crude extract directly in a dialysis experiment without subjecting the sample to heat and ethanol (for formation of an azeotrope as required for concentration *in vacuo*). In addition, it was thought to be important that certain precautions be made if one might be extracting a sensitive high molecular weight proteinaceous Mo species. The four potential hazards recognized[35] when extracting proteins are as follows: 1) the pH of the extract, which if acidic enough could denature a protein-based complex. Extraction with a suitable buffer instead of water will control the pH at neutrality 2) phenolases liberated can react with phenol in the presence of oxygen to give quinones, which may react irreversible with proteins. 3) natural tannins can also cause a similar problem during extraction. 4) proteolytic enzymes can also be a concern in some cases. These potential problems can be avoided by the preparation of an acetone powder[35]. This can be done by macerating the plant tissue in cold acetone (-20°C).

If done with leaf tissue the acetone removes chlorophyll and most of the low molecular weight constituents, including the above-mentioned substances, which can irreversibly bind proteins. The dried powder produced can then be extracted with a buffer. For purposes of a qualitative experiment, 0.5 g of alfalfa was subjected to the above-mentioned procedure. The acetone powder was then extracted with 0.1 M NH_4OAc (pH of 7.5). The extract was not concentrated but transferred directly to a 20 cm Spectrum molecular porous membrane tubing with a molecular cutoff of 3500 g/mol, which was tied at one end. The other end was then tied off and the sausage shaped tubing was then hung from a clamp suspended into the dialysis medium (300 mL of 0.1 mM NH_4OAc) with continuous mixing. The experiment was carried out at 4 °C for 28 hours to ensure equilibrium was achieved. The dialysis medium was then changed to 300 mL of H_2O and again left for 28 hours. The dialysate samples along with the undialysed extract were then concentrated *in vacuo* and the weights recorded. The samples were then subjected to the qualitative visual dithiol Mo assay to locate the Mo. Details of the qualitative Mo dithiol assay is described in the experimental section. The results were quite obvious and are shown in Table 2.4. There was no Mo detected in the undialysed extract, which indicated that the Mo species had a molecular weight of less than 3500 g/mol. These results support earlier evidence obtained by gel filtration chromatography experiments and therefore it is concluded that the Mo exists as a low molecular weight species. It is worth noting that the majority of the sample weight is found in the dialyzed low molecular weight fraction, which is not conducive to eventual purification of a pure molybdenum-containing single

Table 2.4 Molecular weight determination by dialysis

Fraction	Dithiol Mo assay (+) relative [Mo]	Weight (mg)	% Weight
100 mM NH4Oac	+ + + + +	178	70.1
distilled H2O	+ +	29	11.5
undialysised extract	—	45	18
total weight		252	

compound. Given this and the time required to carry out a small-scale experiment, it was decided that dialysis could not be applied effectively for the purification of the Mo species on a large scale.

2.2.2 Ion exchange chromatography

Molybdenum as it is found most commonly in the environment, exists in its highest oxidation state-Mo(VI) as the MoO_4^{2-} oxy anion. Synthetically prepared complexes[28] have been reported to be anionic species in aqueous solutions. Therefore, the ionic properties of the Mo species in alfalfa were screened on cellulose based anion and cation exchange resins DE-32 and CM-32. The resins are described as weak ion exchangers[36] and the active functional groups are shown in Figure 2.3.

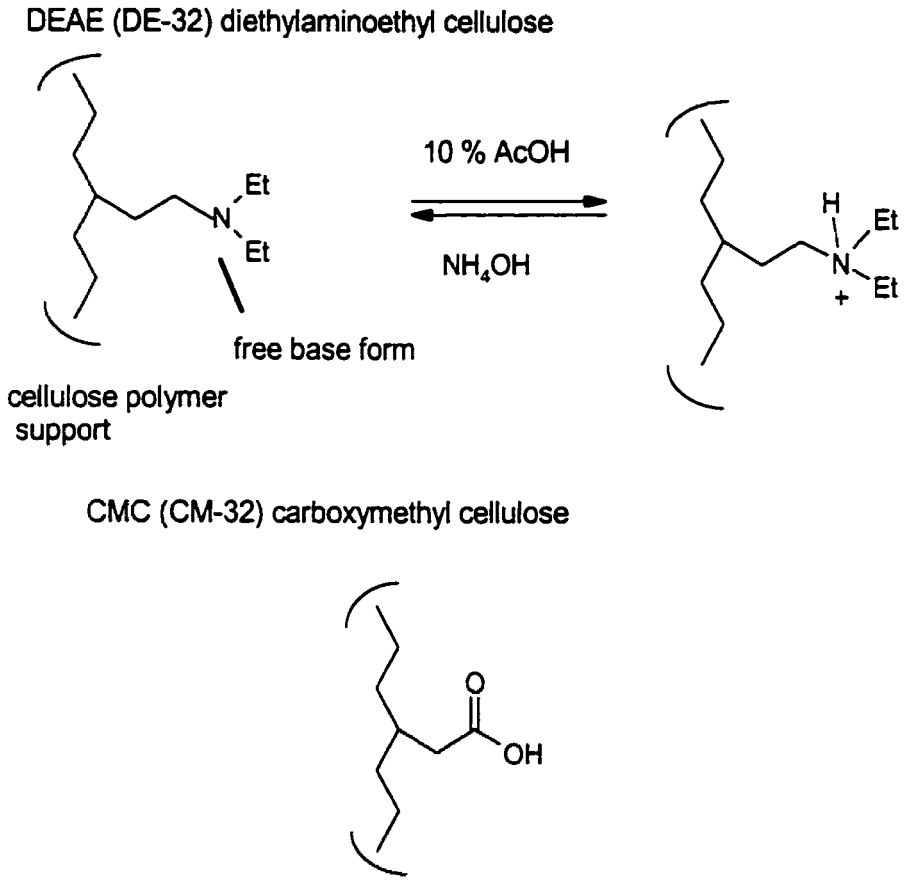


Figure 2.3 Functional sites of ion exchange resins used

Weak anion exchangers[36] typically involve primary, secondary, and tertiary amino functionalities, which may have their ion exchange sites removed by adjustment of the pH of the solution. Therefore, base is a method of eluting as the charge on the exchanger can be removed. Weak anion exchangers do not have as high an affinity for anions as some strong anion exchangers (SAX). SAX utilizes a quaternary amino group, which retains its ion exchange site despite changes in solution pH. The carboxyl group is considered a weak cation exchanger, whereas a strong cation exchanger typically will involve a sulfonate group as the exchange functionality.

The anion resin (Whatman, DE-32, micro granular form) was prepared as outlined in the experimental section. The cation exchange resin (Whatman, CM32, micro granular form) was prepared as follows. An appropriate amount of resin was suspended in 10 % acetic acid, stirred and then washed with water on Buchner funnel until the water was neutral. A previously prepared H_2O extract was applied equally to the cation and anion resin under slight vacuum. The following elution scheme

Table 2.5 Evaluation of ion exchange resins

Resin	Fraction	Dithiol Mo assay
DE-32 (Anion resin)	water wash	-----
	1 % NH4OH	+++++
	sorbent	++
CM-32 (cation resin)	water wash	+++++
	5 % AcOH	++
	sorbent	-----

(Table 2.5) was used for the cation and anion resin. Approximately 100 mL of solvent was used for each of the wash fractions. To summarize, elution of the DE-32 resin with distilled H_2O left the Mo retained on the anion resin. Complementary to this the Mo species was not retained on the CM-32 cation exchange resin and was eluted off with water. This experiment demonstrates the anionic characteristics of the Mo species. However, further experiments were required to optimize the elution of the Mo species from the anion exchange column for purification purposes.

2.3 Method development

It was realized at an early point that the purification of Mo would require a quantitative approach to determine the molybdenum recovery yields at each step. Therefore, attempts were made to obtain a total Mo value by ICP or dithiol analyse (see experimental for details) for major chromatography fractions as often as possible. A qualitative molybdenum dithiol assay was used for quick checks to follow the Mo through a fractionation step. To our knowledge, methods for the purification of low molecular weight molybdenum components from a natural source have not been reported in the literature. A recent study[37] describes inducing the formation of high molecular weight heavy metal-phytochelatin-complexes with Cu and Cd, which could be characterized by HPLC/ICP-MS. Zn and Pb were reported[37] to bind to low molecular weight compounds, which could not be purified adequately for characterization.

To summarize, an adequate aqueous extraction procedure was developed, yielding a crude Mo-containing extract (0.385 mg Mo/360 mg residue derived from 1g of alfalfa). Several purification procedures were evaluated next in attempt to further purify the Mo-containing material for spectroscopic characterization. Work to this end will now be discussed.

2.3.1 Development of cleanup steps

Before a purification procedure can be adopted individual techniques must be tested at each point in the scheme. Chromatography sorbents that were ineffective will be reviewed initially followed by a discussion of the successful chromatography sorbents. An effective sorbent for purification should achieve one of two objectives. Either retain the Mo species, with elution of interfering material, or retain interferences with initial elution of the Mo.

Activated and deactivated forms of coconut charcoal were first tested for their capacity as sorbents for removal of polyphenolic components (indicated by a

yellow color with alkali), but were found to irreversibly bind significant amounts of the Mo species. Another form, Norit decolorizing carbon neutral, was found to retain smaller amounts of the Mo complex. The decolorizing carbon powder (0.75g/g dry PM) was added directly to the H_2O extract and stirred. The mixture was then filtered on a Buchner funnel. If most of the polyphenolics were not removed, then the filtrate was passed on through the bed again or more decolorizing carbon was added. Finally the bed was washed with H_20. This step was reasonably successful in removing the colored material from H_2O extracts except periodic binding of a significant amount of Mo occurred, which could not be desorbed. For these reasons the use of decolorizing carbon as a cleanup step was abandoned. In an attempt to explain this behavior, 50 mL of a 100 ppm Mo Na_2MoO_4 standard in H_2O was treated with 1g of Norit decolorizing carbon. Approximately 78% of the Mo used was retained on the decolorizing carbon after washing with distilled H_2O. It is expected that the high ionic strength of the aqueous extract would influence the degree of Mo binding and cause some variation.

Polyamide resin was tested as an adsorbent for the Mo species and these experiments were carried out as described in the experimental section. As expected organic solvents did not elute the Mo from the polyamide. To obtain reproducible results and complete elution of the Mo, the polyamide was initially deactivated with water containing 5 % EtOH. Partial elution of the Mo species was obtained with 5 % EtOH in H_2O, but aqueous 1 % NH_4OH solution was required for complete elution. However, it was determined that purification of the Mo-containing material was not achieved on polyamide and the method was abandoned.

Various sorbents were screened using TLC (thin layer chromatography). Of the vast array of TLC sorbents commercially available certain requirements were obvious. It was expected that a polar solvent system would be required and would require H_2O as a component. Therefore, the TLC sorbent would require compatibility with H_2O. One can immediately rule out unmodified silica gel.

Solvent systems were tested to see if migration could be achieved using both Na_2MoO_4 standards and purified Mo containing plant extracts. Qualitative applications were tested before preparative separations were attempted. A spray method was developed using the Zn-dithiol Mo assay reagents. The technique required spraying the developed plate with an acid solution (6 N HCl) followed by spraying the Zn-dithiol reagent (preparation given in the experimental section). On cellulose layers the Mo species could be located as a quenching spot by viewing at 254 nm. Table 2.6 highlights some of the solvent systems and commercially available sorbents that were tested. Impetus for some of the three-component solvent systems was found in the literature[38]. The cellulose layer was found to provide the best migration characteristics, which include shape of spot and distance traveled. Therefore, it was thought that preparative cellulose TLC might be used as a last step in the purification scheme. The chromatography systems evaluated were unable to separate a Na_2MoO_4 standard from a synthetically prepared Na-MoO_2(malate)$_2$ complex. This work was carried out at a later point when the Na-MoO_2(malate)$_2$ complex was a possible candidate for the Mo species. Also a solvent system was found for the K_5 silica gel, however no advantages were found over using cellulose, therefore this system was not explored further.

Table 2.6 TLC system screening

Layer	Developing solvent	Comments
cellulose		
	60 % EtOH in H_2O	spots were unsymmetrical
	60 % EtOH with 0.1 M NH_4OAc	spots were symmetrical (NH_4OAc is volatile)
	n-BuOH – AcOH- H_2O (4:1:5)	no migration
	n-BuOH – EtOH – H_2O (4:1:5)	no migration
	n-BuOH – HCO_2H – H_2O (35:15:5)	low R_f
C18-bonded phase	80 % MeOH in H_2O	Very high R_f
	50 % MeOH in H_2O	"
	EtOH – H_2O (95:5)	Streaking spot
	(70:5)	"
	n-PrOH – H_2O (80:20)	"
K5- silica gel	CH_3CN - H_2O (6:4)	good spots R_f -0.6
	(7:3)	R_f -0.4
silica gel 60	MeOH (glass distilled)	no migration
	MeOH with 1% AcOH	streaking
alumina Type e (neutral)	MeOH	no migration

A cellulose preparative TLC system was developed, however the technique was found not to be effective for several reasons. Viewing of a developed plate under 254 nm UV indicated separation of fluorescing bands from the Mo band, but only minor purification was achieved. The amount of Mo recovered was small in comparison with the total weight of the eluted fraction. In addition the amount of sample that could be applied to a single plate was small (<30mg), and when coupled with a development time for a 20 cm x 40 cm plate of 7-12 hours, made this technique impractical. Therefore, another more selective procedure was required for further purification of the Mo fraction. Before discussing this, it is of interest to note that the Mo fraction off of TLC was subjected

to spectroscopic analysis and found to contain an excess of a salt of malic acid. Malic acid complexes with MoO_4^{2-} are known in the literature[28c] and have been prepared (see Appendix) to aid in developing purification and identification techniques. However, no evidence was obtained to indicate that the Mo in its purified form was forming a complex with malate.

The anion exchange resin that was used to determine the ionic characteristics (page 24) of the Mo species was further examined to determine optimal conditions for its application as a potential separation technique. Elution experiments were carried out to determine the amount of 1 % NH_4OH required to achieve elution of Mo from the resin. One gram of alfalfa was extracted using the optimized conditions described earlier (Figure 2.1.). The aqueous extract was then concentrated *in vacuo*. The extract was redissolved in 5 mL of H_2O and applied under vacuum to DE-32 in a Buchner funnel. Results shown in Table 2.7 indicated that a stronger base was required. A solution of 0.05 N NaOH was found to be more effective in eluting the Mo species, but a large volume was required for elution. A 0.1 N NaOH solution was found to elute the Mo species more efficiently. A stronger alkali was not used for fear of altering the chemical state of the Mo species. It was anticipated that the excess NaOH present in the Mo fraction could be removed by treatment with a cation exchanger, thereby scavenging the Na^+ ions and generating H_2O. CM-32 was prepared as described earlier (page 24) and successfully used for the removal of excess NaOH from the basic Mo fraction.

Development of a HPLC procedure for the final purification step for the Mo species will now be summarized. Generally, the separation power that can be achieved in liquid chromatography is profoundly

Table 2.7 Elution of DE-32 with 1 % NH_4OH

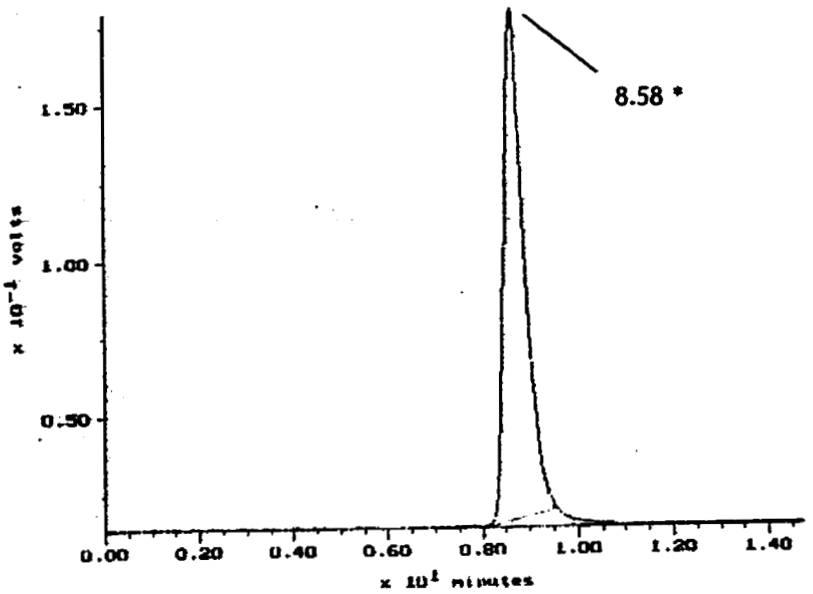
Solvent	Volume (mL)	Dithiol assay
H2O	50	-----
1 % NH4OH	25	-----
(2)	25	++++++
(3)	25	+++++
(4)	25	++++
(5)	100	+++
sorbent	(ashed)	+++

improved when using HPLC columns. This is in part a direct consequence[39] of decreased particle size in column packing material. The following factors were considered for selecting the HPLC procedure: columns that were available in-house, applicable methods for the purification of an anionic Mo compound in a complex matrix, and method of detection. It was expected that an anion exchange system could be employed given the demonstrated anionic properties exhibited by the Mo species. A Hamilton PRP-X100 anion exchange analytical column was initially investigated. The UV spectrum of Na_2MoO_4 was initially determined so as to ascertain if UV detection could be used. A broad end absorption was found starting at 260 nm. The molar absorptivity (ε) value was determined to be 4375 $^{L}/_{cm\ mol}$ at 235 nm. For the purposes of purification, the buffer for the mobile phase had to be volatile in order to remove it from the Mo fraction. Ammonium bicarbonate (NH_4HCO_3) and ammonium acetate (NH_4OAc) are often used in chromatography systems for this purpose. NH_4OAc was used to develop optimal HPLC conditions, which included: buffer concentration, buffer pH, flow rate, and wavelength for UV detection. Optimization of chromatographic parameters was carried out as suggested by the Hamilton HPLC applications handbook[40]. It was found that as the buffer concentration and pH were increased the retention time decreased for the MoO_4^{2-} anion. These results were in agreement with the general trends given in the literature[40] for the PRP-X100 anion exchange column. The pH was adjusted with diluted NH_4OH solutions. A run time less than 10 minutes was obtained using a 0.1 M NH_4OAc (pH 7.5) buffer with a flow rate of 2.0 mL/min. One percent

acetonitrile (CH_3CN) was added as a "organic modifier" to prevent microbial growth. In addition,an increased proportion of CH_3CN increased the retention time of the MoO_4^{2-} anion. Since NH_4OAc is a UV absorbing buffer, wavelength selection required determining where molybdate absorbed the strongest over background absorption. Detection at 235 nm gave satisfactory sensitivity.

Using the above-mentioned HPLC conditions, qualitative comparisons were made between Na_2MoO_4, synthetic Na-MoO_2(malate)$_2$, ammonium malate, and the semipurified Mo species. Example chromatograms are illustrated in Figure 2.4. An injection volume of 50 µL was used for all runs. An injection of a 50 ppm Mo solution (as Na_2MoO_4) yielded a retention time of 8.57 minutes, which was similar to that of the synthetically prepared Na-MoO_2(malate)$_2$ complex. Na-MoO_2(malate)$_2$ was a candidate for the Mo complex in the plant. However, ammonium malate was retained less and eluted at 7.98 minutes. Finally, examination of the chromatogram of a semi-purified Mo-fraction off DE-32 indicated the presence of both the Mo species (R_t=9.05) and sodium malate (R_t = 7.82) by comparison of their R_t's to standards. This supports NMR data, which indicated the presence of malate in purified Mo fractions. In conclusion, the HPLC system described did not have the required selectivity to differentiate between complexed and uncomplexed forms of MoO_4^{2-}. Also, the retention times of Na_2MoO_4 and Na-MoO_2(malate)$_2$

Na_2MoO_4 (50 ppm Mo)



Na-MoO_2(mal)$_2$ (50 ppm Mo)

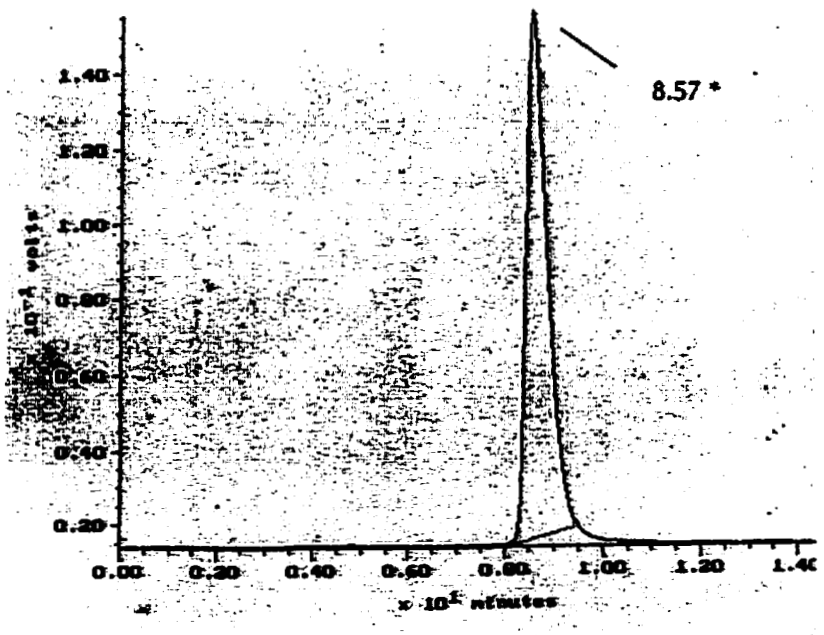
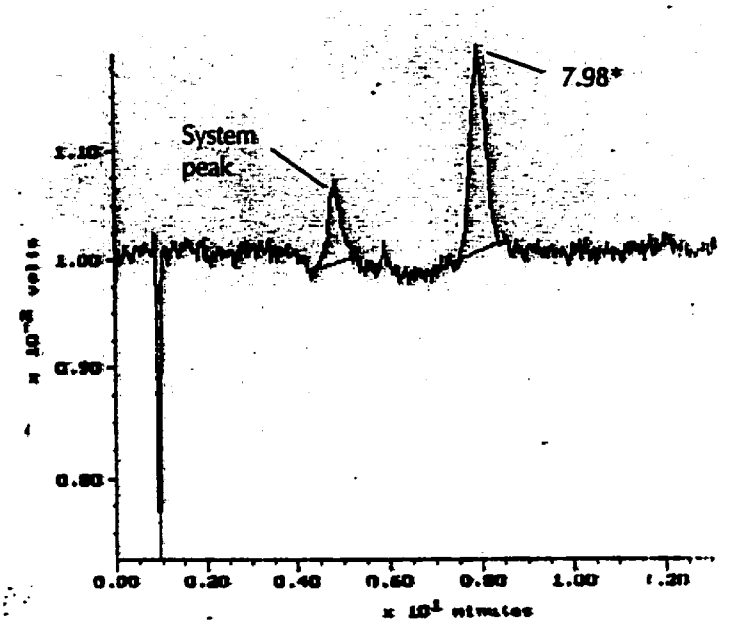


Figure 2.4 HPLC chromatograms, retention times (R_t) shown in minutes (*)

Ammonium malate (50 ppm)



Mo-fraction from DE-32 (semipurified)

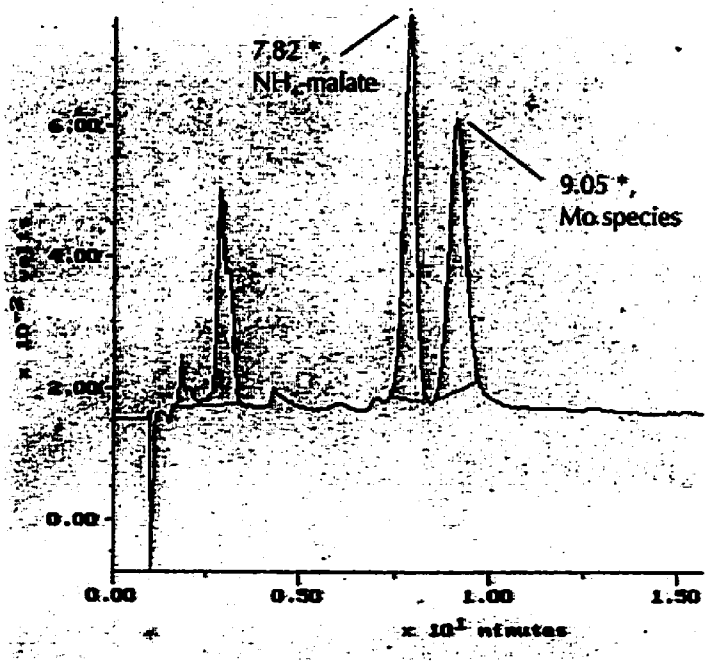
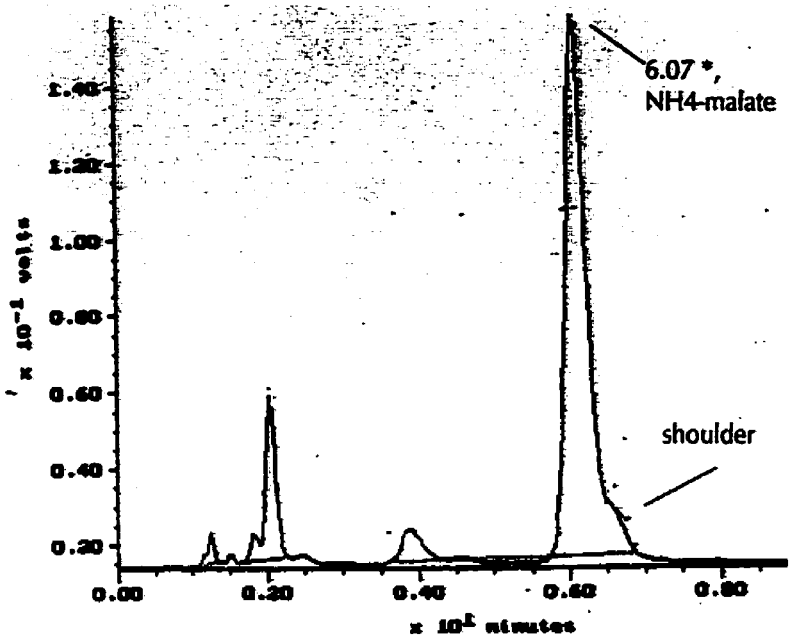


Figure 2.4 (continued) HPLC chromatograms, R_t in minutes (*)

Test sample, using 100 mM NH_4HCO_3 mobile phase buffer



Test sample, using 100 mM NH_4OAc mobile phase buffer

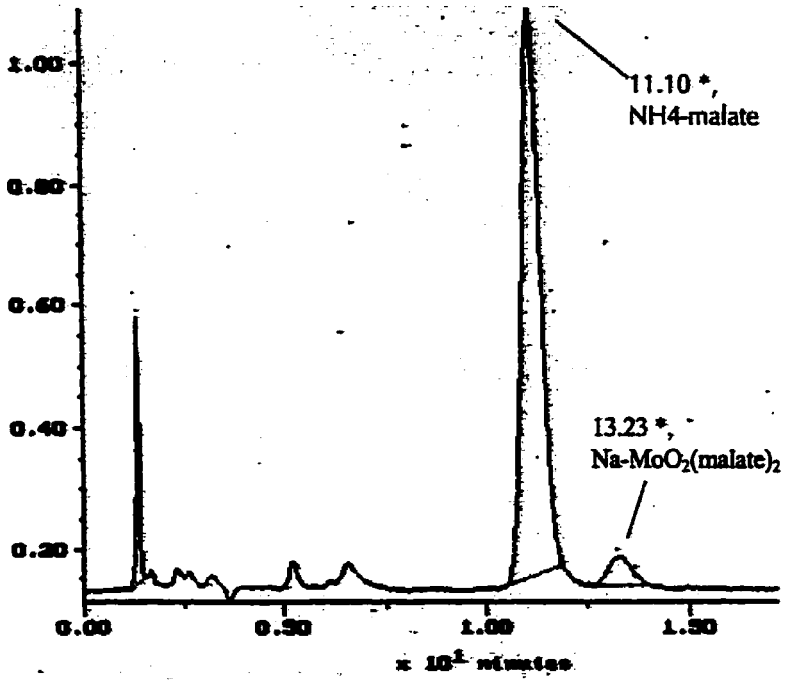


Figure 2.5 HPLC chromatograms for buffer comparison, R_t in minutes (*)

Na_2MoO_4 (50 ppm Mo) are similar to the Mo species derived from alfalfa within experimental error.

Ammonium bicarbonate (NH_4HCO_3) was examined as a mobile phase buffer replacing NH_4OAc in attempt to provide increased separation or resolution between the various Mo species. Results from a comparative study on the separation performance when using NH_4HCO_3 versus NH_4OAc as buffers, will now be discussed. A test mixture containing Na-MoO_2(malate)$_2$ species and ammonium malate, which have different R_t's, was used to evaluate the buffers. The chromatograms are illustrated in Figure 2.5. The buffers strength was 100 mM with 2% CH_3CN using a flow rate of 1.8 mL/min for both runs to allow for direct comparison. A greater separation is achieved when using NH_4OAc as the mobile phase buffer. This could in part be due to the slightly higher pH of the NH_4HCO_3 buffer at equal concentrations. Previous work has shown that increasing pH leads to decreased retention. A search for other buffer systems that might resolve a mixture of free uncomplexed MoO_4^{2-} from a Mo-malate complex was not conducted.

Two possible explanations exist for the similar R_t's between Na_2MoO_4 and Na-MoO_2(malate)$_2$. As mentioned earlier the anion exchange column may lack the required selectivity to separate the closely related Mo species. Another possibility is that Na-MoO_2(mal)$_2$ dissociates during the chromatography process. If a synthetic complex of Mo disassociates during chromatography then a complex of Mo derived from the plant might also. This was examined further by both HPLC and ^{1}H NMR spectroscopy. Since the HPLC system can separate ammonium malate from Na-MoO_2(mal)$_2$ one would expect to see the malate peak for Na-MoO_2(mal)$_2$,if it is dissociating, however this was not the case. It should be noted that the measured molar absorptivity (ε) of sodium malate (7.46 mM-pH 8) is 21 L/cm mol, which is significantly lower than Na_2MoO_4- 4375 L/cm mol. Therefore, the detection limit for malate had to be taken into consideration before any conclusions were made. An experiment was carried out first injecting a molar amount of Na-MoO_2(mal)$_2$,

which did not show a peak for malate at the expected R_t. Next a solution containing two molar equivalents of malate was injected to determine if a signal could be detected at these concentrations (see Figure 2.4 ammonium malate). Results showed that detection was not a factor and Na-MoO_2(malate)$_2$ did not appear to be dissociating during chromatography. Complementary to this, the Na-MoO_2(mal)$_2$ complex was fractionated under the same conditions and various fractions analyzed by ^{1}H NMR. Experiments showed that the Mo containing fraction was the only fraction containing malate. Therefore, it was concluded that if MoO_4^{2-} is present as a complex with a hydroxy acid or sugar it would not dissociate during the HPLC step.

Other HPLC systems were investigated in hope of finding a system that could differentiate between $Na_2MoO_4$and Na-MoO_2(mal)$_2$. If a system was found then the purified Mo species would be tested for differences in R_t by comparison with standards. Reverse phase and normal phase HPLC columns were examined. A (150 mm x 4.1mm) Hamilton PRP-1 polymeric reverse phase column was tested. Due to the strong ionic nature of the Mo-species, reduced retention was expected on reverse phase packings, therefore chromatography was carried out in ion suppression[41] mode. A low pH mobile phase is sometimes required to suppress secondary ionization. The following solvent systems and flow rates in Table 2.8 were evaluated. A 1:1 mixture of Na_2MoO_4 and Na-MoO_2(mal)$_2$ was used as a test mixture. Attempts at finding a suitable solvent system for chromatographing the test mixture were unsuccessful. No conditions were found producing significant retention and separation of the Mo-species. Retention times were typically less than one minute.

Table 2.8 Development of HPLC system for PRP-1 HPLC column

Eluant % A	Composition % B	Flow rate (mL/min)	Comments
100 mM NH4OAc (pH5)	CH_3CN		
30	70	2	peak tailing off
40	60	1.6	"
10 mM AcOH	CH_3CN		
100	0	2	broad at 1min
80	20	2	at front (Rt < 1min)
97	3	2	"
H_2O	CH_3CN		
100	0	2	at front (Rt < 1min.)
80	20	2	"
20	80	2	"
5	95	2	"

Therefore it was concluded that the Mo-species was not interacting with the reverse phase packing using ion-suppressed conditions.

No attempts at this point had been made to chromatograph the Mo species using a normal phase HPLC system. Therefore a Supelco LC NH_2-5 (4.1cm x 25 cm) normal phase HPLC analytical column was evaluated for its ability in the separation of the Mo species. A series of solvent systems were screened as shown in Table 2.9. It was expected that the Mo species would be very strongly retained on the normal phase system. Again, pH was considered an important variable in the solvent system. If the ionization could be suppressed so as to make the Mo species less polar, then satisfactory chromatography might be achieved on a normal phase column. As with the reverse phase system, a 1:1 mixture of Na_2MoO_4 and $Na\text{-}MoO_2(malate)_2$ was used as a test mixture.

Table 2.9 Solvent screening for NH_2-5 HPLC column

Eluant %A	Composition %B	Flow rate (mL/min)	Comments
H_2O	CH_3CN		
100	0	2	broad peak at 5 minutes
100 m M NH4OAc	CH_3CN		
100	0	2	broad peak at 6 minutes
10 m M AcOH	CH_3CN		
100	0	2	peak tailed off
50	50	2	broad peak
30	70	2.6	"
50	50	3	"

Results indicated that under all conditions tested the Mo species were too strongly bound to the stationary phase.

The previously described purification using the optimized HPLC anion exchange system (p.31) for the last step was finally accepted as the best choice. Regardless of the lack of resolution between different Mo species, it was anticipated that the anion HPLC system would be able to provide a purified Mo fraction.

2.3.2 Development of a purification procedure

Development of a purification procedure requires a sequence, which typically involves a high capacity cleanup at the beginning to achieve the greatest purification at an early stage, then using the most selective chromatography step to obtain a pure fraction. An approach similar to that reported in the literature[33] was initially attempted. The reported strategy involved partitioning an aqueous extract against $CHCl_3$-n-BuOH (10:1) to remove high molecular weight compounds of

moderate polarity. The aqueous fraction was then subjected to chromatography over Sephadex G-10. The investigators then further purified fractions using a weak cation exchanger. The compounds of interest were then identified using various spectroscopic techniques.

Our purification scheme is shown in Figure 2.6. Advantages and disadvantages as well as suggestions for improvements are discussed below. The alfalfa was prewashed with hexane and EtOH and an aqueous extract was obtained after grinding the PM in mortar and pestle and washing with H_2O. The pH of the aqueous extract was about 5.5, which might need to be buffered. The extract was partitioned with n-BuOH to remove polar organic material. Care was required, as stable suspensions would form if the separatory funnel was shaken too vigorously. Negligible amounts of Mo were lost in process. The aqueous fraction was concentrated to dryness on a rotorotary evaporator at 40 ^{0}C. The sample was then chromatographed over Sephadex G-10 using H_2O as the eluting solvent. A problem with insoluble material clogging up the top of the G-10 column was encountered. Gentle stirring of the top of the bed permitted continued elution. A brown colored band would elute in the exclusion volume, however this fraction did not represent a major portion of the overall weight. The Mo-containing fractions eluted after the exclusion volume as determined using the Mo dithiol visual assay. Molybdenum fractions were combined and applied under vacuum to a DE-32 bed in a Buchner funnel. The resin was then washed with H_2O. After concentration the H_2O wash was found to contain a large portion

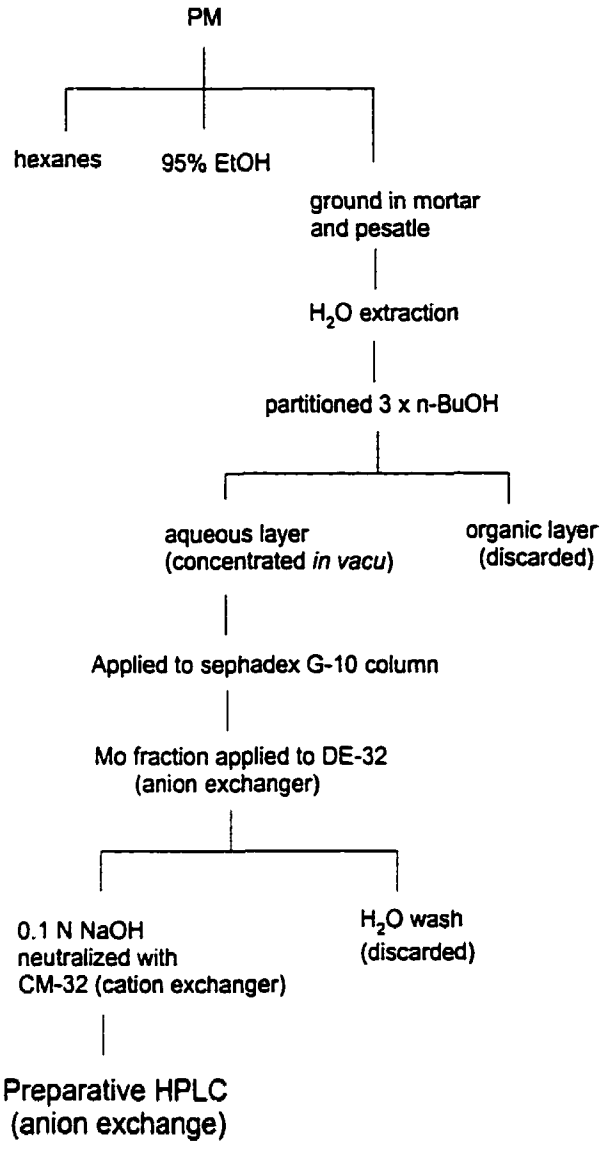


Figure 2.6 Initial purification sequence for Mo from alfalfa

of the overall weight (> %50), which meant a significant purification had been achieved. Elution of the Mo species from DE-32 anion exchange resin was carried out with 0.1 N NaOH. Elution was determined to be complete when Mo was shown to be absent in the eluant using the Mo dithiol assay. CM-32 (H^+) was added to the alkali fraction until a neutral pH was achieved. The CM-32 was removed by filtration and washed with H_2O on a buchner funnel. The filtrate was

concentrated to dryness *in vacuo*. This fraction was then used for purification by HPLC. The ion exchange cleanup step was effective in removal of interfering material in the water wash step, while the Mo species was retained on the resin. However, the step was very time consuming due to the large volumes of solvents used, which required a great deal of time for concentration.

Moderate success was found after attempts were made at preparative HPLC anion exchange (7.1 mm x 305 mm) of the DE-32 Mo fraction. Sample preparation included filtration through a 0.45 μm syringe filter to remove particulate. The above-described optimized conditions were used for the preparative HPLC runs. A series of runs to determine maximum injection volume and sample concentration were required for the preparative HPLC step. It was found that large backpressures were generated in the system. This normally occurs from particulate buildup after injecting many samples. In this case it was occurring at a much faster rate. It was determined that the PRP-X100 Hamilton guard column was becoming fouled. The columns could be partially regenerated using the regeneration solvent (see Experimental). It was thought that certain sample components were irreversibly binding the packing material. More importantly, the Mo fraction required repeated purification by HPLC, which diminished the total recovery of purified Mo. For a large-scale isolation of the Mo species it was realized that modifications to the purification sequence were required.

2.3.3 Modifications to purification sequence

The use of Sephadex G-10 at an early stage in the purification sequence was reexamined. The amount of material in the exclusion volume was negligible and it was believed that the majority of the material was eluting with the Mo species, however this needed to be verified. An experiment was carried out extracting 10 g of alfalfa and then taking half of the aqueous extract for partitioning with n-BuOH. The aqueous layer was concentrated and applied to a 43 cm x 3 cm Sephadex G-10 column. Fractionation was carried out as shown in Table 2.10. The fraction

Table 2.10 Fractionation of Mo fraction on G-10

Fraction	Volume (mL)	Elution volume (mL)	Fraction wt. $X10^1$ mg	[Mo] (ug/mL)
void volume	83	83		0.0
exclusion volume	19	102	7.5	0.8
1	12	114	13.2	20.0
2	17	131	35.4	36.9
3	17	148	40.8	25.8
4	18	166	9.3	8.1
5	20	186	3.6	2.0
6	20	206	2.5	0.6

weight and Mo concentration versus the elution volume is shown in Figure 2.7. It was concluded that the majority of the weight is chromatographing with the Mo on the Sephadex G-10 column. Therefore, it was decided that G-10 be used at a later point in the purification.

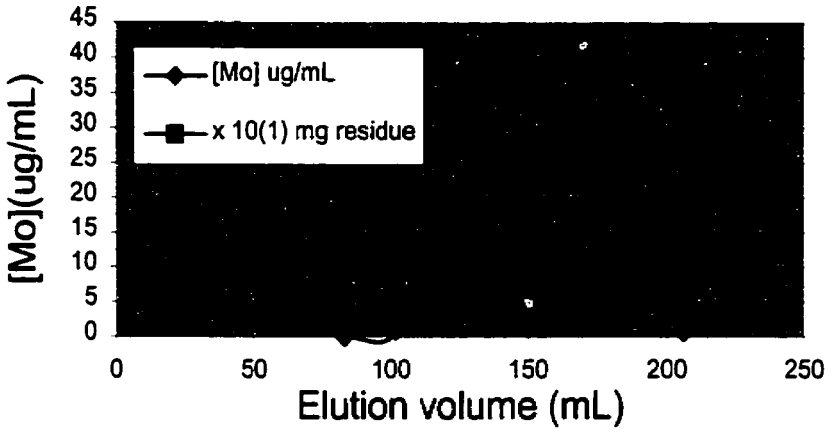


Figure 2.7 Elution-weight distribution curve on Sephadex G-10 of purified Mo species

Modifications were made to the DE-32 anion exchange step. In the initial procedure elution was being carried out by removing the ion-exchange site with alkali, however it was thought that using an ion-exchange mechanism would be more effective and have the potential of several benefits. A more selective elution would provide a cleaner Mo fraction. In addition, use of an exchange buffer would negate the use of base for elution in the purification sequence, therefore providing a milder procedure leading to the purified product. However, there are also difficulties associated with this approach. Again a buffer solution, which can be removed from the Mo fraction, would be required for this step to be successful. Other possibilities included finding a buffer wash to selectively remove interferences, therefore allowing elution of a cleaner Mo fraction. Preliminary experiments involved elution on a small scale while monitoring Mo with the dithiol assay. A solid phase extraction (SPE) technique was used for this purpose. SPE uses small cartridges packed with a particular sorbent that can be used on a vacuum manifold, allowing for the processing of many samples or used conveniently for method development. The following procedure was used for the preparation of DE-32 SPE cartridges. DE-32 was made up in the –OAc form from the free base, using

the procedure outlined in the Experimental section. A 3 mL syringe, fitted with a glass wool plug at the base, was used as the cartridge. DE-32 was placed into the cartridge under vacuum using a manifold until a 1cm bed was obtained. The bed was washed three times with H_2O to ensure a uniform tightly packed bed. A previously prepared H_2O extract was used for the elution experiments. An elution trial is illustrated in Table 2.11. Elution volume of 4 mL was collected and tested by the Mo dithiol assay. Comments are given to indicate where interfering material is

Table 2.11 DE-32 SPE (1X1cm) purification of aqueous extract

Solvent	Mo dithiol assay (+) relative [Mo]	Comments
H_2O	—	Clear
0.1M NH_4OAc	—	"
0.3M NH_4-CO_2H	—	slight color
0.4M NaCl	+ + + +	"
"	+ + +	"
"	—	
"	—	
0.2 M NaOH	—	Yellow

eluting. The volatile buffers, NH_4OAc and ammonium formate (NH_4-HCO_2), did not have the exchange strength to elute the Mo species. Also, very little color was eluted, therefore their utility as wash solvents was limited. However, a 0.4 M NaCl solution eluted the Mo species in 8 mL, but if NaCl was to be used as the eluting buffer, desalting of the Mo fraction would be required before the HPLC step. A strong anion exchanger (SAX) was also tested for its ability for clean up. Details of sorbent brand and conditioning can be found in the Experimental section. It should

be noted that SAX resin is silica based and has a pore diameter of 60 Å compared to 250 Å for DE-32. The smaller pore diameter does not allow for the migration of larger molecules (molecular weights > 2000 g/mol) to enter into the pores of the ion exchange resin[36]. There was a potential for size exclusion if using the smaller pore diameter SAX resin, therefore carrying out two steps in one. Finding a suitable eluant for the Mo species from a crude H_2O extract was required. Elution trials were conducted and the results are shown in Table 2.12. Elution volumes of 4 mL were collected and tested by the Mo dithiol assay. Alkali elution was

Table 2.12 SAX purification of aqueous extract

Solvent	Mo dithiol Assay (+) relative [Mo]	Comments
Trial 1		
H_2O	—	
CH_3CN	—	
2.5 %$NaNO_2$	+ + +	Colored
5% $ZnSO_3$	+ +	"
0.1 M NaOH	—	Yellow
Trial 2		
H_2O	—	Light color
0.3 M NH_4-HCO_2	—	
0.4 M NaCl	+ + + +	Light color
0.2 M NaOH	—	Yellow color

carried out in both cases, which destroyed the gel by hydrolysis of the silanol groups on the silica support, allowing the SAX resin to be checked for residual Mo after elutions were made. A H_2O wash was used to remove any neutral components that may be present in the void volume. Washing with CH_3CN seemed to have no benefit in terms of a cleanup. Sodium nitrite ($NaNO_2$) and zinc sulfate

($ZnSO_4$) solutions were found to be able to elute the Mo species effectively. In trial 2, light color was eluted with the H_2O wash, while the NH_4HCO_2 did not elute Mo or provide a cleanup. Elution of Mo with 0.4 M NaCl was achieved using only 4 mL of solvent. High recoveries can be expected as no Mo was found in the 0.2 M NaOH fraction, which indicated complete elution of Mo. It therefore appears possible that a SAX resin can be used in the place of the weak anion exchanger DE-32. The benefits of size exclusion were not investigated. However, it was decided for preparative purposes to continue using DE-32, which was readily available and could be used conveniently in the preparation of home-made SPE columns in a variety of sizes depending on the sample requirements. If NaCl was to be used as an elution solvent its subsequent removal needed to be evaluated. Initially it was thought that desalting could be achieved on Sephadex G-10. Experiments toward this end will now be discussed.

For applications Sephadex G-10 has the capacity to be used in two different types of separation[34]. In one type, compounds of high molecular weight are completely excluded from the gel and are eluted in the exclusion volume of the column, while components of lower molecular weight are eluted later. This type of separation is referred to as group separation and is typically used for desalting or buffer exchange. The other type of application is separation by fractionation, which separates substances that are similar in molecular weight. This is a more difficult separation to achieve. Attempts were made to desalt the Mo fraction on Sephadex G-10 using the fractionation method of separation. A solution of 20 mM NH4OAc was used as the eluting solvent for desalting experiments. It is reccommended[34] to use a buffer when the samples have a high ionic strength to avoid zone broadening. The Mo containing DE-32 fraction was applied to a 5.0 cm x 48 cm Sephadex G-10 column in 20 mL of 20 mM NH_4OAc. The Sephadex column was prepared as described in the experimental section. The fractionation was carried out as shown in Table 2.13. The fractions where tested for Mo using the dithiol assay and weighed to allow identification of the salt fractions. Partial separation of the Mo fractions from the salt was achieved. A decrease in the fractionation volumes in

this particular run may have allowed for more complete desalting of the Mo species. Nevertheless a separation based on molecular weight was achieved.

Table 2.13 Desalting of purified Mo DE-32 fraction on Sephadex G-10

Fraction	Volume (mL)	Dithiol Mo Assay	Weights (mg)
Vo	335	—	discarded
Vexcl	47	—	"
1	60	—	"
2	78	+ + + + +	150
3	48	+ + +	140
4	60	+ +	680
5	110	+	6.8 g
6	130	—	190
7	200	—	—
8	200	—	—

Encouraged by the ability to desalt the Mo fraction, a large-scale purification was attempted using a modified procedure (Figure 2.8). The exact conditions and details for extraction and purification of 30 g of PM can be found in the Experimental section. All Mo values were determined by ICP for the various fractions tested.

Modifications that were made to the cleanup procedures included extraction with a buffer instead of water. A 20 mM NH4OAc solution buffered the extract at pH 7.0 and was removed under high vacuum by a freeze-drier. Boiling EtOH solublized more interfering components (5.39 g) prior to extraction with an aqueous buffer. After partitioning with n-BuOH the concentrated aqueous soluble fraction was clarified using Celite to remove insoluble material before chromatography. A

negligible amount (0.037 mg) of Mo was found in the n-BuOH soluble fraction. The clarified fraction was applied to a 6 x 25cm DE-32 column without a concentration step. The DE-32 SPE cleanup step was used at this point for two reasons: 1) it has already been shown that DE-32 can be used as an efficient cleanup step, i.e. DE-32 has a high capacity, while still being an effective purification step (note that 7.15g of material was found in the wash fraction, Figure 2.8). 2) Sephadex G-10 was required to follow the DE-32 cleanup step for desalting the Mo fraction, while also removing 1.3g of high molecular weight components. Therefore, DE-32 followed by Sephadex G-10 is a logical sequence for purification. A drawback when using Sephadex G-10 for desalting includes low sample capacity. The sample volume was limited to 5-10% the void volume for optimal desalting, which required using sample volumes of 20 mL for the 48 x 5 cm column. If any larger volumes were used the salt fraction overlapped with the Mo fraction. Therefore, numerous runs were required to desalt the Mo fraction and the column required flushing between runs, which was time consuming. Modifications made to the preparative anion exchange HPLC step

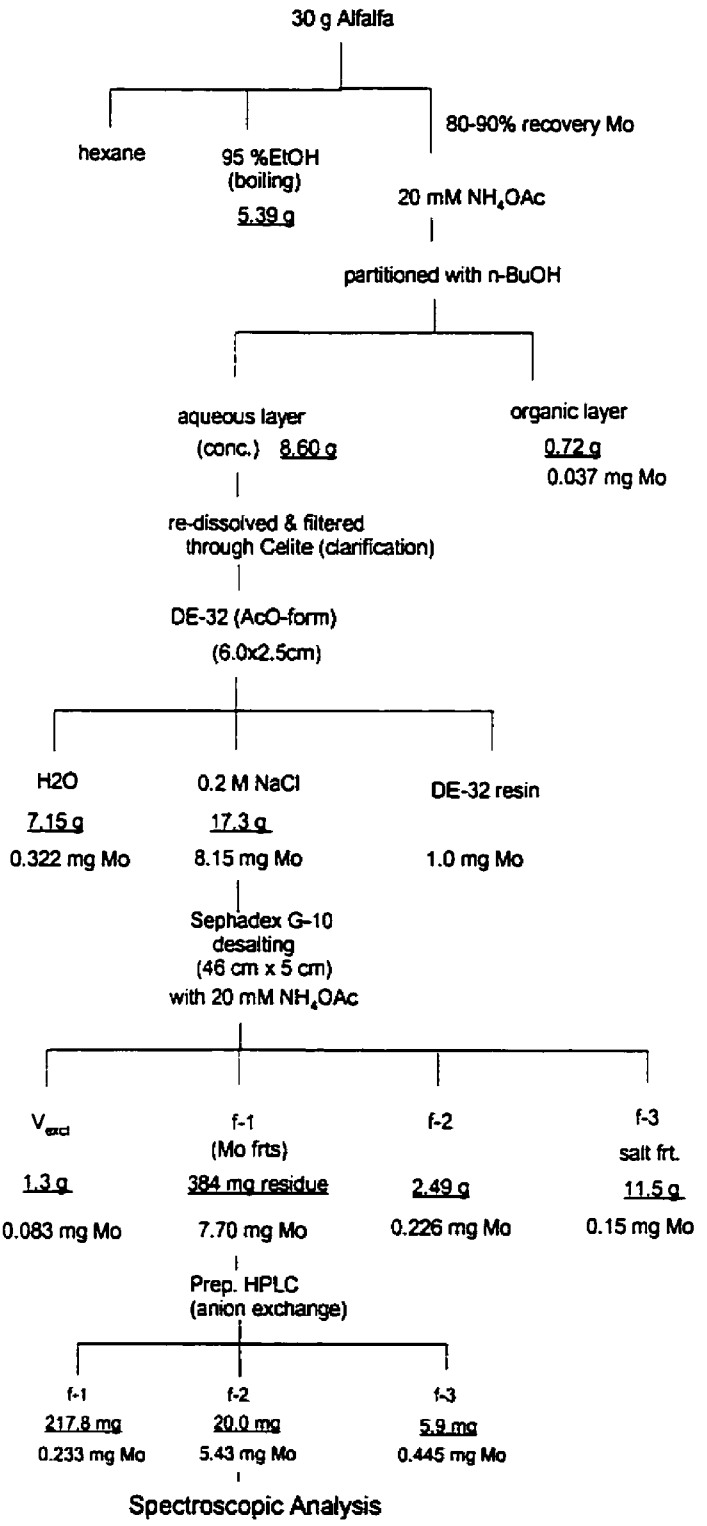


Figure 2.8 Modified purification procedure for Mo in alfalfa

involved increasing the CH_3CN composition in the mobile phase to 10% providing increased retention of the Mo species and better separation from interfering sample components.

Recoveries of Mo through the various steps are presented in Table 2.14. A theoretical amount of approximately 12 mg of Mo in 30g of plant material was based on an average of 400 ppm Mo in the alfalfa sample. The amount of Mo prior to DE-32 was a summation of Mo in all the DE-32 fractions and resin. All other values were measured directly by

Table 2.14 Recoveries of Mo through the various purification steps

Purification stage	Mo before (mg)	Mo after (mg)	% Mo recovery
Extraction and cleanup	12	9.47	78 9
DE-32	9.47	8.15	86
Sephadex G-10	8.15	7.7	94.5
Prep. HPLC	7.7	5.43	70.5

ICP. Satisfactory Mo recoveries were obtained throughout the various steps, ranging from 70.5 to 94.5% with the poorest being preparative HPLC. It was thought that there may be tailing occurring on the HPLC column and therefore not all the Mo was being collected, which would lower the recovery. However, it was more likely that loss occurred during the sample preparation and injection steps due to the number of sample runs required to purify all the material.

The optimized purification procedure shown in Figure 2.8 gave material, which had 5.43 mg Mo in 20 mg total weight of purified sample and was the cleanest Mo fraction achieved to this date. Note that the weight is expressed as Mo, so that a MoO_4^{2-} complex would have additional weight, and a counterion

would also be present. The physical and spectroscopic characterization of the purified Mo fraction will now be considered.

2.4 Physical and Spectroscopic Characterization

The purified material was a slightly brown colored material, possibly due to trace polyphenolic impurities. The sample was initially concentrated to dryness and the residue weighed 20.5 mg. The sample was redissolved in a minimal amount of H_2O and transferred to a small sample vial. The sample was frozen and lyophilized, yielding 20.0 mg. An accurate weight of the purified material was required at this point to permit determination of the purity of the sample to the best extent possible and to provide information as to presence of a complex. Knowing the Mo content from ICP to be 5.43 mg, the possibility of their being a ligand associated with the Mo can be predicted. First by adding on four oxygen's to Mo yields 9.05 mg for the MoO_4^{2-} anion. This does not include the counter-ion or any H_2O if present as a hydrated material. If a molar equivalent of low molecular weight (< 300 g/mol) organic ligand was associated with the Mo then ^1H and ^{13}C NMR would permit detection and provide information leading to its identity. For unambiguous characterization of a complex of Mo the use of ^{95}Mo NMR was required and was investigated as follows.

2.4.1 ^{95}Mo NMR optimization

Molybdenum has two NMR active isotopes[42]. Both ^{95}Mo and ^{97}Mo have a spin I = $^5/_2$ and low detection sensitivities. Comparatively, ^{95}Mo is more readily detected with a relative receptivity to ^{13}C of 2.9 while that for ^{97}Mo is 1.8. In addition[43], ^{95}Mo and ^{97}Mo have a natural abundance of 15.72 % and 9.46 % respectively. The quadrupole moment for ^{95}Mo is 0.12 X10^{-24} cm^2, while that for ^{97}Mo is 1.1 X10^{-24} cm^2. In light of this, very wide lines are expected for all but the most symmetric environments. Given the above outlined NMR properties, ^{95}Mo is the more commonly used Mo NMR nuclei. Information from ^{95}Mo NMR is obtained from the chemical shift (using Na_2MoO_4 as external reference), the line width, and less commonly spin-spin coupling.

Due to the limited amount of Mo in purified samples, optimization of various NMR parameters was required to shorten experimentation time. It was also important to determine the minimal amount of Mo required to obtain a ^{95}Mo NMR spectrum using Na_2MoO_4 and Na-MoO_2(malate)$_2$ as standards. The following optimal parameters were determined from optimization experiments (Table 2.15). The main parameters under

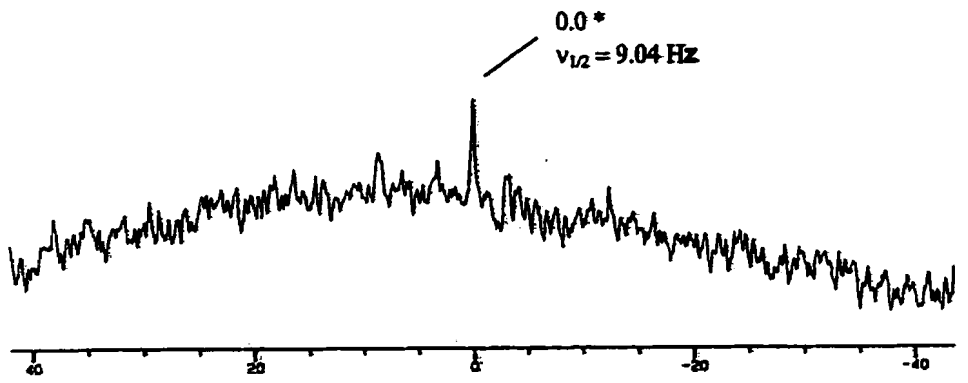
Table 2.15 Important NMR parameters

Parameters	Abbreviations	Optimized values
sweep width	SWH	25000 Hz
acquisition time	AQ	0.0817 sec
pre-scan delay	DE	50 usec
delay time	D1	50usec
# of data points	TD	4.0 K
receiver gain	RG	2298.8

consideration were SWH and TD, while attempting to minimize AQ for the experiment. The following considerations were observed. Increasing SWH, decreased AQ, however the larger SWH the larger TD was required to adequately define the spectral window. If SWH was made large and TD was not increased then the potential existed that a sharp signal, if present, would not appear because of an inadequate # of data points (TD) defining the spectral window. It should be noted that the SWH given above was not always used on purified Mo samples. Not knowing where the Mo signal may resonate, SWH was increased so as to cover a larger region. Typical[44] T_1 relaxation times for ^{95}Mo range from 0.2 to 6 x 10^{-3} sec, which is determined by the symmetry of the Mo species being measured. Implications of this were that short delays could be employed given the inherently short T_1 relaxation time. The pre-scan delay (DE) and the delay time (D1) were both set to 50 μsec.

It was determined that Na_2MoO_4 was relatively sensitive after finding that only 6000 scans were required to adequately detect a Mo signal in a sample containing only 0.5 mg Mo as Na_2MoO_4 (see Figure 2.9). It should be pointed out that this experiment could be carried out in less than ten minutes. The line width ($\nu_{1/2}$) for Na_2MoO_4 was determined to be 9.04 Hz, which is quite narrow for a Mo NMR line. Increased sensitivity is a direct consequence of a narrow line width. A narrow line width arises from a long T_2 relaxation time (as $T_2 = T_1$, when considering line-widths), which is characteristic of the symmetrical tetrahedral environment of Mo found in the MoO_4^{2-} anion. These results are in agreement with literature findings[43], in which structural changes have been correlated with line width. Initially this was promising because if a purified Mo sample contained 0.5 mg of Mo as a MoO_4^{2-} salt then ^{95}Mo NMR could detect the Mo signal. On the other hand, no signal detection would provide

0.5 mg Mo of Na_2MoO_4



1 mg Mo of Na-Mo $(malate)_2$

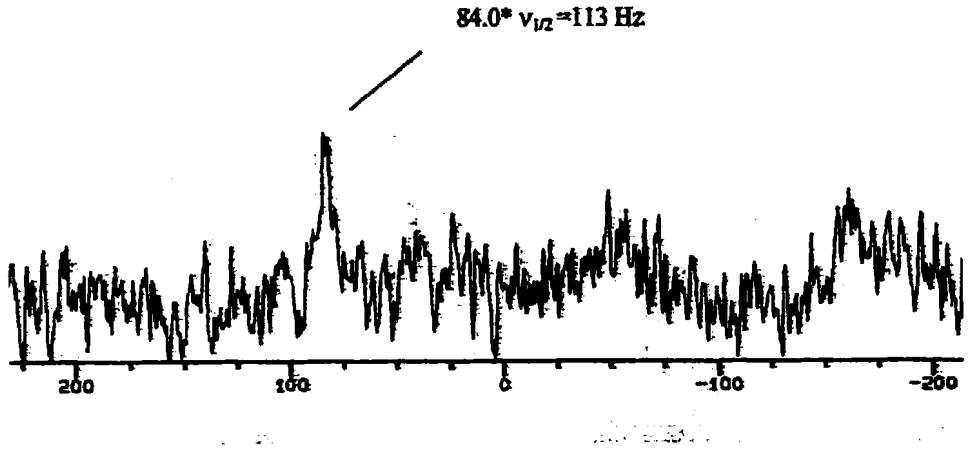


Figure 2.9 ^{95}Mo NMR spectra, chemical shift in ppm (*)

negative evidence that the Mo was not occurring as free MoO_4^{2-}, but as a Mo species with a wider peak width (and perhaps different chemical shift). This was investigated by determining the ^{95}Mo NMR sensitively for a Na-MoO_2(malate)$_2$ standard (a plausible complex of the MoO_4^{2-} anion since malate is a common plant hydroxy acid. Results (Figure 2.9) showed that a sample containing 1mg of Mo required three hours of collecting 100,000 scans to detect a signal. The line width was 113 Hz, which was a result of the decreased symmetry in the Na-MoO_2(malate)$_2$ species. Since the solution contained twice the Mo concentration as used for the MoO_4^{2-} detection this was a significant increase in experiment time. Knowing that the signal to noise ratio in FT-NMR is proportional to the square root of the number of scans it was calculated that if 0.5 mg Mo as Na-MoO_2(malate)$_2$ had been used then approximately 12 hours of NMR time would be required to detect a signal. It was concluded that a larger Mo concentration was necessary since the unknown ^{95}Mo resonance had to be considerably broader then that of the Na_2MoO_4 signal.

Other factors[42] that can influence the ^{95}Mo NMR behavior of MoO_4^{2-}include the cation, solvent and pH. Of these pH was considered the most important. A previously study[43] reported ^{95}Mo T_1 relaxation times as a function of solution pH. The study involved measuring the rate of polymerization of MoO_4^{2-} as a function of decreasing the solution pH. These line-width measurements reflect structural differences at the different pH values. It was noted that between pH 9-12 Mo relaxation times were independent of hydrogen ion concentration. However, below pH 9 protonation of the Mo species caused line broadening due to the formation of less symmetrical polymolybdates. In light of this information, solution pH was considered to be an important factor for our work. If no ^{95}Mo NMR signal was detected in our purified Mo sample, then conclusions could only be made after the pH of the sample was known. Therefore, the absence of a NMR signal in our Mo fraction could be due to the presence of an unsymmetrical Mo complex, which has an inherently large line width, or the sample pH could be low and the formation of polymolybdates could cause the line broadening.

A MoO_4^{2-} pH study was then carried out to examine the effect on the ^{95}Mo line-width. The goal was to determine the pH where the Mo could no longer be detected due to NMR line broadening, using the adjusted NMR parameters (page 52). A solution of 0.1 M Na_2MoO_4 in 25 ml of H_2O with 30 % D_2O (for NMR lock) was used. The pH was measured with a standard laboratory pH meter calibrated with pH 4.00, 7.00, and 10.00 aqueous buffers. The pH adjustments to the MoO_4^{2-} solutions were made with a 0.6 N HCL solution and 0.5 mL aliquots were taken for NMR analysis as shown in Table 2.16. To allow comparison between spectra, 900 acquisitions were obtained for each of the following measurements. All other parameters were kept constant.

Table 2.16 ^{95}Mo NMR pH study on Na_2MoO_4 solution

NMR sample	pH	δ(ppm)	$\nu_{1/2}$(Hz)	S/N
1	8.44	0.0	6.4	19.9
2	6.72	0.0	6.4	18.6
3	6.12	0.0	6.4	17.1
4	5.73	0.1	11.0	8.3
5	4.98	—	—	—
6	4.73	—	—	—
7	4.48	—	—	—
8	8.36	0.0	6.4	13.7

The pH of a 0.1 M Na_2MoO_4 solution is 8.44, which was used as the starting point for the experiment. The important factors are the line width ($\nu_{1/2}$) and the signal to noise ratio (S/N). The S/N dropped off as the solution became more diluted from the addition of acid, which is seen in the first three samples. There is no change in the $\nu_{1/2}$ until the fourth solution, which at pH 5.7 had a line width of 10.9 Hz. More

noticeable is the decrease in S/N, which drops from 17.1 to 8.3. These changes are indicative of polymerization due to the protonation of the MoO_4^{2-} species. After further decreasing the pH to 4.98 in sample five, no signal was detected after acquiring 900 scans. Also, no signal was detected after further decreases in the pH were made. It is important to note that adjusting the pH up to 8.36 with a 0.2 N NaOH solution regenerated the MoO_4^{2-} anion. It is concluded that major structural changes caused by polymerization take place below pH 6 and below pH 5 no signal was detected using the conditions described above. More experiments with solutions between pH 5 and 6 would have better defined this region, but since the pH effects on the line-width of ^{95}Mo peaks in MoO_4^{2-} solutions have been previously studied[43] a modified experiment was not attempted.

The above experiments have established the following: 1) the optimal NMR parameters needed to minimize experiment time and maximize the S/N ratio. 2) that a minimum of 1 mg Mo in our purified Mo fraction was required for ^{95}Mo NMR analysis, and 3) the importance of knowing the solution's pH. In conclusion ^{95}Mo NMR can provide evidence as to the existence of a complex of the MoO_4^{2-} species, as has been shown by studies of synthetic MoO_4^{2-} complexes[28]. However, establishing the presence and identity of such a possible ligand is better carried out using ^1H and ^{13}C NMR. Work to this end will now be discussed.

2.4.2 Spectroscopic studies on the purified Mo fraction

After the weight of the Mo containing fraction from prep HPLC (Figure 2.8) had been properly established for the isolated material, the sample was dissolved in D_2O and the pH determined to be 5.65. A 2 mm micro glass pH probe connected to a Corning pH meter was used to directly measure the pH in a 5 mm NMR tube. The sample was then subjected to ^1H, ^{13}C, and ^{95}Mo NMR. The ^1H NMR spectra revealed three single peaks in the 1.8 – 2.5 ppm range as well as the solvent HOD peak, which was used as the reference signal (4.80 ppm). The methyl group signal from acetate occurs in this region and therefore was a likely candidate as it was used

as the mobile phase buffer for HPLC. Other minor peaks were detected. After acquiring 34,544 scans no signals were detected in the ^{13}C NMR spectrum. Similarly, after acquiring 103,230 scans no signals were detected in the ^{95}Mo NMR spectrum. The pH of the sample was adjusted with 50 % NH4OH in D_2O to pH 9.2 in an attempt to obtain a ^{95}Mo NMR signal. A very strong signal was found after collecting 492,125 scans (refer to Figure 2.10). The chemical shift and line-width of this spectrum requires some discussion. The signal occurs at a chemical shift of 0.1 ppm, which suggests that the Mo is in a similar chemical environment to that of MoO_4^{2-}, however the linewidth is much larger at 109 Hz. At a sample pH of 9.2

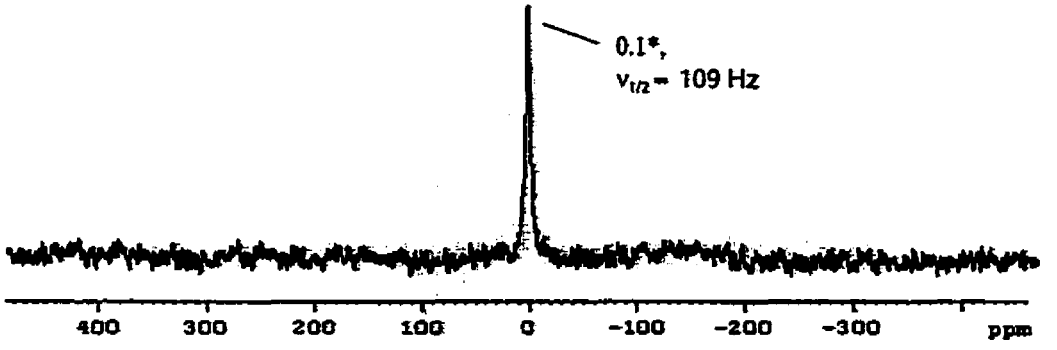


Figure 2.10 ^{95}Mo NMR spectrum of the purified Mo species, chemical shift in ppm(*)

free MoO_4^{2-} would have a much narrower line width. Therefore it is concluded that some other sample component is interacting with the MoO_4^{2-} anion, which alters the chemical environment resulting in a broadened NMR line.

It was decided to investigate more thoroughly the possibility of there being an organic component, which might be involved in some mobile equilibrium with the Mo species, since there were four proton signals in the ^1H NMR spectrum. However, this was somewhat unlikely because a molar equivalent (to the [Mo]) of any organic species containing carbons and hydrogens would have been detected

by ^{13}C NMR. To provide unambiguous proof, the apparent methyl peaks (the singlets in the ^{1}H NMR spectrum) were quantified to determine their absolute amounts. This was achieved by spiking the sample with a known amount of methanol as the standard. The resulting spectrum is shown in

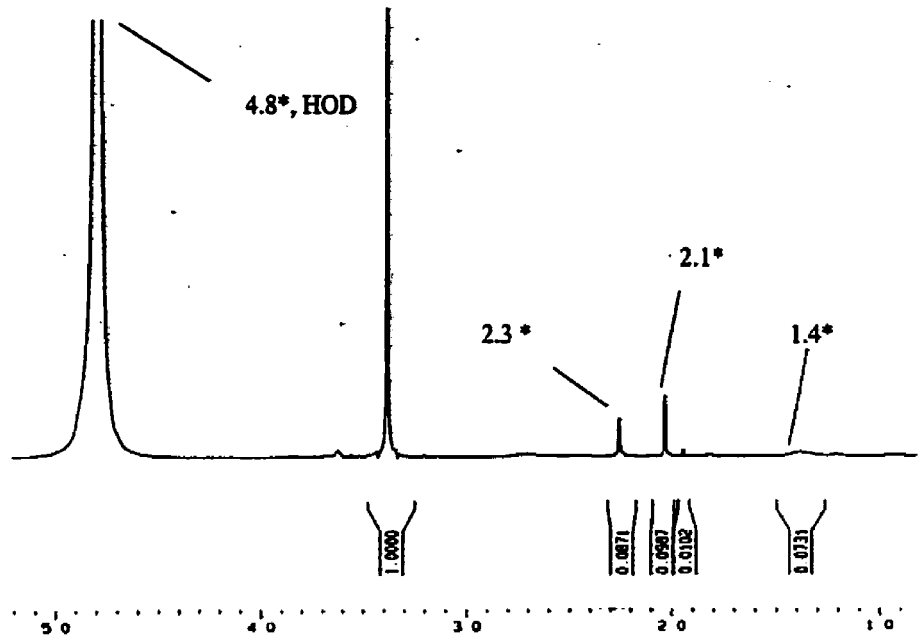


Figure 2.11 Quantitative ^{1}H NMR of purified Mo species, chemical shifts given in ppm (*), integration values shown above ppm scale

Figure 2.11. Methanol was chosen because the methyl group signal occurs at 3.83 ppm, which was in a region free of any other interfering resonances. Reagent grade (99.8%) methanol was used for the experiment. Using a 5 μL glass syringe, 2.52 μL (2.0 mg, 0.062 mmol) was added to the pH adjusted NMR sample containing the purified Mo fraction. Too ensure that strong signals were obtained, 128 scans were collected on a 400 MHz NMR spectrometer. It was immediately apparent that the methanol signal was much larger than the other signals, which indicated that the unknown signals arose from a relatively small amount of some unknown

substance(s). In addition, calculations were made and the results are given in Table 2.17. The calculations are based on the assumption the unknown signals result in each case from three equivalent hydrogens of a methyl group. The integrations were referenced to the methyl group signal of methanol. Table 2.17 provides the calculated amounts if the signals were to belong to ammonium acetate (NH_4OAc), acetone ($(CH_3)_2CO$), or an unknown methyl group containing substance with a molecular weight of 400 g/mol.

Table 2.17 Quantitative ^1H NMR calculated amounts

δ (ppm)	Integration	$AcONH_4$ (mg)	$(CH_3)_2CO$ (mg)	400 g/mol (mg)
3.4	1.00 (ref.)			
2.3	0.09	0.4	0.3	2.2
2.1	0.10	0.5	0.4	2.4
2.0	0.01	0.0	—	0.12
1.4 (brd)	0.07	—	—	1.8

Based on the assumption of the low molecular weight species being involved, then only submiligram amounts of these would be present in the sample containing 5.4 mg of Mo. What this means is that there does not appear to be enough organic compound, as indicated by ^1H NMR integrations, to complex with Mo on a 1:1 manner. The organic material present is believed to be impurities that have tracked along with the Mo through the purification process. Therefore, another explanation was sought to explain the ^{95}Mo NMR line broadening.

Besides pH and complexation with a ligand, paramagnetic metals can effect NMR line widths[45]. Of the possible metals that can cause this effect, copper was deemed most likely as it is found in slightly elevated levels in the plant material.

To test this, a NMR solution containing Na_2MoO_4 (13.5 mg, 0.056 mmol) was spiked with 9 µL of an aqueous 1µg/µL $CuSO_4$ (5.63×10^{-5} mmol) solution. Surprisingly a signal at 1.7 ppm was detected with a line width of 85 Hz. A very small addition of $CuSO_4$ caused a significant broadening of the NMR signal. The next step was to obtain an accurate measurement of Cu in the purified Mo sample to see whether this could affect the ^{95}Mo NMR spectrum. The Cu analysis was carried out by atomic absorption as described in the Experimental section. ICP Mo analysis was also carried out to determine the Mo concentration so the exact amount of Mo and Cu were known. Results from two separate analyses averaged 62.8 µg of Cu in the Mo sample. The Mo analysis found 4.57 mg of Mo present in the sample. To demonstrate that Cu was causing the ^{95}Mo NMR line broadening, a reference NMR solution containing the reported amounts of Cu and Mo was prepared. Approximately 4.57 mg Mo as $(NH_4)_6Mo_7O_{24}$ (8.43 mg, 0.00682 mmol) was dissolved in 0.5 mL of D_2O and transferred to an NMR tube. The pH was then adjusted to 10.2 so as to eliminate formation of polymolybdates. The ^{95}Mo NMR spectrum was then obtained (Figure 2.12). The sample was then spiked with 155 µL of a 1 µg/µL $CuSO_4$ solution (62.0 µg of Cu) and the spectrum recorded (Figure

$(NH4)_6Mo_7O_{24}$, pH adjusted to 10.2 with 50 %NH_4OH

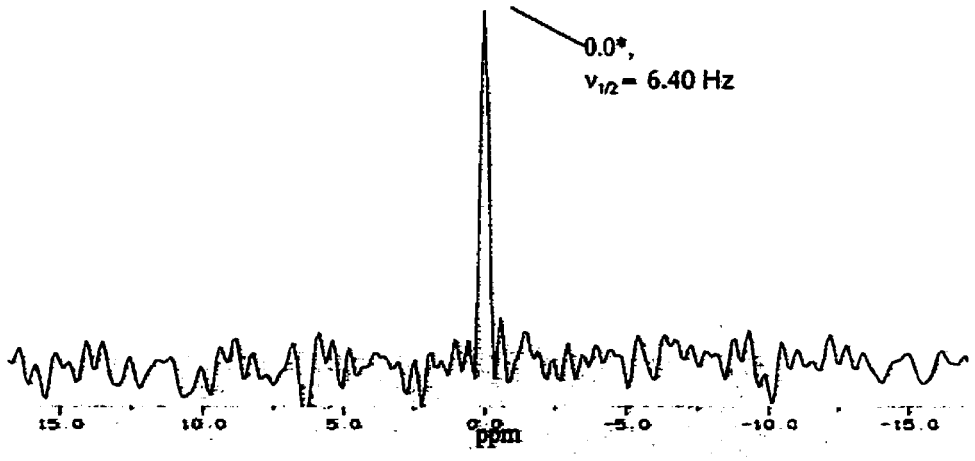


Figure 2.12 ^{95}Mo NMR spectra, chemical shift in ppm(*)

$(NH4)_6Mo_7O_{24}$, pH adjusted to 10.2 with NH_4OH, 155 µg of $CuSO_4$ (reference solution)

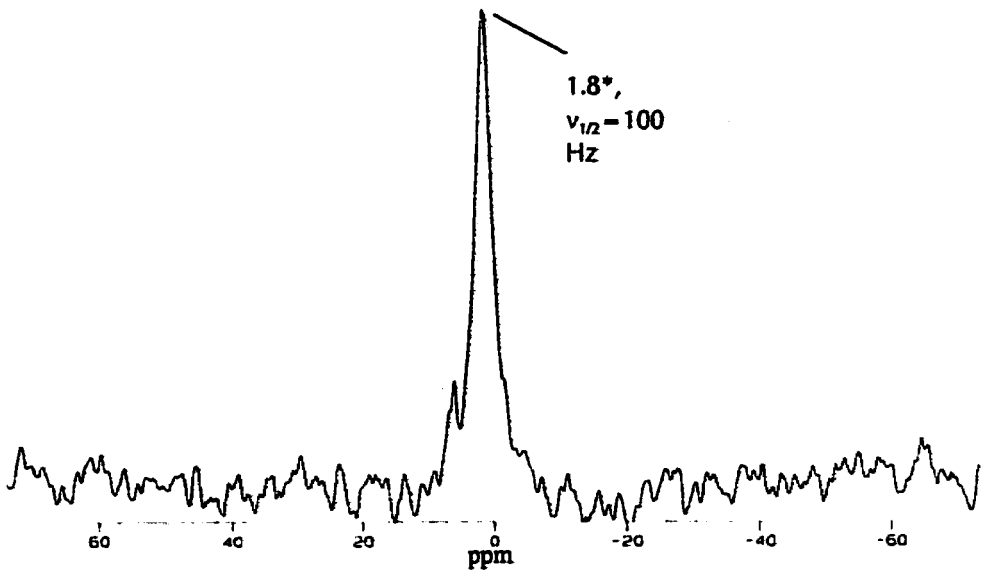


Figure 2.12 (cont.) ^{95}Mo NMR spectra, chemical shift in ppm (*)

2.12). The NMR solution contained the same amount of Mo and Cu as the purified Mo sample. $(NH_4)_6Mo_7O_{24}$ was used because the Mo species would exist after the HPLC ion exchange step, as the NH_4^+ salt. As expected, a sharp signal centered on zero was observed for the pH adjusted $(NH4)_6Mo_7O_{24}$ solution. The addition of the $CuSO_4$ caused a significant increase in the line width from 6 Hz to 100 Hz. The line width of the reference Mo/Cu solution ($v_{½}$ = 100 Hz) was very similar to the line width value of the purified Mo solution ($v_{½}$ = 109 Hz). The chemical shift of the reference solution was 1.8 ppm, which was found to vary slightly based on the shimming of the signal. This was also the case for the broad signal from the purified Mo fraction. The chemical shifts were therefore the same within experimental error, suggesting the presence of the same Mo species. Based on the above findings, it was concluded that the purified Mo species was the MoO_4^{2-} anion and 62.5 µg of Cu^{2+} caused paramagnetic line broadening of the ^{95}Mo NMR signal.

2.5 Summary and Conclusions

With the intent of determining the chemical form of Mo in alfalfa (*Medicago sativa*) grown on mine tailings, extraction, cleanup, and purification procedures were developed. Determination of optimal parameters for detecting Mo by ^{95}Mo NMR was also achieved. Mo was found to be present in its purified form as the MoO_4^{2-} anion. This was based primarily on multinuclear {^{1}H, ^{13}C, and ^{95}Mo} NMR studies.

A model Na-MoO_2(malate)$_2$ complex was prepared to aid in the purification and identification studies, but it was not detected in purified Mo fractions. It was found that Na_2MoO_4, Na-MoO_2(malate)$_2$, and the purified Mo species behaved similarly on the various TLC and HPLC systems tested. The stability of Na-MoO_2(malate)$_2$ on the preparative HPLC system was evaluated. After testing fractions by ^{1}H NMR it was concluded that the model Na-MoO_2(malate)$_2$ complex was not dissociating during chromatography. The anion exchange HPLC system did not possess the selectivity to separate very similar Mo species such as Na_2MoO_4 and Na-MoO_2(malate)$_2$.

No model extraction and purification procedures for Mo compounds were available in the literature to our knowledge, therefore development of new procedures was required and various purification schemes were tested. Physical characteristics of the Mo species included: highly water soluble; anionic; and a molecular weight of less than 2000 g/mol. Various extracting solvents and conditions were examined. Hexane and boiling ethanol washes of the plant material were used to increase extraction efficiency. A 20 mM NH_4OAc aqueous buffer was found to extract more than 80 % of the Mo from the plant material. The aqueous extract was partitioned with n-butanol and clarified through Celite. The weak anion exchanger DE-32 was employed as a cleanup step. Optimal elution of the Mo species was carried out with 0.4 M NaCl. Desalting of the Mo fraction was achieved using gel filtration by chromatography over Sephadex G-10. High molecular weight components were also removed in the exclusion volume. A

significant purification had been achieved at this point (eg 7.70 mg Mo in 384 mg of residue). An anion exchange HPLC method was developed for the final step using 100 mM NH4OAc buffer with 10 % acetonitrile as the mobile phase. The NH_4OAc could be successfully volatilized from the Mo fraction under high vacuum using a freeze drier, after the fraction had been concentrated to dryness. Recoveries at each of the purification steps ranged from 70.5 to 94.5% Mo.

Quantitative ^1H NMR along with ^{13}C NMR indicated no organic material was present in significant amounts to complex the purified Mo in a 1:1 ratio. Trace amounts of copper in the isolate resulted in paramagnetic line broadening of the ^{95}Mo NMR signal, which was demonstrated using reference solutions of ammonium molybdate containing copper sulfate. All spectroscopic data obtained indicated the occurrence of the MoO_4^{2-} anion in the final purified fraction derived from alfalfa plant material.

The developed purification procedure follows a logical and efficient sequence. Care was taken to use mild conditions and solvents so as to not alter the chemical form of the Mo species during purification. The Mo may exist as a variety of complexes in the plant, however a complex of MoO_4^{2-} was not observed after purification using the above-described conditions.

It is worth noting that similar work[50] being carried out at Agriculture and Agri-Food Canada on orchard grass (*Dactylis glomerata*) has also found Mo in its purified form to be present as MoO_4^{2-}.

2.6 Future Work

An explanation for the absence of molybdenosis in grazing livestock has yet to be determined. The present work on the chemical form of Mo in plants grown on the Highmont site does not provide an answer and different approach to the problem is required. Molybdenosis has been shown to involve a three-way interaction between Cu-S-Mo. The dependency of Cu absorption on dietary sulfur levels has been studied[46] under the conditions of moderately elevated levels of Mo

in the diet (1- 10 mg/kg DM) of sheep. This work established that a range of 2-5 g/kg sulfur in DM decreased copper absorption. In other words, 1000X more S than Mo was required in the diet to decrease Cu absorption. If the Mo is elevated by a factor of 100 without an increase in S, then S is no longer in excess and Cu absorption remains unaffected. Studies on extremely elevated Mo diets have not been reported to our knowledge. A quantitative approach to the amount of S, Cu, and Mo present in the diet and in the rumen is required to justify further work in this direction.

CHAPTER 3 EXPERIMENTAL

3.1 Plant material

All experimental procedures were carried out using aerial portions of *Medicago sativa* L. collected in the vegetative to bud stage from the Highmont tailings site on August 28, 1996. The plant material was freeze-dried using a Labconco cascade freeze-drier 8. The plant material was then ground to pass through a 1mm steel sieve using a Wiley Mill.

3.2 Molybdenum and Copper Analysis

Molybdenum dithiol Analysis [47]

Total Mo values (calc. in ppm Mo) in plant samples and in chromatography fractions were obtained in part using the dithiol procedure as described in the literature[47]. The following modified version of the dithiol procedure was used as a qualitative visual assay of Mo in chromatography fractions. The Zn-dithiol reagent was prepared by suspending 0.1g of 3,4-toluenedithiolato (2-)]-Zn hydrate (Aldrich 30,926-5) and 0.1g of ascorbic acid in 50 mL of 0.2 N NaOH followed by gentle heating with mixing to give a pink solution. The visualization procedure requires placing 3-5 drops of test solution in a spotting dish to which 3-5 drops of the dithiol solution and 6 N HCl are added. A green precipitate is indicative of Mo. The visual assay's detection limit was determined to be 1 ppm Mo.

Molybdenum ICP (Inductively Coupled Plasma) Analysis

A Thermo Jarrell Ash Atomscan 25 sequential inductively coupled argon plasma atomic emission spectrophotometer was used for all ICP Mo analyses. The molybdenum emission spectrum was monitored at λ-203.844 nm. A 2 kW crystal controlled radio frequency operating at 27.12 MHz powered the emission source. The peristaltic pump rate was set to 100 rpm (1.3mL/min). A 10 ppm Mo standard was prepared from dilution of a 100 ppm Mo stock solution prepared from Na_2MoO_4. A distilled water blank was used as the second point for calibration. ICP analysis was carried out directly on diluted chromatography fractions. Plant sample preparation required ashing in a muffle furnace at 475 ^{0}C followed by acid digestion with 0.5 N HCl before ICP.

Cu determination by Flame Atomic Absorption

An Instrumentation Laboratory IL Video II flame atomic absorption spectrophotometer was used for the Cu determination. The source for atomic absorption measurements was a Hollow Cathode Lamp irradiating at 324.7 nm. An air/acetylene mixture used a flow rate 1.5 mL/min at 10 psi. The resulting sensitivity for the Cu analysis was 0.03 ug/mL. The following Cu standards were used (in ppm Cu) 0.1 0.75, 1.5, and 2 ppm. Cu standards were prepared from a 100 ppm Cu stock HNO_3 solution, which was prepared from Cu pellets.

3.3 Chromatography

Ion Exchange Chromatography[48]

Two types of ion exchange resins were investigated during the course of the project. The commercially available (bond elute analylitichem) strong anion exchanger (SAX) was used as the pre-made solid phase extraction (SPE) cartridge. A vacuum manifold was used for the elution. Cartridge conditioning included activation (wetting) with 2 mL of CH_3CN followed by a wash with 2 mL H_2O. A weak anion exchanger Diethylaminoethyl cellulose (Whatman, DE-32, micro granular form) was prepared as follows. The required amount of resin was allowed to swell in 0.5 N NaOH for 30 minutes and then washed with distilled water on a Buchner funnel until the filtrate was neutral. It was converted to the acetate form by treatment with 10 % acetic acid, and then washed to neutral pH. The following optimized procedure was used for the DE-32 anion exchanger. The sample was dissolved in a minimal amount of H_2O and applied to the top of the resin bed under slight vacuum without letting the bed go dry. Once the entire sample had been applied the bed was washed with distilled H_2O corresponding to 10 times the bed volume. Elution of the Mo species was carried out with 0.1 M NaCl. The qualitative dithiol Mo assay was used to determine when Mo elution was near completion. This Mo containing salt fraction was then further purified.

Gel Filtration Chromatography[14]

Sephadex G-10 (Aldrich) was employed in all gel filtration experiments. Gel bed dimensions varied with sample size. The dry gel powder was suspended in 20 mM NH_4OAc with gentle mixing for at least 30 minutes. The solution was then degassed under reduced pressure. The slurry was then poured carefully down a tilted gel filtration column to prevent the formation of air bubbles. Void (V_0) and exclusion volumes (V_{excl}) were determined using a 2 mg/mL blue dextran solution.

Blue Dextran 2000 (Pharmacia Fine Chemicals) has a weight average molecular weight of 2 million and is therefore completely excluded from the gel bed. The blue dye also allowed visual inspection of the gel bed. The top of the bed was fitted with a piece of cheesecloth to prevent disruption of the bed surface upon the addition of liquids. The sample was dissolved in a minimal amount of 20 mM NH_4OAc and applied carefully to the top of the bed while eluting. Care is taken to avoid letting the bed go dry. Elution was continued with 20 mM NH_4OAc. Fractionation proceeded after collection of V_o and V_{excl}. The qualitative dithiol Mo assay was used to determine the Mo-containing fractions, which were pooled and taken on for further purification.

Regeneration of the G-10 bed was carried out by treating the used gel with 0.2 N NaOH in a large beaker with gentle stirring. The gel was then transferred to a large Buchner funnel and further washed with 0.2 N NaOH until all the pigments were removed from the gel. The gel was then washed with H_2O to a neutral pH.

Absorption chromatography on Polyamide

Column chromatography grade polyamide was used for all experiments. The eluotropic series for polyamide is: water, alcohol, acetone, formamide solution of sodium hydroxide. The following procedure was used. Using an appropriate amount of polyamide, a slurry in 5 % EtOH in H_2O was poured carefully into a glass column. The sample was slowly added to the top of the bed to avoid disturbance. Further elution was carried out with 5 % EtOH in H_2O under gravity. Fractions were then collected by eluting with 1 % NH_4OH.

Absorption Chromatography on decolorizing charcoal

All experiments were carried out with Norit decolorizing carbon (neutral and activated and deactivated forms of coconut charcoal). Deactivation of the coconut charcoal was achieved by treatment with 0.5 N HCl overnight, followed by washing

until a neutral pH was obtained. The other charcoals were applied directly to aqueous extracts.

Preparative cellulose thin-layer chromatography

The following systems were used over the duration of the project for preparative cellulose TLC. Ethanol (EtOH)-H_2O (1:1) and 60 % EtOH in H_2O with 1% NH_4OH and 0.25 M NH_4OAc. Preparative 1mm TLC plates were prepared using the following procedure. For three 20 x 40 cm plates, 37 g of Avicel PH 101 (Fluka) sorbent was added to 170 mL of H_2O in a blender with a variable speed control. Blending was done at 80 % speed for 20 sec then 100% for 10 sec, the slurry was applied to the glass plates with a Desaga spreader. The coated plates were then tapped on the bench to even out the applied layer. The coated plates were left to air dry for a minimum of 48 hours in a non-drafty area. The long edges of the plates were then scraped so solvent development would proceed evenly. Samples were dissolved in a small amount of H_2O and applied as a narrow band to one end of the plate (usually the best looking end) with a pipette or capillary. Usually two or three applications were made, allowing for the bed to dry between applications. The plate was then set in the tank containing the solvent for development.

High Performance Liquid Chromatography (HPLC)[41,49]

All preparative HPLC isolation was carried out using a Varian 9012 Liquid Chromatograph coupled to a Varian 2550 UV/visible detector or a Waters 510 pump, 712 sample processor coupled to a Waters 490 programmable multiwavelength detector. A variety of HPLC conditions and analytical columns

were tested. A Hamilton PRP-X100 4.1 mm x 250 mm analytical anion-exchange column was used for method development. A Hamilton PRPX- 100 7.1 mm x 305 mm semi-preparative anion exchange column was used for all preparative work. Optimized conditions for preparative work are as follows. The mobile phase consisted of 100 mM NH_4OAc with 10 % CH_3CN with a flow rate of 2.5 mL/min and UV detection at 246 μm. A PRP-X100 guard column was used in some cases. Sample preparation included filtering a diluted sample through a 0.45 μm filter before concentration. Sample concentrations ranged from 0.12 mg/μL to 0.2 mg/μL with a maximum sample size of 500 μL for injection into the HPLC system.

3.4 Spectroscopic Techniques

Molybdenum-95 Nuclear Magnetic Resonance (^{95}Mo-NMR) Spectra

^{95}Mo NMR spectra were obtained at 19.56 or 26.07 MHz using a Bruker AMX 2 300 or a Bruker AM-400 spectrometer respectively. The spectra were all collected in 99.9 % D_2O solutions at room temperature with 0. 1 M Na_2MoO_4 used as an external standard. The chemical shifts are all reported in parts per million (ppm) and the line widths at half height ($\upsilon_{1/2}$) are in Hz. For purified samples pH was measured directly on the NMR sample in a 5 mm tube. pH was adjusted directly with a 30 % NH_4OH solution in D_2O using a Corning 140 pH meter equipped with a 2 mm micro glass capillary probe.

Proton Nuclear Magnetic Resonance (^{1}H-NMR) Spectra

^{1}H NMR spectra were obtained at 200 or 400 MHz with Varian XL-200 or Bruker AM-400 spectrometers respectively. Spectra were recorded in D_2O with an internal reference signal of δ 4.80 ppm (residual HOD). The chemical shifts (δ) are reported in parts per million and the coupling constants (J) are given in Hz. The

multiplicities of signals are indicated by br d (broad doublet), d (doublet), t (triplet), dd (doublet of a doublet).

Carbon-13 Nuclear Magnetic Resonance (^{13}C-NMR) Spectra

^{13}C NMR spectra were determined at 50.3 or 100.6 MHz with Varian XL-200 or Bruker AM-400 spectrometers. Spectra were collected in D_2O using methanol (δ 49.0 ppm) as an internal reference. The chemical shifts (δ) of carbon were obtained using the broadband proton-decoupled mode, and are reported in ppm.

3.5 Purification Scheme

The optimized purification scheme is given below in Figure 3.1. Thirty grams of freeze-dried ground plant material (*Medicago sativa* L.) from the Highmont mine site 1996 was pre-washed on a sintered glass funnel with approximately 600 mL of Hexanes (drum) and boiling 95 % EtOH before extraction. The plant material was transferred to a mortar and pestle with 20 mM NH_4OAc and ground into a slurry. The slurry was then transferred to a glass column and extracted further with approximately 1900 mL of buffer. The aqueous extract was partitioned with 2 x 300 mL of n-BuOH. Both the aqueous and organic layer were concentrated to dryness on a roto-evaporator at 40 °C. EtOH was added to the sample to aid in the evaporation. The aqueous extract was then redissolved and clarified by filtration over celite. The sample was then directly applied to a 6 x 2.5 cm DE-32 (-OAc) anion exchanger under slight vacuum. The bed was then washed with approximately 500 mL of distilled water. A portion of this fraction was taken for Mo-ICP analysis. The remainder was concentrated to dryness on a roto-evaporator yielding 7.15 g of residue. Elution of the Mo species was carried out using 1.5 L of 0.2 M NaCl. Various fractions were collected and assayed for Mo using the visual dithiol procedure. Elution was complete when no Mo was detected in the eluant. The DE-32 anion exchange resin was ashed at 475° for 24 hours, digested in 0.5 N

HCL, then diluted with water to 50 mL for Mo ICP analysis. The Mo salt fraction was taken to dryness and weighed. A 5cm x 46 cm Sephadex G-10 column was prepared as described above for desalting and for further purification by molecular weight fractionation. The column was equilibrated with 20 mM NH_4OAc. The V_{void} and V_{excl} volumes were determined to be 300 mL and 20 mL respectively using a 2 mg/mL Blue dextran 2000 solution. The maximum soluble amount of the Mo containing salt fraction in 20 mL of buffer was slowly applied to the top of the gel bed. After the entire sample had entered the bed elution was continued with the buffer solution. Further

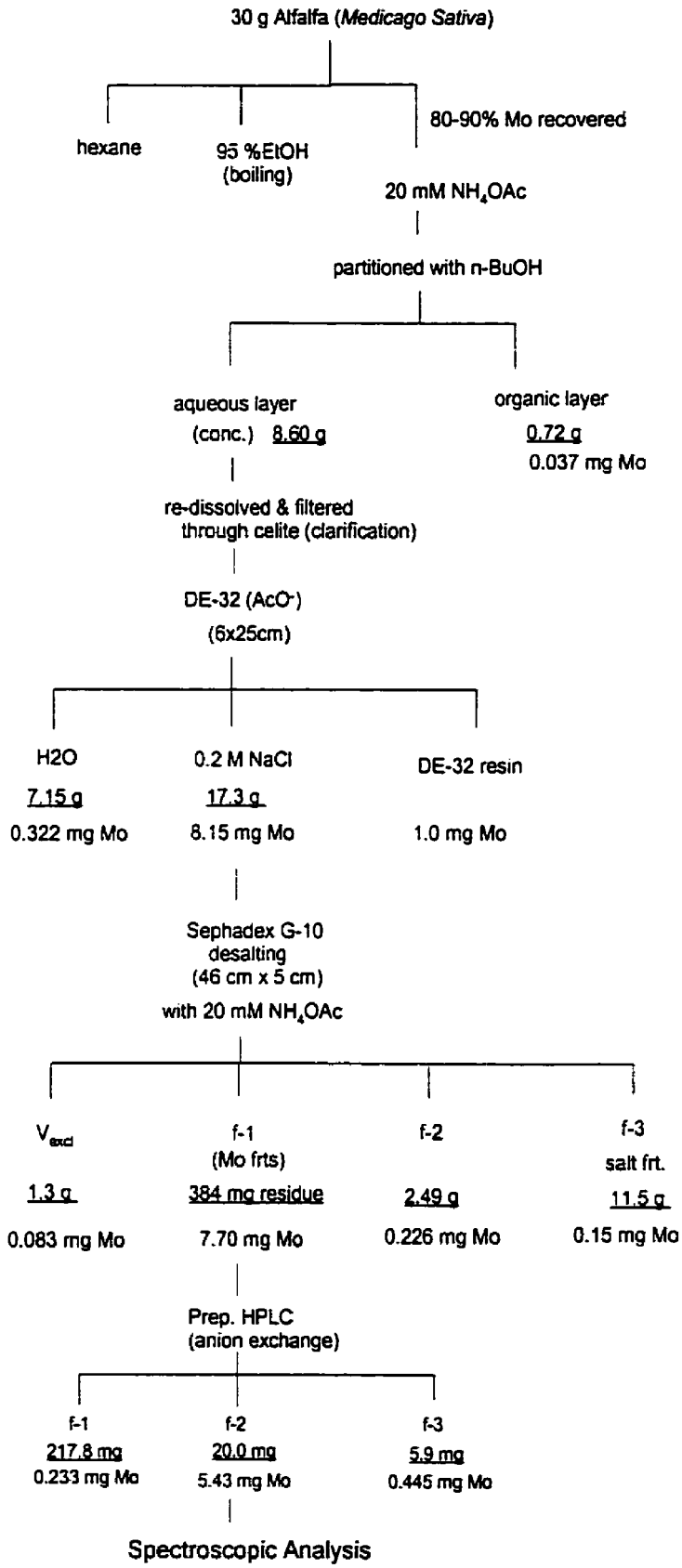


Figure 3.1 Optimized purification procedure for Mo from alfalfa

Table 3.1 Example of desalting of Mo-fraction on G-10 Sephadex

Fraction	Volume (mL)	Mo dithiol assay (+) relative [Mo]	Groupings
void volume	300	—	
exclusion volume	20	—	
1	40	+	1-4
2	40	+ + + + + +	
3	40	+ + +	
4	20	+	
5	20	—	
6	20	—	6-9
7	100	—	
8	120	—	
9	120		

fractions were collected and assayed as shown in Table 3.1. The Mo-containing fractions (1-4) free of NaCl were pooled and concentrated. The void and exclusion volumes were collected and tested for Mo using the visual dithiol assay. The salt containing fractions (6-9) were pooled and concentrated for Mo-ICP analysis. The above desalting procedure was repeated on the remaining Mo salt fraction from DE-32 (previous step) until the entire sample was desalted. These were carried out in a similar manner to those shown in Table 3.1. It should be noted that no regeneration of the Sephadex was required during desalting. The desalted Mo-

containing sample (7.70 mg Mo, ca 384 mg residue) was further purified by preparative anion exchange HPLC.

A Hamilton semi-preparative LC-column was used for the purification step using the above-mentioned optimized conditions. Sample preparation was carried out as described above. A subsample of the desalted Mo fraction was diluted to 2 mL with mobile phase. A trial run with the diluted subsample was carried out using the following conditions: 0.1 M NH_4OAc with 10 % CH_3CN, 2.0 mL/min, UV detection at 235 nm and a 50 uL injection volume. The remaining Mo sample was dissolved in approximately 1 mL of mobile phase for purification. Three fractions were collected during each preparative run. The fractionation run times are as shown in Table 3.2. Similarly numbered fractions from the

Table 3.2 Preparative- HPLC purification of Mo fraction

Runs	Fractions (mins.)		
	1	2	3
1	3.50-36.7	36.7-73.0	73.0-90.0
2	3.00-33.5	33.5-76.0	76.0-90.0
3	4.50-32.0	32.0-80.0	80.0-90.0
4	9.00-33.0	33.0-66.0	66.0-90.0
5	7.00-33.0	33.0-65.0	65.0-90.0
6	5.00-33.0	33.0-66.0	66.0-90.0
7	4.50-33.0	33.0-55.0	55.0-90.0

runs were pooled and concentrated to dryness on a roto-evaporator. The samples were then dissolved in a minimal amount of H_2O and transferred into preweighed vials and freeze-dried. The samples were analyzed by ICP to determine Mo content. Spectroscopic analysis of the Mo containing fraction has been discussed in the text.

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Appendix

Preparation of $Mo(VI)O_4^{2-}$ (l)-malic acid complexes in a 1:2 ratio

During the method development stage malate was identified by both HPLC and ^1H NMR as a component in various semi-purified fractions. Synthetic complexes of this type have been reported in the literature[28c]. Therefore, a Mo-malate complex was a candidate, which required further investigation. To act as an aid in developing purification procedures and to investigate the Mo-malate candidate, synthetic complexes of this type were prepared.

Literature precedence for the preparation of the NH_4^+ salt is available[28c]. Examples of preparations are given below. The sodium (Na) salt was prepared as follows. (l)-malic acid (2.68 g, 20 mmol) was dissolved in 20 mL of distilled H_2O. $Na_2MoO_4.2H_2O$ (2.41 g, 10 mmol) of was added and mixed using a magnetic stirrer. The pH was immediately adjusted to 5-6 using 3 N NaOH. The solution was allowed to evaporate overnight producing a viscous liquid. Ethanolwas added until cloudiness persisted. water was then added dropwise until the cloudiness faded. The solution was then put into the fridge (1 °C) overnight. The fine crystals that formed were collected on a Buchner funnel and washed with cold 90 % EtOH. Yield 3.187 g, 73 % yield

^1H NMR: 2.875 (d, 4H, 5.74 Hz), 5.12 (br d, 2 H)

^{13}C NMR: 38.84 (CH_2), 80.70 (CH), 175.30 (C=O), 184.00 (C=O)

^{95}Mo NMR: 83.50 ($\upsilon_{1/2}$- 113 Hz)

The ammonium salt was prepared using the following procedure. Ammonium molybdate ($(NH_4)_6Mo_7O_{24}.4H_2O$) (1.76 g, 1.42mmol) and (l)-malic acid (2.68 g, 20 mmol) were dissolved in a minimum amount of H_2O while mixing with a magnetic stir bar. The pH was immediately adjusted to approximately 5 with a 5

% NH_4OH solution. EtOH was added until the solution turned slightly cloudy. Fine crystals formed after slow evaporation over time. The crystals were collected on a Buchner funnel and air dried. Yield 1.303 g, 30.6 % yield

^1H NMR: 2.82 (d, 4H, 5.81 Hz), 5.12 (br d, 2H)

^{13}C NMR: 1.95 (CH_2), 82.47 (CH), 179.24 (C=O), 184.86 (C=O)

^{95}Mo NMR: 83.40 ($\upsilon_{1/2}$- 175 Hz)

Multinuclear NMR experiments determined unambiguously that a complex had been formed. A broad peak (113 Hz) at 83 ppm in the ^{95}Mo NMR spectrum indicated that a complex existed, which complemented ^1H and ^{13}C NMR evidence. It was important to know the stoichiometry of the prepared complexes. The amount of Mo in a known amount of the Na-MoO_2(malate)$_2$ complex was determined by Mo ICP analysis. A theoretical weight for a 2: 1 malate – Mo complex was determined from this value, which agreed with the known amount that was initially weighed out. The synthetically prepared Na-MoO_2(mal)$_2$ complex was used throughout the purification and characterization protocol as a test standard.