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To my family

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CHEMICAL STUDIES ON SOME PLANTS THAT HYPERACCUMULATE NICKEL

A thesis presented in partial fulfilment of the requirements

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ABSTRACT

Following the discovery of the hyperaccumulation of nickel by the Philippine plants Dichapetalum gelonioides subsp. tuberculatum and Phyllanthus 'palawanensis', the nature of the nickel in aqueous extracts has been investigated by gel filtration chromatography, ion-exchange chromatography, high-voltage electrophoresis and GC-MS.

Nickel in D.gelonioides subsp. tuberculatum was shown to associate mainly with compounds of high polarity and low molar mass. In P.'palawanensis' only about 50 % of the nickel demonstrated this association, while 25 % of the metal appeared to be in the form of pectate or bound to proteins. In both plants, nickel was shown to exist in anionic and cationic forms. A discussion of the usefulness of assigning portions of nickel to these forms is presented in the light of changes in the relative amounts of cationic and anionic nickel observed during ion-exchange chromatography and high-voltage electrophoresis.

Nickel , citric acid and malic acid comprised 95 % of the purified extract from D. gelonioides subsp. tuberculatum. Only 25 % of the low molar mass, high polarity nickel-rich fraction from P.'palawanensis' was accounted for by these constituents. Small amounts of Ca, Mg, K and Na were detected in each extract. The nickel:citric acid:malic acid mole ratios were 1:0.4:1 and 1:0.4:0.4 for D.gelonioides subsp. tuberculatum and P.'palawanensis' respectively. These observations are discussed in terms of the stabilities of the nickel citrate and nickel malate complexes. Tartaric acid was identified in both extracts, while 4-oxopentanoic acid and 2-furylacetic acid were identified in the nickel-rich fraction from P.'palawanensis' only. The role of these acids in the plant is discussed in an attempt to explain their presence in the nickel-rich material.

By using X-ray crystallography, it was shown that crystals obtained from a nickel-citrate-malate solution simulating the extract from D. gelonioides subsp. tuberculatm, contained nickel exclusively in the form of an anionic Ni(II)-citrato complex. It was assumed that a crystal obtained from a nickel-citrate-malate solution of mole ratio 1:0.4:0.4, as in the nickel-rich fraction from P.'palawanensis', would have yielded similar results given the greater stability of the Ni-citrate complex over the Ni-malate complex.

Pot trials carried out on Alyssum troodii confirmed its hyperaccumulating status, and showed it to be a cobalt hyperaccumulator as well. The amount of cobalt taken up by the plant was an order of magnitude lower than that of nickel. It was observed that A.troodii survived soils with

available concentrations of nickel and cobalt at least five times higher than those commonly found in serpentine soils. Possible reasons for this behaviour are presented. Alyssum troodii also co-accumulated nickel and cobalt. However, while the cobalt concentration in plant organs showed little difference from that obtained when the plant was cultivated in soil enriched with cobalt only, the nickel levels were lower.

Aurinia saxatilis did not hyperaccumulate nickel and cobalt. The levels of the metals found in the plant were one-tenth of those observed in A.troodii. As in the Ni-hyperaccumulating plant, cobalt uptake appeared to suppress nickel uptake when the plant was cultivated in media containing added nickel and cobalt. A possible uptake mechanism giving rise to this differential uptake is discussed. Very little difference was discerned in the tolerance to, and uptake of, copper in the two plants. The levels of this metal in A.troodii were about one-tenth those of cobalt, while in Au.saxatilis the levels of copper and cobalt were comparable.

Low concentrations of nickel exerted a stimulatory effect on the germination of A.troodii seeds. Cobalt appeared to exert this effect on Au saxatilis seeds at higher concentrations. Copper was not observed to be stimulatory to either plant.

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CHAPTER I

General Introduction

I.1. Introduction

The association between substrate and vegetative cover was recognised as early as the sixteenth century when Agricola (1556) noted unusual colouration and growth of plants associated with mineralised soils (Woolhouse, 1980). During the following century, Pope Pius II discovered an aluminium deposit in Italy by simple observation of the distribution of the Holly tree Ilex aquifolium (Brooks and Malaisse, 1985). Several taxa have since been found on heavily mineralised soils containing metals such as Ni, Cu, Co, Pb and Zn. A review of the distribution of these taxa, their ecology and phytochemistry is given by Linstow (1929), Ernst (1974a, 1974b, 1975) and Antonovics et al. (1971). Plants that appear to be restricted to heavily mineralised soils are referred to as **metallophytes.**

Numerous metallophyte communities can be located on ultramafic ("serpentine") areas throughout the world (Table I.1). It has been hypothesised that these substrates may be unsuitable for the proliferation of most plants; metallophytes are therefore exceptions (Brooks and Malaisse, 1985). One third of New Caledonia is covered with ultramafic soil which supports a substantial number of metallophytes. Metallophytes are also found on ultramafic soil in Greece, Turkey, Italy, Ukraine, North West USA, Japan, Philippines, Australia, Brazil, South Africa and United Kingdom (Brooks, 1987). Serpentine is produced by the hydration of the ferromagnesian silicate - olivine. Similarities in ionic radii and charge facilitate isomorphic substitution such that Co^{2+}, Ni^{2+} and Cu^{2+} ions can replace Mg^{2+} ions while Cr^{3+} ions can replace Fe^{3+} ions. Thus in general, the siderophiles nickel, cobalt, chromium and copper are strongly enriched in ultramafic soils.

Apart from the high concentrations of potentially phytotoxic elements [Ni : 3000-5000 $\mu g\ g^{-1}$; Co: 500 $\mu g\ g^{-1}$; Cr: 200-2000 $\mu g\ g^{-1}$and Cu: 50- 5000 $\mu g\ g^{-1}$], serpentine is also characterised by a high magnesium content and low concentrations of Ca, N,P and K. These factors are responsible for the relative infertility of serpentine soils. The general view is that a combination of all three factors influences soil fertility and therefore also dictates the nature of the vegetative cover. However, various workers have shown that the addition of lime to serpentine soils improves their fertility for crops (Hunter and Vergnano, 1952; Crooke and Inkson, 1955; Jowett, 1958; Gregory and Bradshaw, 1965; Proctor, 1971).

Table I.1 World-wide Distribution of Serpentine Vegetation

COUNTRY/REGION	LOCATION
New Zealand	South Island (west coast)
Australia	Western Australia, New South Wales, South-central Queensland
Northwest Europe	United Kingdom, Norway, Sweden, Finland
Southcentral Europe	Spain, Portugal, France, Germany, Austria, Italy, Corsica, Yugoslavia, Albania, Greece, Cyprus
Continental Asia	Russia, Turkey, Iran, Oman, India, Sri Lanka
Southeast Asia	Japan, Philippines, Indonesia, Malaysia, Celebes, New Guinea, Solomon Islands
Tropical America	Brazil, Puerto Rico, Cuba
North America	Northern California,Pacific Northwest, USA (east coast from Alabama to New Jersey), Eastern Canada (Quebec, Gaspe Peninsula, Newfoundland)
Southern Africa	Zambia, Zimbabwe, South Africa

(Compiled from Brooks, 1987; Brooks et al., 1990; Morrey et al., 1989; Batianoff, et al., 1990)

Large areas of non-serpentine soil have also been successfully colonised by metallophytes. In this respect, the species that inhabit soils in volcanic areas which contain high levels of S and B (Hewitt and Smith, 1975), the Zn (Calamine) flora of Western Europe and Britain (Brooks, 1987), the Se flora of Western USA, and the Cu and Co tolerant vegetation of Southcentral Africa (Brooks andMalaisse,1985) are noteworthy. Cognisance should also be taken of the Na tolerant plants (halophytes) which inhabit salt marshes and deserts (Chapman, 1975). No doubt the increase in spatial separation of metal refining and metal processing industries over the past 150 years has resulted in a rather wide distribution of heavily contaminated non-serpentine soil available for colonisation by and evolution of metal-tolerant vegetation (Ernst, 1989). The vegetation in many of these soils is still to be studied.

I.1.I. Metallophytes Containing Elevated Levels of Nickel

Generally, the supply of nutrients in soils is scarcely ever optimal for plant growth owing to a deficiency of one or more elements. This situation is augmented by the occasionally high concentration of a phytotoxic element. Under such conditions most species die but a small minority can survive through adaptation. Metallophytes constitute this minority.

In 1948, Minguzzi and Vergnano reported >1000 $\mu g\ g^{-1}$(0.1 %) Ni in dry tissues of Alyssum bertolonii Desvaux found in Tuscany, Italy. This inordinately high concentration of Ni contrasted markedly with ~1 $\mu g\ g^{-1}$ usually found in plants growing over non-nickeliferous soils and 10 -100 µg/g in plants growing over Ni- rich substrates. Some 13 years later, >1% Ni was reported in dry leaves of Alyssum murale Waldst.and Kit. from Turkey (Doksopulo, 1961). Menezes de Sequeira (1969) discovered a third Ni metallophyte belonging to the genus Alyssum :- A.serpyllifolium subsp. lusitanicum T.R Dudley and P. Silva (= A. pintodasilvae Dudley,1986) from Northeast Portugal, was subsequently shown to contain ~9000 $\mu g\ g^{-1}$ Ni by Brooks et al. (1979). The first Ni metallophyte discovered in Africa was Dicoma niccolifera Wild (formerly D. macrocephala subsp. Wild, 1970, 1971). Severne and Brooks (1972) and Cole (1973) independently reported elevated levels of Ni (~10000 $\mu g\ g^{-1}$) in specimens of Hybanthus floribundus (Lindl.) F. Muell. supported by Ni-rich substrates of Western Australia. Similar

observations were made for three subspecies (Bennett, 1969; Severne, 1972). During the period 1974-1980, a substantial number of plants growing over Ni-rich serpentine in New Caledonia were found to contain high concentrations of Ni (Brooks et al., 1977; Lee et al., 1977; Kersten et al., 1979; Jaffré, 1980). The New Caledonian tree Sebertia acuminata Pierre ex Baill. is noteworthy as it contained > 20 % Ni in its dry blue-green latex (Jaffré et al., 1976)

In 1977, Brooks et al., applied the term "hyperaccumulator" to plants found to have accumulated Ni to such a degree that dry matter concentrations were in excess of 1000 $\mu g\ g^{-1}$. The discovery of three Ni-hyperaccumulating species within the genus Alyssum naturally stimulated an interest in other members of the genus. Subsequently, nearly 50 additional species of Alyssum were found to possess hyperaccumulator status (Brooks and Radford,1978; Brooks et al.,1979). The term has also been applied to plants containing Cu and Co concentrations >1000$\mu g\ g^{-1}$in dry tissue (Brooks et al., 1980), and to those containing > 10000 $\mu g\ g^{-1}$ Zn and Mn (Baker and Brooks, 1989). The concentrations of Zn and Mn in plants growing over soils containing normal levels of these metals is about one to two orders of magnitude higher than the concentration of Ni in plants growing over non-nickeliferous soils (Hewitt and Smith, 1975). An African serpentine endemic - Pearsonia metallifera Wild was shown to contain 1.53% Ni by Brooks and Yang (1984). As a result of recent expeditions, several Ni-hyperaccumulating plants have been discovered in Philippines [Palawan] (Baker and Proctor, 1988) and Brazil [Goiás] (Brooks et al., 1990). Among the Brazilian taxa, the low forest shrub Jatropha sp. was of particular interest owing to the high level of Ni (1.35 % on a dry weight basis) found in its white latex (Brooks et al., 1990). These workers also discovered the first Ni-hyperaccumulating fern - Adiantum sp. during their expedition. Studies carried out on soils and vegetation associated with ultramafic rocks in Transvaal, South Africa revealed yet another Ni-hyperaccumulating plant. Berkheya codii Roessl. was shown to have dry a leaf Ni concentration of 11637 $\mu g\ g^{-1}$, while dry stems and roots contained 4344 and 2127 $\mu g\ g^{-1}$ Ni respectively (Morrey et al., 1989). Batianoff et al. (1990) are credited with the discovery of the Ni-hyperaccumulating plant Stackhousia tyronii Bailey from Queensland, Australia. Leaves were reported to contain 1-2 % Ni. Other parts of the plant were shown to have Ni concentrations ranging from 0.1-1 %.

The low levels of Co, Cr and Cu in Ni hyperaccumulators can be related to their availability to the plant as evidenced by extractability tests. Chromium is the least available of the three elements, followed by copper and then cobalt. Chromium is virtually unavailable in the soil because of its existence as insoluble Cr(III) oxides and hydroxides (McGrath and Smith, 1990) which may also be occluded in hydrous oxides of iron (Cary et al., 1977). The concentration of Co in Ni-rich serpentine is usually five to ten times lower than that of Ni (Brooks et al., 1977). Since Ni and Co are chemically similar, it is reasonable to assume that the percentages of the metals available to the plant will be similar. A Co concentration one order of magnitude lower than that of Ni is therefore not unlikely in a Ni-hyperaccumulating plant. The Ni-poor soils of Southcentral Africa are an exception. The interaction between hydrous oxides of Fe and Mn, and organic matter is the principal means through which Cu is rendered non-exchangeable in the soil (Loneragan et al., 1981). Nonetheless, a significant pool of diffusible Cu exists in organic complexes which is in equilibrium with a low level of Cu in the soil solution.

I.1.II. The Essentiality of Nickel to Plants

While the essentiality of Cu for plants has been firmly established (Sommer, 1931), this has not been the case for Ni, Co and Cr. There have however been reports of apparent stimulation of plant growth by low concentrations in solution culture (Bollard, 1983).

According to Mishra and Kar (1974), Ni could be important to the activity of several enzymes, the majority of which are found in higher plants. The first definite assignment of Ni to a role in plant metabolism came from Dixon et al. (1975) who provided evidence that Ni was an active site component of urease from the jack bean (*Canavalia ensiformis*). Based on this work the concentration of Ni in the enzyme was found to be 0.12 %. Polacco (1977a) observed that the synthesis of urease in soybean, tobacco and rice callus was stimulated by Ni and suggested that the metal might be a component of all plant ureases. Eskew et al. (1983) showed that Ni was essential for soybeans growing in urea free media irrespective of whether they were allowed to fix nitrogen or were supplied with inorganic nitrogen. Similar findings were reported for cowpeas by Eskew et al. (1984). Using tomato plants, Checkai et al. (1986) observed that meristems of Ni-deficient specimens developed necrosis following chlorosis of their newest leaves. Brown et al. (1987)

showed that the life-cycle of barley was not completed in the absence of Ni. In all of these studies, Ni was required at the nanogram level. This is the first time strong evidence has been amassed in support of the essentiality of Ni in higher plants. Welch's view (1981) that Ni might be required for the mobilization of stored seed-nitrogen through ureides or arginine during early stages of seedling growth is noteworthy. At present there is no experimental evidence for Ni as an essential element in hyperaccumulator plants. A Ni requirement has been reported for the growth of some microbes (Bartha and Ordal, 1965; Bertrand and De Wolf, 1967; Van Baalen and O'Donnell, 1978).

Nickel is ubiquitous in the lithosphere and biosphere, constituting ~0.008% of the earth's crust. The largest fraction can be found in igneous rocks, which contain about 0.01 % Ni. Therefore should Ni be classified as an essential element, deficiency symptoms are expected to be rare since background levels of the element in natural soil and experimental media will inevitably be adequate for the plant needs. Symptoms of Ni toxicity include chlorosis, stunted growth of roots and shoots and growth abnormalities (Mishra and Kar, 1974). The major growth-inhibiting effect of toxic levels of Ni appears to be depressed enzyme activity (De Kock et al., 1960; Kratochvil et al., 1967).

Cobalt has been reported to be essential for growth of nodulated legumes (Ahmed and Evans, 1960,1961; Hallsworth et al., 1960; Delwiche et al., 1961; Wilson and Reisenauer, 1963). There appears to be no evidence of essentiality of Cr for plants (McGrath and Smith, 1990).

I.1.III. Distribution of Nickel Hyperaccumulators in the Plant Kingdom

Nickel hyperaccumulators existing as herbs, shrubs and trees now comprise about 170 taxa representing a relatively small number compared to the thousands of taxa found on nickeliferous substrates (Baker and Brooks, 1989; Batianoff et al., 1990; Brooks et al., 1990). Hyperaccumulators are distributed among many families of the dicotyledons and a few monocotyledons. Of these, 62 % are confined to the Brassicaceae family in which only a few genera in the Northern temperate zone contain many of the Ni-hyperaccumulating species. Tropical genera in which Ni-hyperaccumulation occurs are members of many families including the Flacourtiaceae, Violaceae, Cunoniaceae and Euphorbiaceae (Table I.2). To date, 24 Cu

hyperaccumulators and 26 Co hyperaccumulators have been identified. Most of these taxa and those that hyperaccumulate both metals belong to the Lamiaceae and Scrophulariaceae families (Baker and Brooks, 1989).

Brooks et al. (1979) suggested the promotion of section Odontarrhena of the genus Alyssum (Brassicaceae) to a distinct genus separate from Alyssum, based on the Ni-accumulating capability of the group and its unique combination of morphological characters. Recently, a study of Ni-hyperaccumulation in other genera of the Brassicaceae led Reeves (1988) to conclude that metal-accumulating potential may be confined to certain sections of the genera in which it occurs. He also noted that separate generic status had at some stage been accorded all those sections of genera in which Ni-hyperaccumulation is known to occur. Metal-accumulation may therefore be of taxonomic significance.

I.2. Evolution

The observation that some taxa can be found on metalliferous soils while others are not, raises interesting questions regarding evolutionary processes involved in adaptation to metalliferous environments. Bradshaw (1984) suggested that evolutionary processes and the level of adaptation were dictated by genetic variability. According to him, most species were in a state of genostasis that prevented evolutionary change. Thus, the minority of species that are not in this state may be precursors of metal-tolerant species. In addition to the Ni-hyperaccumulating plants, other metal-tolerants can be found among the families listed in Table I.2. Heavy metal tolerance therefore appears to have arisen independently; no obvious phylogenetic relationship is apparent.

Table I.2. Families Containing Nickel-hyperaccumulating Plants
(Using the Classification of Cronquist ,1981; Updated from Brooks, 1989)

CLASS	SUBCLASS	ORDER	FAMILY
Filicopsida	Leptosporangiatae	Filicales	Adiantaceae
Magnoliopsida	Magnoliidae	Magnoliales	Myristaceae
	Caryophyllidae	Caryophyllales	Caryophyllaceae
			Amaranthaceae
	Dilleniidae	Theales	Dipterocarpaceae
			Ochnaceae
		Malvales	Tiliaceae
		Violales	Flacourtiaceae
			Turneraceae
			Violaceae
		Capparales	Brassicaceae
		Ebenales	Sapotaceae
			Ebenaceae
			Oncothaceae
	Rosidae	Rosales	Cunoniaceae
			Grossulariaceae
			Saxifragaceae
		Fabales	Fabaceae
		Celestrales	Dichapetalaceae
			Stackhousiaceae
		Euphorbiales	Buxaceae
			Euphorbiaceae
		Sapindales	Anacardiaceae
	Asteridae	Gentianales	Asclepiadaceae
		Lamiales	Lamiaceae
			Verbenaceae
		Scrophulariales	Scrophulariaceae
			Acanthaceae
		Rubiales	Rubiaceae
		Asterales	Asteraceae
Liliopsida	Commelinidae	Juncales	Juncaceae
	Liliidae	Liliales	Velloziaceae

The highly disjunct distribution of metal-tolerant species (Table I.1) poses questions about the time-scale over which evolution occurred. The substrates that support the endemic Cu and Co tolerant flora of Southcentral Africa contain a graduation of metal concentrations, ranging from background to heavy mineralisation. Thus the area affords the opportunity of studying various stages of the evolution of the metallophytes (Brooks and Malaisse, 1985). Wild (1978) postulated that Cu and Co metallophytes, and serpentinophytes evolved in Southern Africa in the Mesozoic period (135 million years ago). Some metal deposits are believed to have been formed during the Pre-Cambrian period (3000 million years ago), thus the substrates were already available to facilitate evolution of metal-tolerant flora. Brooks and Malaisse (1985) concluded that other metallophytes in the temperate zone also evolved during the Mesozoic period. Their conclusion was based on the premise that in Canada and Northern Europe, where metallophytes were thought to be virtually absent, sufficient time would not have elapsed since the last glaciation (10000-20000 years ago) to facilitate comparable evolution. However, many metal-tolerant plants have been found in Canada and Northern Europe (Brooks, 1987). Further, there is increasing evidence to show that metal-tolerance can occur in a few generations on newly contaminated environments such as mine spoils (Antonovics et al., 1971; Wu and Bradshaw, 1972; Proctor and Woodell, 1975; Bradshaw, 1984). According to Wu and Bradshaw (1972) and Bradshaw (1984), evolution of tolerance can occur within one generation in plant populations as a result of the selective forces of metal toxicity and appropriate genetic variability.

MacArthur and Wilson's Theory of island biogeography states that metalliferous sites could be viewed as being isolated from each other by large expanses of non-metalliferous soil. This kind of distribution can provide the type of geographical environment in which quite restricted or endemic species can emerge. Stebbins (1942) and Turrill (1951) defined two kinds of endemism:- **paleoendemism** and **neoendemism**. Paleoendemic plants are considered relics of once widely spread taxa which became restricted in their distribution owing to climatic changes and/or competiton from other taxa. Metallophytes derived from neighbouring non-metallophytes in response to adverse edaphic or environmental conditins are neoendemic. Nickel-hyperaccumulating plants are considered to be paleoendemic (Kruckeberg, 1954; Brooks and Malaisse, 1985; Brooks, 1987). The metal-tolerant strains that evolved over heavily mineralised areas, produced as a result of industrial mining activity are classified as neoendemic (Antonovics et al., 1971). It has been

observed that neoendemic taxa are often morphologically indistinguishable from their relatives from which they are not reproductively isolated (Antonovics et al., 1971). Brooks and Malaisse (1985) noted that distinctions may, at times, only be discernible at the varietal level or from pot trials involving metal-tolerant and non-tolerant strains. A detailed discussion of the evolutionary processes that mediate the selection of tolerant individuals, and ecotypic differentiation of adapted populations growing on metalliferous soils is presented by Baker and Proctor (1990).

I.3. Adaptation to Toxic Metals

Just as plants have developed the means to combat nutrient deficiencies (e.g phosphorus acquisition by mycorrhizal association, nitrogen fixation by Rhizobium bacteria in root nodules of leguminous plants and the ingestion of insects by insectivorous plants), there is evidence that plants have the capacity to deal with toxic concentrations of certain elements. This is manifested by a series of biochemical and physiological adaptations either for the purpose of metal exclusion or metal accumulation (Baker, 1987).

The hallmark of metal exclusion is restricted uptake and transport. Increased exudation of metal-chelation compounds and mycorrhizal associations have proved effective as restrictive mechanisms (Vancura and Hovadik, 1965; Rovira, 1969; Bradley et al., 1981; Bradley et al., 1982). Accumulated metals are rendered non-toxic by deposition in cell vacuoles, formation of insoluble precipitates and complexation by organic acids, amino acids and their derivatives. In some Zn metallophytes, chelation by oxalic and malic acids has been reported (Mathys, 1977). Several workers have demonstrated an association of Ni with various carboxylic and hydroxycarboxylic acids in Ni hyperaccumulators (Pancaro et al., 1978; Lee et al., 1978; Kersten et al., 1980). It has been observed that synthesis of potential metal-binding compounds is increased in plants exposed to elevated levels of heavy metals (Torii and Laties, 1966; Shaw, 1989). Tolerance may also be mediated by enzymic adaptations, alterations of cellular metabolism and changes in the permeability of cellular and sub-cellular membranes (Wainwright and Woolhouse, 1975; Woolhouse, 1980). The recent isolation of **phytochelatins**, a group of polypeptides consisting of γ-glutamylcysteinyl glycine residues from plants exposed to heavy metals has

attracted wide attention (Grill et al., 1985; Robinson and Jackson, 1986). The role (if any) of this family of compounds in Ni hyperaccumulators is yet to be assessed.

I.4. Use Of Metallophytes

Over the past 50-60 years, metallophytes have attracted significant attention from scientists in the areas of biosystematics, ecology, molecular biology, physiology, chemistry, biochemistry, and geology. They may be of potential benefit to mankind in following ways:-

a) Location of previously unrecorded or poorly recorded ultramafic substrates can result in the production of more up to date geological maps. Brooks and Wither (1977) and Wither and Brooks (1977) successfully used herbarium specimens to locate unknown ultramafic areas in Southeast Asia. These workers also suggested that the Ni hyperaccumulator Rinorea bengalensis (Wall.) O.K. of the Violaceae family could be ideal for defining ultramafic sites in Southeast Asia and Northern Australia due to its ability to colonise a variety of such substrates in these areas.

b) Metal prospecting by the observation of vegetative cover, thereby using metallophytes as indicator plants (i.e geobotanical prospecting), and carrying out chemical analysis to detect mineralisation at depth (i.e biogeochemical prospecting). A few metal-tolerant plants from Southcentral Africa (Brooks, 1978) and North America (Wickland, 1989) have been used for these purposes.

c) Metallophytes are also of phytoarchaeological interest. A study of their distribution and remains can result in the location of buried archaeological features (Brooks, 1989).

d) Extreme hyperaccumulators may be used for vegetating sterile mine dumps which are potential environmental hazards. Recovery of the metal would then be the next logical step. Haumaniastrum katangense (S. Moore) Duvign. et Plancke, an endemic Cu/Co hyperaccumulator from Zaïre is presently being considered for decontaminating soil containing radioactive Co via continual cropping (Baker and Brooks, 1989). Land polluted by being used for disposal of sewage sludges of industrial origin may be reclaimed in like manner.

sludges of industrial origin may be reclaimed in like manner.

I.5. Research Aims

The full use of hyperaccumulators can only be realised if a better understanding of their behaviour is achieved. In order to do so, greater knowledge must be gained about the nature of compounds associated with metals in vivo, metal uptake characteristics and the mechanisms of evolution. Previous research has provided valuable information regarding the nature of Ni complexes in Ni hyperaccumulating plants (Farago et al., 1975; Lee et al., 1977; Pancaro et al., 1978 (a&b); Stockley, 1980). Some studies on uptake characteristics have also been carried out (Brooks et al., 1979; Morrison, 1980; Hajar, 1987). Baker (1987) and Baker and Proctor (1990) have discussed some evolutionary aspects of metal tolerance. The aims of this research are as follows:-

a) To ascertain the nature of compounds that associate with Ni in selected Ni-hyperaccumulating plants from the Philippines.

b) To investigate a possible relationship between Ni and F accumulation in a Philippine Ni-hyperaccumulating plant.

c) To ascertain whether a relationship exists between Ni-hyperaccumulation and urease activity in Mediterranean and Philippine Ni-hyperaccumulators.

d) To compare the metal uptake characteristics of a Mediterranean Ni-hyperaccumulating plant with those of a non-accumulator and study the effect of different levels of Ni, Co and Cu on their germination.

This thesis is divided into two parts. Part 1 comprises research and findings pertaining to the first three aims, while work relating to the fourth aim is considered in Part 2. It is hoped that the outcome of this research will be useful in gaining a better insight into the hyperaccumulation of Ni by plants.

PART 1

PHYTOCHEMICAL STUDIES

CHAPTER II

Extraction and Isolation of Nickel Species from Two Philippine Nickel-hyperaccumulating Plants

II.1 Introduction

The Republic of the Philippines lies between 20°-40° N and 116°-127° E. The country comprises about 7000 islands located east of the Asian Coast. Geologically, the Philippines are derived from Post-Tertiary volcanic eruptions. Small outcrops of tertiary sedimentary rock including shales, sandstone and limestone, and metamorphic rock are present. Ultramafic rocks containing as much as 50% Fe and 2% Ni can be found on the island of Palawan (Fig. II.1) (Podzorski, 1985). Soils overlying these have been reported to contain 8700 $\mu g\ g^{-1}$ total Ni (Baker and Proctor, 1988), compared to 400-1500 $\mu g\ g^{-1}$ in New Caledonian ultramafic soils (Lee, 1977). Other ultramafic sites can be found in Mindoro, Mindanao and Sibuyan.

The variation in the length of the dry season or its absence, and the timing of the maximum period of rainfall, interact to produce variable climatic conditions. These, coupled with altitudinal changes and the range of microclimates created on the multiplicity of islands, have resulted in substantial variation in vegetation. The islands of the Philippines have a very rich flora, a high percentage (~75%) of which is thought to be endemic (Merrill, 1923; Merrill, 1946).

Podzorski (1985) observed that Mount Bloomfield on the west coast of Palawan supported a rain forest growing over ultramafic rocks that was both stunted and species poor. He noted that at 100 m altitude, there was a sharp transition from lowland forest (up to 25 m in height) to stunted pole forest (<5 m in height). The limited depth of soil development and consequent hydrological problems resulting from drought conditions were the likely reasons for this discontinuity. Mount Bloomfield's lowland forest traverses a distinct geological boundary between Ni-rich serpentine and surrounding sedimentary (greywacke) rocks. The forest system is therefore of ecological interest because it provides an ideal situation for a study of the effect of parent rock on soil and forest development.

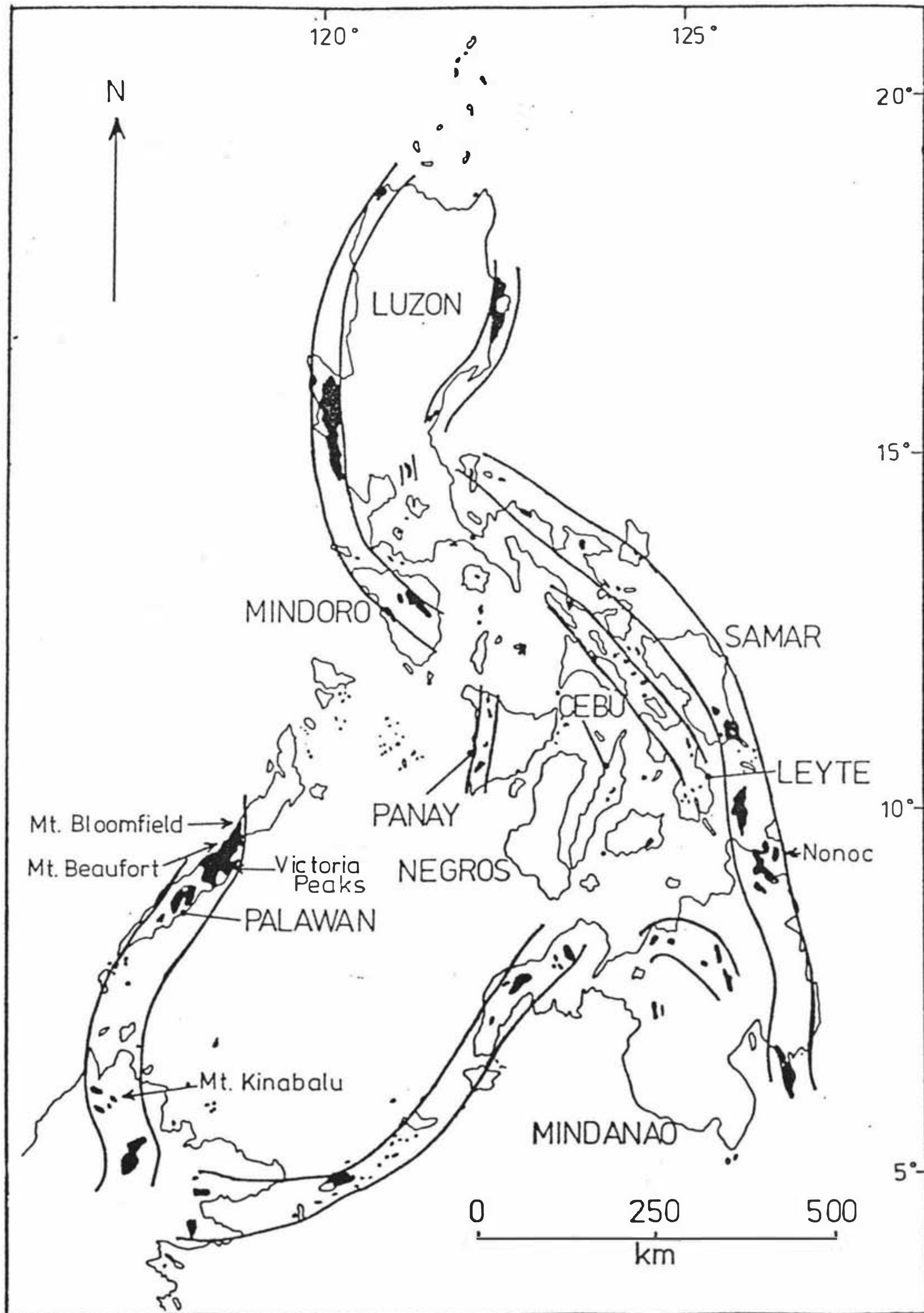


Fig. II.1. Ultramafic outcrops of the Philippines. Heavy lines demarcate assemblages of mafic and ultramafic igneous rocks (Source: Bureau of Mines and Geosciences, Philippines, 1981)

While carrying out ecological studies on the forest types referred to above, Baker and Proctor (1988) discovered four species which hyperaccumulated nickel. The highest concentrations (up to 30000 μg g^{-1}) were found in dry leaves of Dichapetalum gelonioides (Roxb.) Engl. subsp. tuberculatum Leenh. (Dichapetalaceae). Phyllanthus 'palawanensis' Merr. & Quis. (in ed.) (Euphorbiaceae) contained >10000 μg g^{-1} Ni in dry tissue. In one field sample of this plant, Ni was present at the level of 16226, 88583 and 10792 μg g^{-1} in leaves, green tissue beneath the bark of the main stem, and secondary roots respectively (Baker and Proctor, unpublished).The hyperaccumulators Brackenridgea palustris Bartell. ssp. foxworthii (Elmer) Kanis (Ochnaceae) and Walsura monophylla Elmer (Meliaceae) contained 4000-7000 μg g^{-1} nickel. It is of interest to note that several species of Phyllanthus in New Caledonia have been reported as Ni hyperaccumulators (Kersten et al., 1980), with concentrations in P. serpentinus reaching 38100 μg g^{-1}. Nickel concentrations ranging from 146-526 μg g^{-1} were found in dry leaves of Diospyros ferrea (Ebenaceae) and Hemigraphis sp. (Acanthaceae). These plants were therefore not Ni hyperaccumulators.

The variation in foliar Ni concentrations of plants growing over Ni-rich serpentine supporting the forests on Mount Bloomfield indicates that tolerance to Ni is mediated by both metal exclusion and metal accumulation (Baker and Proctor, 1988). Baker et al. (1985) observed similar tolerance strategies in closely-related and sympatric species in New Caledonia. In view of the strong floristic affinities that the Philippines have for Southeast Asia (Brooks, 1987), it should be mentioned that unlike Mount Bloomfield, the ultramafic mountain of Gunung Silam (854 m) in Sabah, Malaysia, supports a species-rich forest of large stature in which the dominant tree species are nickel excluders (Proctor et al.,1988; Proctor et al., 1989). Dichapetalum gelonioides subsp. tuberculatum and several species of Phyllanthus that hyperaccumulate Ni can be found on ultramafics elsewhere in Sabah.

The relative ease with which milligram quantities of metal complexes can be isolated from metal hyperaccumulators for further study as opposed to microgram quantities expected from non-accumulators, has served to encourage phytochemical studies. Many such studies of Ni hyperaccumulators using aqueous extracts of leaf material have revealed associations between Ni and various carboxylic and hydroxycarboxylic acids. Malic and malonic acids have been found

with Ni in extracts from species of Alyssum from Southern and Eastern Europe, the Mediterranean and Turkey (Pancaro et al., 1978a, 1978b; Morrison, 1980; Brooks et al., 1978). Nickel in several New Caledonian hyperaccumulators appears to be bound to citrate (Lee et al., 1977, 1978). Homocitric acid has been reported in Ni-rich extracts of Pearsonia metallifera from Zimbabwe (Stockley, 1980). The significance of these associations is still rather obscure. However, Torii and Laties (1966) have suggested that malic acid may act as transport ligand for Ni in the cytoplasm, and Lee (1977) suggested that citric acid may do likewise in the cell vacuole. Stockley (1980) proposed that malonic acid inhibited the formation of fumarate from succinate in Krebs Cycle. In contrast, studies of Hybanthus floribundus from Western Australia have indicated probable binding of Ni to pectic carbohydrates (Farago et al., 1975; Farago and Mahmoud, 1984). In 1989, Freeman identified malic and citric acids in the Ni-rich extract from Walsura monophylla of the Philippines.As very little phytochemical work appears to have been carried out on Philippine Ni hyperaccumulators, Dichapetalum gelonioides subsp. tuberculatum and Phyllanthus 'palawanensis' were selected for initial study in view of their extreme nickel uptake.

II.2 Nickel Hyperaccumulation in the Dichapetalum and Phyllanthus Genera

The genus Dichapetalum is a member of the Dichapetalaceae family which is pantropical, comprising some 200 species in four genera. Over 150 species of Dichapetalum exist, most of which are found in Africa. There appears to be no documentation of the precise distribution of D. gelonioides but based on inspection of herbarium and field collections, D. gelonioides subsp. tuberculatum occurs mainly on ultramafic and possibly other metalliferous soils in Southeast Asia, notably the Philippines, Malaya, Sabah and Sumatra. Evidence of this has been obtained from analysis of foliage samples from herbarium collections at the Rijksherbarium, Leiden and the Bishop Museum, Honolulu. In the field, Ni is detected by moistening dry filter-paper saturated with 0.1% ethanolic dimethylglyoxime and applying this to the leaf tissue. The appearance of a red colour on the paper is a rough qualitative indication of Ni being present at a level of at least 1000 $\mu g\ g^{-1}$. Thus in addition to providing information regarding the distribution of D. gelonioides subsp. tuberculatum, the analysis served as a means of confirming the hyperaccumulator status of the plant.

Apart from Ni, foliage samples were analysed for Cd and Zn. The results (Table II.1) indicated that all specimens with Ni concentrations > 1000 μg g^{-1} were collected from known ultramafic regions. The highest values were similar to that recorded for the specimen from Mt. Bloomfield, Palawan. None of the specimens from the other locations accumulated Ni to such a high degree. Two other subspecies of D. gelonioides were found to be Ni hyperaccumulators; D.gelonioides subsp. pilosum Leenh. (Philippines) and D. gelonioides subsp. andamanicum (King) Leenh. (Andaman Island).

Enhanced Cd and Zn uptake was observed for specimens of D. gelonioides subsp. tuberculatum from Sumatra and Malaya. Similar behaviour was observed for a specimen of D. gelonioides subsp. gelonioides (Bedd.) Engl. from Sumatra. Zinc hyperaccumulation (i.e Zn uptake to a level >10000 μg g^{-1} in dry tissue), was exhibited by D.gelonioides subsp. tuberculatum, D. gelonioides subsp. sumatranum and D.gelonioides subsp. pilosum, suggesting that the plants may have been growing over sulphide mineral ores as opposed to ultramafics. Dichapetalum gelonioides therefore appears to be both Zn and Ni accumulating depending on the nature of the underlying rocks. It is not certain whether D.gelonioides subsp. pilosum is restricted to non-ultramafic soil (Baker and Proctor, unpublished). If it is, then its ability to hyperaccumulate Ni cannot be viewed as a tolerance mechanism, thus implying that Ni hyperaccumulation may not be an adaptation to nickeliferous soils.

Table II.1 Foliar Analysis of Dichapetalum gelonioides Samples from Field and Herbarium Collections from Philippines and Elsewhere in Southeast Asia. All Elemental Concentrations are Expressed in $\mu g\ g^{-1}$ Leaf Dry Matter

(Baker and Proctor, unpublished)

LOCATION/SITE	COLLECTOR AND NUMBER	Cd	Ni	Zn
D. gelonioides subsp. tuberculatum				
Philippines				
Palawan, Mt. Bloomfield	PFR 526	<10	24817	973
Palawan, Brooks Point	Elmer 12616	<3	7769	220
Palawan, Mt. Victoria	PNH 12434	<4	17257	44
Palawan, Tinitian River	Curran 3815	<5	20613	109
Mindoro, Mt. Calavite	Bur. Sc.39365	<3	12406	103
Mindoro, Boliran, Naujan	Celest. & Castro	-	2112	775
Mindoro, Pimalayan	Bur. Sc. 40913	<4	<39	2575
Sibuyan, Mt. Giting-Giting	Elmer 12121	<3	14307	104
Sibuyan, Mt. Giting-Giting	Elmer 12211	<3	12596	135
Malaysia				
Sabah, Bukit Silam, Lahad Datu	SAN 57302	<3	26647	257
Sabah, Silam Buock10, Lahad Datu	SAN 3609	7	14117	166
Sabah, Segama Rd, Lahad Datu	SAN 21684	<3	21445	319
Sabah, Scaian-Mostyn Rd, Lahad Datu	SAN 68326	<3	18234	88
Sabah, Kinabatangan, Lamag Distr.	SAN 23692	12	3509	645
Sabah, Kinabatangan, Lamag Distr.	SAN 32879	1	<9	7740
Malaya,Lepar, Pahang	FRI Kep 104989	10	480	11572
Malaya, Ulu Langat, Selangor	Millard (KL) 1693	79	270	17895
Malaya, Kedah, Kuah Lankawi	Kerr 21695	<2	9	86
Indonesia				
W. Sumatra, Padang Sidempuan	Kosterm. 22021	9	243	30000
Thailand				
Doi Lang Ka, Chiangmai	Put.3339	3	7	1
D. gelonioides subsp. sumatranum				
Malaysia				
Sabah, Lahad Datu (Rd to Sungei Taun)	SAN 16060	1	7	15657
Sabah, Mt. Kintabalu, Dallas 3000	Clemens 27027	3	6	2553
Sabah, Talau Distr., Ulu Sungai	SAN 30460	<3	<5	14056

Continued overleaf

Table II.1.Continued

LOCATION/SITE	COLLECTOR AND NUMBER	Cd	Ni	Zn
Indonesia				
Kalimantan, Nanukan Is.	BOG 107740	<3	3	14004
Kalimantan, Nanukan Is.	Meijer 2164	<1	6	6291
Kalimantan, E. Kutei, Sg. Susuk	Kosterm. 5472	1	3	427
Kalimantan, Central Kutei	Kosterm. 10544A	4	7	4793
Sumatra, Sarak Palembang	Teysmann	7	<5	14994
D.gelonioides subsp. pilosum				
Philippines				
Mindanao,Zamboanga	Bur. Sc. 37284	<7	16	15286
Mindanao, Zamboanga	PNH 37994	<4	9	26360
Palawan, Mt. Beaufort	SMHI 34	_	20295	390
Malaysia				
Sabah, Ranau	SAN 49206	<3	91	635
Sabah, Sandakan	BISH 139508	_	9	_
Sabah, Kota Belud, Kelawat F. R.	SAN 41188	<4	6	859
Malaya				
Selangor, Ulu Langat F. R.	FRI Kep. 0101	25	158	1841
D.gelonioides subsp. gelonioides				
Indonesia				
W. Sumatra, Sidjundjung region	Meijer 4431	3	72	76
Thailand				
Kao Lunag, N. Sritamarat	Kerr 15410	4	149	303
India				
Assam, Khasi Hills, Cherrapunjee	MICH. 6387	2	14	126
D.gelonioides subsp. andamanicum				
S.Andaman Is.	Hort. Calcutt.	6	3157	1027

The genus Dichapetalum is also of phytochemical interest because of the ability of some African species (D. cymosum and D. toxicarium) to accumulate fluorine to levels in excess of 200 $\mu g\ g^{-1}$ on a dry weight basis. The fluorine exists as fluoroacetate, ω-fluoro-oleate and ω-fluoropalmitate in these plants (Marais, 1944; Peters et al., 1960; Ward, 1964). These fluoro-compounds are toxic to animals because they can be converted to fluorocitrate, a potent inhibitor of Krebs Cycle

The genus Phyllanthus (Euphorbiaceae) is one of the 20 genera throughout the world containing >1000 species. Some 50 of these are endemic to New Caledonia (Guillaumin, 1911;1929;1948). Characterisation of Phyllanthus is a difficult task; a given specimen is seldom characterised beyond the section level. Kersten (1979) reported that 30 species of Phyllanthus accumulated Ni to levels exceeding 100 $\mu g\ g^{-1}$. Ten were Ni hyperaccumulators which were distributed among seven of the ten sections comprising the genus. Twenty-six (26) species were found to accumulate Co to concentrations > 10 $\mu g\ g^{-1}$. This concentration is one order of magnitude higher than that usually found in plants not growing over Ni-rich serpentine soil.

Leaf samples of specimens of Phyllanthus spp. from both field and herbarium collections from Southeast Asia, were analysed for Ni. The highest concentrations were found in specimens of P. securinegoides Merr. and P. cf securinegoides Merr from Philippines (Table II.2). With the exception of specimens of P.'palawanensis' and Phyllanthus sp., both from Palawan, the concentration of Ni in the other Ni-hyperaccumulating plants was one order of magnitude lower. Like D.gelonioides, specimens containing >1000 $\mu g\ g^{-1}$ Ni were collected from known ultramafic regions. It is noteworthy that one specimen of P. buxifolius (Bl.) Muell. Arg. var. was shown to hyperaccumulate Ni despite its observed restriction to non-ultramafics (Baker and Proctor, unpublished). This kind of behaviour is reminescent of that of D.gelonioides subsp. pilosum. It was observed that ~~that~~ the green tissue beneath the bark of the main stem in P.'palawanensis' (PFR 520), which was shown to contain the highest Ni concentration (88583 $\mu g\ g^{-1}$) compared to other plant parts, also contained the highest level of Co (152 $\mu g\ g^{-1}$). Whether Zn hyperaccumulation is exhibited within the genus Phyllanthus is yet to be determined.

Table II.2 Nickel Analysis of Phyllanthus spp. Samples from Collections from Philippines and Elsewhere in Southeast Asia. All Nickel Concentrations are Expressed in $\mu g\ g^{-1}$ Leaf Dry Matter.

(Baker and Proctor, unpublished)

LOCATION/SITE	COLLECTOR AND NUMBER	Ni
P.'palawanensis'		
Philippines		
Palawan, Mt. Bloomfield	PFR 520	16230
Palawan, Mt. Bloomfield	SMHI 1584	5248
Palawan, Bacuñga	Edaño 178	3919
Indonesia		
Sabah, Ranau, Bukit Ampuan, 3000'	SAN 20911	9207
Sabah, Labuk Sugit, Mt. Meliau	SAN 51578	5140
P. lamprophyllus		
Philippines		
Palawan, R. Irawan Valley	SMHI 183	71
Palawan, Mt. Pulgar	Elmer 12733	<13
Palawan	Curran 4156	<19
Palawan, Samar, Mt. Sohoton	PNH 117502	<13
Malaysia		
Sabah, Ranau, Nahumad, 1700'	SAN 77279	5480
Sabah, Labuk Sugit, Mt. Meliau, 2600'	SAN 91613	8035
Indonesia		
Talaud Is., Gunung Piapi	LAM 3279	38
Celebes, Pangkadjene	Teysmann 11877	<32
Java, Madura Is.	LEI 1808	<4
Papua & New Guinea		
Port Moresby, Veimauri River	LAE 51507	13
Western Province., Nomad River	LAE 76125	<8

Continued overleaf

Table II.2 Continued

LOCATION/SITE	COLLECTOR AND NUMBER	Ni
Australia (Queensland)		
Atherton, McLeod River	F&T Bur. 7977	<4
Cape York Peninsula, Coen R.	Brass 19819	<2
P.cf phyllanthus		
Philippines		
Palawan, Langen Is. (karst)	SMHI 435A	8
P.securinegoides		
Philippines		
Mindanao, Mt. Urdaneta	Elmer 13936	24839
Mindanao, Mt. Urdaneta	Elmer 14163	34751
P. cf securinegoides		
Philippines		
Palawan, Victoria Mts.	PNH 16268	27468
Palawan, Narra, Victoria Peaks	SMHI 1770	4815
P. aff securinegoides		
Philippines		
Palawan, R. Irawan Valley	SMHI 96	9390
P. buxifolius		
Philippines		
Palawan, (Is. of Paragua)	Vidal (1886)	9310
Luzon, Rizal Province	Bur. Sc. 19278	<4
Luzon, Mt. Bawa Cagayan	Bur. Sc. 78457	<4
Leyte	MISS 800357	17
Phyllanthus sp.		
Philippines		
Palawan, Mt. Beaufort	SMHI 1874	14496

II.3. Survey of Techniques Used for Locating Nickel-binding Sites in Plant Tissue.

A preliminary step in the characterisation of Ni complexes in Ni hyperaccumulators is the determination of possible Ni-binding sites in leaf tissue. Knowledge of these gives some insight into the kinds of ligands with which Ni associates. Techniques that have proved useful for this purpose are differential centrifugation, proton microprobe analysis, microscopic examination and sequential solvent extraction.

II.3.1 Differential Centrifugation

This technique involves centrifugation of suitably buffered tissue homogenates for increasingly longer periods at increasingly higher centrifugal forces. Both the centrifugation period and the magnitude of the force are specific for different cell components(e.g cell wall, microsomes, chloroplasts, mitochondria and ribosomes). Determination of Ni in the various supernatants and the final residue reveals which components bind the metal. By the use of this technique, Lee (1977) showed that most of the Ni present in leaf material of the New Caledonian hyperaccumulator Homalium kanaliense was in the form of a readily soluble, polar compound of low molar mass which was located mainly in the cell vacuole.

II.3.II. Proton Microprobe Analysis and Microscopic Examination.

Proton microprobe analysis (Horowitz and Grodzins, 1975; Horowitz et al., 1976) is a more direct technique. Essentially, a microsection of the sample is bombarded with a microbeam of protons which causes the nuclei to become excited. As the nuclei return to the unexcited state, characteristic x-rays are emitted. The elemental composition of the sample can then be determined by analysing the x-rays. Samples are usually scanned past a fixed microbeam; thus the final result is a plot showing the spatial distribution of the elements detected. Nuclei can also be excited by electrons, but proton microprobe analysis (PIXE) is advantageous because of the higher sensitivities (x 100-1000) that can be achieved under atmospheric conditions. In the absence of such expensive equipment, visualisation of the element or compound of interest by staining and the

use of a high powered microscope can also provide a distribution map.

These micro-techniques have proved extremely useful in phytochemical studies of metal hyperaccumulating plants. In 1967, Vergnano-Gambi showed some Ni in Alyssum bertolonii to be localised in epidermal regions and between wascular bundles. In other European heavy metal accumulators Turner (1970) observed that a high proportion of Zn and Co was deposited in the cell wall. Ernst (1972) found high concentrations of Ni , but no Co, in the cell sap of Dicoma niccolifera (Southcentral Africa) but no cobalt. Kelly et al. (1975) showed the distribution of Ni in leaves of some Hybanthus species (New Caledonia) to be quite uniform.

II.3.III. Sequential Solvent Extraction

In 1962, Bowen et al. developed an extraction scheme for trace elements in plants in an attempt to improve the knowledge of their chemical states. This work was a sequel to the experiments of Epstein (1956), Fried and Noggle (1958) and Nason (1958) who provided evidence for the existence of metal ions in uncharged complexes for the purpose of ion transport, enzyme activity and in major macromolecules e.g chlorophyll. Bowen et al., (1962) used a series of extractants (both inorganic and organic compounds), each of which was considered to be capable of dissolving specific groups of compounds. In this way, he was able to associate metal ions with specific ligand types. In 1969, Peterson, using a modified version of the procedure, reported an association between ^{65}Zn and pectate in the metal-tolerant grass Agrostis tenuis. He also observed a higher concentration of pectate in tolerant plants compared to non-tolerant plants. Lee (1977) demonstrated that >80% of the Ni present in Homalium kanaliense, Hybanthus austrocaledonicus and Psychotria douarrei was readily extracted into water and dilute acid using a modified version of the technique of Bowen et al. (1962). This was an indication of the high solubility and polarity of the Ni complexes present. Working on taxa from Southcentral Africa, Morrison et al. (1981) also used the extraction scheme to ascertain the chemical form of Cu and Co in tissues.

Despite the apparent usefulness of the above three techniques, an important consideration in the interpretation of their results is that these may be artifactual. The elemental distribution may change during sample preparation for storage and/or during storage itself. Lee (1977) observed that

when fresh material was subjected to differential centrifugation, a higher percentage of vacuolar Ni was obtained compared to freeze-dried material. The converse was true of Ni apparently bound to the cell wall. These findings demonstrated that the higher concentration of Ni bound to the cell wall in freeze-dried material was artifactual. Other spurious results could arise during microscopic examination of dimethylglyoxime-treated sections. The Ni-dimethylglyoxime suspension diffuses readily in some media. As a result, red colouration may not always be indicative of natural Ni distribution. In this respect, it would be advantageous to optimise the amount of dimethylglyoxime required for staining to prevent misinterpretation of results.

II.4. Aim and Rationale

Owing to the unavailability of proton microprobe equipment in New Zealand and the possibility of obtaining artifactual results using differential centrifugation, sequential solvent extraction was the method of choice for our air-dried samples. An atomic absorption spectrophotometer, commonly used for metal analysis, was available in the laboratory, and the extraction conditions were considered sufficiently mild to keep artifactual results at a minimum. An attempt was then made to isolate the fraction containing the highest Ni concentration using gel filtration chromatography as a preparatory step in chemical characterisation.

II.5. Sequential Solvent Extraction of Nickel in Some Philippine Nickel Hyperaccumulators.

The solvent extraction scheme of Bowen et al. (1962) which was later modified by Lee (1977), was used to determine the distribution of Ni among the range of various groups of compounds commonly found in plant cells. The procedure was applied to eight enumerated plants collected during the 1987 expedition to Mount Bloomfield (Palawan).

Ground, air-dried leaf material (2g) was shaken with 10 cm^3 of 95 % Analar ethanol in a 12 cm^3 Pyrex centrifuge tube for 20 minutes using a Jank-Kunkel Electrical Shaker. The slurry was then centrifuged for 10 minutes in a Gallenkamp Junior Centrifuge and the supernatant was

transferred to a 15 cm^3 Pyrex test-tube. The residue was subjected to two repeat extractions. All supernatants were combined and thoroughly mixed. The residue was then extracted with two - 10 cm^3 aliquots of distilled deionised water. The supernatant was retained following centrifugation and the aqueous extraction was repeated. Supernatants were again pooled. The residue was then extracted thrice with 5 cm^3 aliquots of 0.2 M HCl prepared form redistilled constant-boiling 6 M Analar acid. After centrifugation and the combination of the supernatants, the volume was carefully noted and an equal volume of Analar acetone was then added. The test-tube was vigorously shaken, then centrifuged. Both the supernatant and the precipitate (P_1) were retained. The residue was similarly extracted with 0.5 M Analar $HClO_4$ for 20 minutes at a temperature of 90-95° C. The slurry was centrifuged and the supernatant allowed to react with acetone as before. The supernatant and precipitate (P_2) were also retained. The residual material was boiled for 10 minutes with 2 M Analar NaOH. The suspension was centrifuged and the supernatant removed. Both the supernatant and the final residue were retained.

Following the removal of ethanol from the first extract by evaporation, the remaining material was ashed in a Mc Gregor SR 5000 Series Muffle Furnace at 500° C for 6 hours. One cm^3 of 2 M HCl was then added and the digest thoroughly mixed. The acetone precipitates (P_1 and P_2), and the final residue were transferred to filter-papers and dried in a Qualtex/Contherm Hot Air Oven for 1hr at 110° C. The filter-papers were then folded, placed in Pyrex test-tubes and ashed as before. The remaining material was redissolved in 1 cm^3 of 2 M HCl. All supernatants and digested samples were analysed for nickel by atomic absorption spectrometry.

II.6. Atomic Absorption Spectrometry

Atomic absorption spectrometry (AAS) is an analytical technique used for the determination of elemental concentrations in samples based on the absorption of radiation by free ground state atoms. The development of the technique is credited to Walsh (1955) and has as its underlying principle Kirchoff's law which states that under certain conditions every chemical

substance can absorb the radiation it emits itself (Pinta, 1978). Since 1955, AAS has been used extensively for quantitative determination of metals. When free atoms absorb or emit energy, only electronic transitions can occur. Owing to the quantised nature of the transitions, line spectra can be observed (Christian, 1978), lending great elemental specificity to atomic absorption spectrometry.

In order to make measurements in AAS, the sample must be converted to a population of ground state free atoms through which resonance radiation of the element to be determined must be passed. In spectrochemical analysis, the concentration of an element is determined according to its absorption of specific monochromatic radiation (Pinta, 1966). Ideally, only the light being absorbed by the atoms should be sensed by the measuring device as other radiation lowers the proportion of absorbed radiation resulting in reduced sensitivity (Price, 1979).

Sample material (usually in solution form) is converted into atomic vapour by aspiration into an air-acetylene flame or less commonly a hotter nitrous oxide-acetylene flame. The source of radiation is a hollow cathode lamp which emits a spectrum of lines. A monochromator is therefore used to isolate the line chosen for measurement. A photomultiplier is used as a measuring device. The instrument is calibrated with element solution standards, thereby allowing the relationship between absorbance readings and elemental concentrations in solution to be determined. Within the linear range, the relationship is of the form $A = \varepsilon cl$ (Beer-Lambert Law) where

A = absorbance

ε = absorption coefficient

c = concentration

l = pathlength

The absorption coefficient can be graphically determined while l is a constant. Thus, the concentration of an element whose absorbance is known can be determined.

Nickel in the extracts and digests described in this study was determined using an IL 457 Instrumentation Laboratory Atomic Absorption Spectrophotometer . Sample solutions were aspirated into an oxidising (fuel-lean) air-acetylene flame. A nickel hollow cathode lamp provided the source of radiation and sampling was manually carried out. Resonance lines used were 232.0,

294.4 and 351.5 nm depending on the magnitude of the Ni absorbances. The instrument was calibrated with multi-element standards over the range of 10-500 $\mu g\ cm^{-3}$ which were prepared by diluting 1000 $\mu g\ cm^{-3}$ of Analar Spectrophotometric standards with 2 M HCl. Other operating conditions are given in Table II.3. Calibration curves were drawn and the concentrations of Ni in the samples were then determined. The concentration of Ni in the original sample which was extracted into each solvent was also calculated and these were expressed as percentages. Calibration curves for other elements were similarly prepared over appropriate concentration ranges when necessary.

Table II.3 Operating Parameters for Nickel Determination by Atomic Absorption Spectrometry

WAVELENGTH (nm)	LAMP CURRENT (mA)	BAND PASS (nm)	SENSITIVITY ($\mu g\ g^{-1}$)
232.0	10	0.15	0.06
294.4	10	0.50	5.0
351.5	10	0.50	0.6

Table II.4. Results of Sequential Solvent Extraction of Nickel in Philippine Hyperaccumulators

SAMPLE	PERCENT NICKEL EXTRACTED (% w/w)							
	a	b	c	d	e	f	g	h
Phyllanthus 'palawanensis' (leaves) [520D]	0.23	40.27	10.06	25.26	10.92	0.25	6.06	6.95
Phyllanthus 'palawanensis' (stems) [520D]	0.23	68.66	7.34	5.81	7.18	0.48	5.03	5.28
Brackenridgea palustris [356D]	0.86	67.04	10.02	4.60	6.15	0.23	9.35	1.76
Brackenridgea palustris [474D]	1.16	74.69	14.78	1.94	4.15	0.41	2.73	0.14
Walsura monophylla [516D]	0.31	66.76	10.54	1.02	9.59	0.29	0.70	4.47
Walsura monophylla [361D]	1.04	66.91	10.54	1.34	7.70	0.14	10.89	1.45
Walsura monophylla [314D]	2.22	68.60	15.01	5.37	4.78	0.42	2.96	0.44
Dichapetalum gelonioides subsp.tuberculatum [526D]	1.08	77.0	14.64	1.65	3.96	0.03	1.48	0.16

a: ethanol extract - small neutral molecules
b: aqueous extract - polar, low molecular weight compounds
c: 0.2 M HCl extract - acid-soluble polar compounds
d: precipitate from c in acetone - proteins and pectates
e: residue in 0.5 M $HClO_4$- lignin, cellulose etc.
f: precipitate from e in acetone - nucleic acids
g: residue in 2 M NaOH - complex proteins and polysaccharides
h: final residue - cellulose, lignins and insoluble material in cell walls

Sample numbers are given in square brackets

II.7. Discussion

The results of sequential solvent extraction are given in Table II.4. About 50-80 % of the Ni was readily extracted with water and 0.2 M HCl indicating that a large portion of the metal existed as a small, highly polar compounds of low molar mass, probably located in the cell vacuole. Similar findings have been reported by Kelly et al. (1975) and Lee (1977). The highest percentage of Ni extracted into water (77 %) was observed for Dichapetalum gelonioides subsp. tuberculatum. Generally, no more than 30 % of the Ni appeared to be associated with macromolecules. Morrison et al. (1981) observed Cu and Co as both polar, low molar mass compounds and also in association with structural materials when the technique was applied to Cu and Co hyperaccumulators of Southcentral Africa. It was observed that when two specimens of Diospyros ferrea - the Ni non-accumulator, were similarly extracted , there was also a high extractibility of Ni into water and dilute HCl. Brooks and Malaisse (1985) noted that 30-60 % of the Co extracted from non-accumulating plants fed Co was removed by treatment with 95 % ethanol.

In Phyllanthus 'palawanensis' the partitioning of Ni in leaves among the various solvents was anomalous. It appeared that ~25 % of the Ni was present as pectates or bound to proteins while just under 50 % was bound to low molecular weight, polar ligands. However, in the stems, most of the Ni was found in the aqueous extract. It is noteworthy that phytochemical studies carried out on at least one member of this genus from New Caledonia (Kersten et al., 1980) did not reveal complexing of Ni by pectates or proteins. However, Farago et al. (1975) have reported a possible association between Ni and pectates in an unrelated hyperaccumulator from Western Australia Hybanthus floribundus.

II.8. Aqueous Extraction of Nickel in Dichapetalum gelonioides subsp. tuberculatum

On account of the high solubility of Ni in water, an aqueous extraction was carried out on leaves of Dichapetalum gelonioides subsp. tuberculatum as the first step in the isolation of the Ni complexes present. The use of water is advantageous as it is not expected to produce artifactual results and its pH lies within the physiological range of 5-7.5 (Mengel and Kirkby, 1987).

Ten gram of dried ground leaf material was extracted with a 50 cm^3 aliquot of distilled deionised water for 35 minutes using a Heidolph MR 2002 Magnetic Stirrer at ~500 rpm. The slurry was filtered by suction through a clean dishwashing sponge as conventional filtration media proved ineffective. The filtrate was retained and the process repeated twice. The final residue was washed with 10 cm^3 of distilled deionised water. After the final filtration, the three filtrates were combined, thoroughly mixed and the volume recorded. Additional extractions were not performed since previous workers showed that 95 % of the Ni was readily removed after two extractions (Lee, 1977; Freeman, 1989). The combined extract was freed of compounds of high molar mass and low polarity such as pigments and lipids (and other structural material) by shaking with three -20 cm^3 aliquots of an Analar chloroform/butanol (10:1; v/v) mixture (henceforth referred to as chloroform/butanol reagent) for 5 min. in a 250 cm^3 separatory funnel. The removal of the undesirable compounds was deemed complete when the lower chloroform/butanol layer remained clear and colourless. At the end of each extraction, a 0.5 cm^3 volume was removed from the organic layer and tested for Ni by adding a few drops of 0.1 % ethanolic dimethylglyoxime (Analar). A faint pink colouration was observed on each occasion indicating that the loss of Ni into the organic layer by direct transfer or adsorption onto the precipitate was negligible. Due to the highly organic nature of the matrix, Ni was not determined by AAS as the instrument was not fitted with a conduit for auxiliary gas. Suitably prepared standards would also have been necessary. The alternative method of evaporating off the reagent (after removal of the precipitate) and redissolving the residue in water or dilute acid prior to AAS determination could have been time-consuming. The clear, brown Ni-rich extract was then transferred to a 250 cm^3 distillation flask and the solvent was removed using a Buchi Rotary Evaporator at a temperature of 33-35° C until the final volume was about 10 cm^3. The concentrated extract contained 145 mg Ni. It was stored in a stoppered plastic vial in the refrigerator at 4° C. The Ni complexes were then isolated by gel filtration chromatography.

II.9. Gel Filtration Chromatography

Gel filtration chromatography is a technique in which separation of sample components is achieved on the basis of molecular size. The stationary phase consists of particles with a fixed pore size and separation of the sample components is dependent on their ability to enter the pores. A sample component that is too large to enter these, moves through the phase the fastest while smaller components that can enter the pores have a longer residence time in the stationary phase. Since molecular size increases with molar mass, components are eluted in order of decreasing molar mass. A wide range of stationary phases or gels is available. They can be described by their exclusion limits ranging from 400 to 50 x 10^6 g/mol. The exclusion limit is the molar mass of the smallest particle which is too large to partially enter the pores of the phase in question. The choice of stationary phase is dictated by the range of molar masses of the components to be separated. The stationary phase is usually held in a vertical column and separation occurs through the passage of a suitable solvent. Gels consist of chemically stable, inert macromolecules that have a low content of ionic groups. Very labile compounds can be readily separated with minimum risk of decomposition. The procedure is affected very little by temperature, and mild eluants such as water and 0.5 M ammonium acetate can be used.

Lee (1977), Kersten (1979) and Freeman (1989) have successfully used Sephadex G-10 to isolate Ni complexes from aqueous extracts of Ni hyperaccumulators. Severne (1972) and Lee (1977) reported Ni complexes of molar mass <700, the lowest, of course, being that of $[Ni(H_2O)_6]^{2+}$ at 167 g/mol. Sephadex G-10 consists of dextran crosslinked with epichlorhydrin and has a molar mass cut-off of 700 g/mol. This gel was employed for the isolation of Ni complexes in the aqueous extract of D. gelonioides subsp. tuberculatum.

II.10. Isolation of Nickel Complexes in the Extract from Dichapetalum gelonioides subsp. tuberculatum

About 100 g of Pharmacia Sephadex G-10 of particle size 40-120 µm was washed with ~150 cm^3 of 0.1 M HCl. The acid was decanted and distilled deionised water (200-300 cm^3) was added until a near neutral pH was achieved. The gel was then allowed to stand overnight in

200-300 cm^3 of distilled deionised water at room temperature. The slurry was then transferred to a glass column (3 cm x 45 cm) then equilibrated by passing water through continuously for about one hour. The void volume (V_0) of the column was determined in order to ensure that the system was working properly and to give some idea of when fraction collection should commence after sample application. The compound used for this purpose was Blue Dextran (Sigma) which has a molar mass of 2 x 10^6 g/mol and is therefore totally excluded from the column. Three cm^3 of aqueous Blue Dextran (2 mg cm^{-3}) was applied to the column. A blue band was observed migrating down the column as the dye eluted with water at a flow rate of 1 cm^3 min^{-1}. As the band approached the bottom of the column, fraction collection commenced using a LKB Bromma 2212 Helirac Fraction Collector. Thirty 4 cm^3 fractions were collected. By use of a Shimadzu 160 UV/VIS Recording Spectrophotometer, the spectrum of some of the original solution showed a maximum absorbance at 612 nm. This wavelength was utilised and showed that the fraction having the highest absorbance reading corresponded to an elution volume of 300 cm^3. This was taken to be the void volume.

A 2 cm^3 aliquot of the concentrated extract from D. gelonioides subsp. tuberculatum containing 28 mg of Ni was applied to the column and eluted with water at the same flow rate. Three distinct bands were observed migrating down the column as separation occurred. A broad green band followed yellow and brown bands. The green band which contained the isolated Ni complexes was collected in thirty-three 4 cm^3 fractions. These were analysed for Ni by AAS and an elution diagram was constructed. The fractions containing the largest amount of metal were combined, the final volume was measured and the eluate stored in a plastic vial at 4° C. Total extraction efficiency was calculated at ~90 %. Two cm^3 of a ten-fold diluted sample of the original concentrated extract was also subjected to gel filtration chromatography under identical conditions.

II.11. Extraction of Nickel in Phyllanthus 'palawanensis'

About 20-gram of dried ground leaf material was extracted twice with 150 cm^3 of 3:1 (v/v) water/acetone mixture in the same manner as for D. gelonioides subsp. tuberculatum. Pure water proved unsuitable as an extractant because the aqueous slurry assumed a gelatinous consistency making filtration extremely difficult. This behaviour could be explained by the anomalous partitioning of Ni among the solvents used in the sequential solvent extraction (Section II.7). The water/acetone slurries were filtered by gravity using Whatman # 1 paper rather than suction owing to the high volatility of acetone. The residue was washed with 50 cm^3 of extractant which was then removed by filtration. The three filtrates were combined and treated with the chloroform/butanol reagent to remove compounds of high molar mass and low polarity. The concentraion of Ni in the purified extract was determined by AAS. The extraction efficiency was 80 %; losses may have occurred during filtration since suction was not used and some of the Ni might have been bound in an insoluble form. The volume of the extract was reduced to 29 cm^3 in vacuo at 33-35° C, and Ni was determined in both the concentrated sample and the distillate. The extract contained 39 mg Ni but none was detected in the distillate. The extract was stored in the refrigerator.

II.12. Isolation of Nickel Complexes in the Extract from Phyllanthus 'palawanensis'

An attempt was made to isolate the Ni complexes in the concentrated extract by gel filtration chromatography. Two cm^3 of the extract (2.7mg Ni) was applied to the Sephadex G-10 column. Distilled deionised water was used as eluant at a flow rate of 1.5 cm^3 min^{-1}. No separation was apparent. Small volumes of the eluant were tested for Ni using 0.1 % ethanolic dimethylglyoxime after the void volume had been exceeded. The results were all negative. The sample remained near the top of the column where it formed a thin brown band. Treatment with dilute acid and ethanol proved to be ineffective so the procedure was terminated and the top 2-3 cm of the gel discarded. Examination of the remainder of the concentrated extract showed the presence

of a precipitate. The extract was therefore divided in half and each portion was centrifuged for ten minutes. The precipitates labelled A_1 and A_2 were retained. The supernatants were then transferred to separate centrifuge tubes and the process was repeated. The resulting supernatants were combined and the volume reduced to 8 cm^3 by rotary evaporation at 33-35° C. The two precipitates labelled A_3 and A_4 were also retained. The concentration of Ni in this extract was 63 % lower than that expected. After overnight storage at 4° C, a precipitate was again observed. The concentrate was therefore divided in half and the centrifugation procedure was repeated. Supernatants were combined (extract B), and the two precipitates labelled A_5 and A_6 were retained.

The six precipitates (A_1 to A_6) were tested for Ni using ethanolic dimethylglyoxime (0.1 %). The most intense red colouration due to the formation of the Ni-dimethylglyoxime complex was observed in precipitates A_1 and A_2. Precipitates A_5 and A_6 exhibited the least intense colouration. Quantitative analysis was not applicable owing to the small amount of wet precipitates obtained. The low concentration of Ni in extract B was probably due to loss via precipitation and inaccurate AAS results owing to matrix effects which became pronounced during the concentration process.

A 3-cm^3 volume of extract B containing 11mg of Ni was subjected to gel filtration using the Sephadex G-10 column. The sample was eluted with distilled deionised water at a flow rate of 1.5 cm^3 min^{-1}. Seventy-four 3 cm^3 fractions were collected and subsequently analysed for Ni by AAS. The eluted Ni represented only 60 % of that applied to the column. The first 27 fractions which contained most of the Ni were combined and thoroughly mixed. The final volume was noted and the eluate (B_1) was stored in the refrigerator.

Due to the unstable nature of the extract as evidenced by the formation of a precipitate during storage, the chloroform/butanol treatment was applied in an attempt to ensure the amenability of the extracts to gel filtration chromatography. Extract B_1 (66 cm^3) which supposedly

contained the isolated Ni complexes was shaken with two-25 cm^3 portions of the reagent in a 250 cm^3 separatory funnel. This purified extract (B_2) was evaporated down to 6.9 cm^3 in vacuo at 33-35° C and the Ni concentration was determined. The remainder of the crude extract (5 cm^3) was shaken with two-10 cm^3aliquots of chloroform/butanol reagent. The volume of this extract (C), was reduced to 3 cm^3 under identical conditions. Extract B_2 was divided into two portions, and each underwent further purification by gel filtration chromatography with a Ni recovery of only 50-60 %. In each case, Ni-rich fractions were combined and refrigerated.

II.13. Results And Discussion

The Ni complexes in aqeous extracts of D.gelonioides subsp.tuberculatum eluted in two poorly resolved peaks; the first being the larger of the two (Fig.II.2). This was an indication that Ni existed in at least two forms in the extract. Only one peak was observed when gel filtration was performed on a ten-fold diluted sample of the original extract (Fig II.3). The elution profile of the P.'palawanensis' extract B_1 also showed Ni to be present in two forms (Fig.II.4). When the isolated Ni complexes were subjected to further purification on Sephadex G-10 only one peak was observed in the elution profile (Fig.II.5).

The above results indicate that in D.gelonioides subsp.tuberculatum, Ni complexes are polar, with low molar mass. This is also true of a portion of the Ni in P.'palawanensis'. It would appear that pectates and/or proteins present in the crude extract of P. 'palawanensis' might have rendered the separation and isolation of the Ni complexes difficult by binding with Ni directly and/or through interaction with the complexes or the stationary phase. The latter interaction would not be surprising owing to the fact that like Sephadex G-10, pectates contain a preponderance of hydroxyl groups. The nature of Ni complexation and/or elution from the column appeared to be influenced by the level of Ni in the extract as evidenced by the reduction in the number of peaks from two to one. This suggests that the extracted Ni complexes may be labile under what are considered to be rather mild sample processing conditions. The significance of these results will be

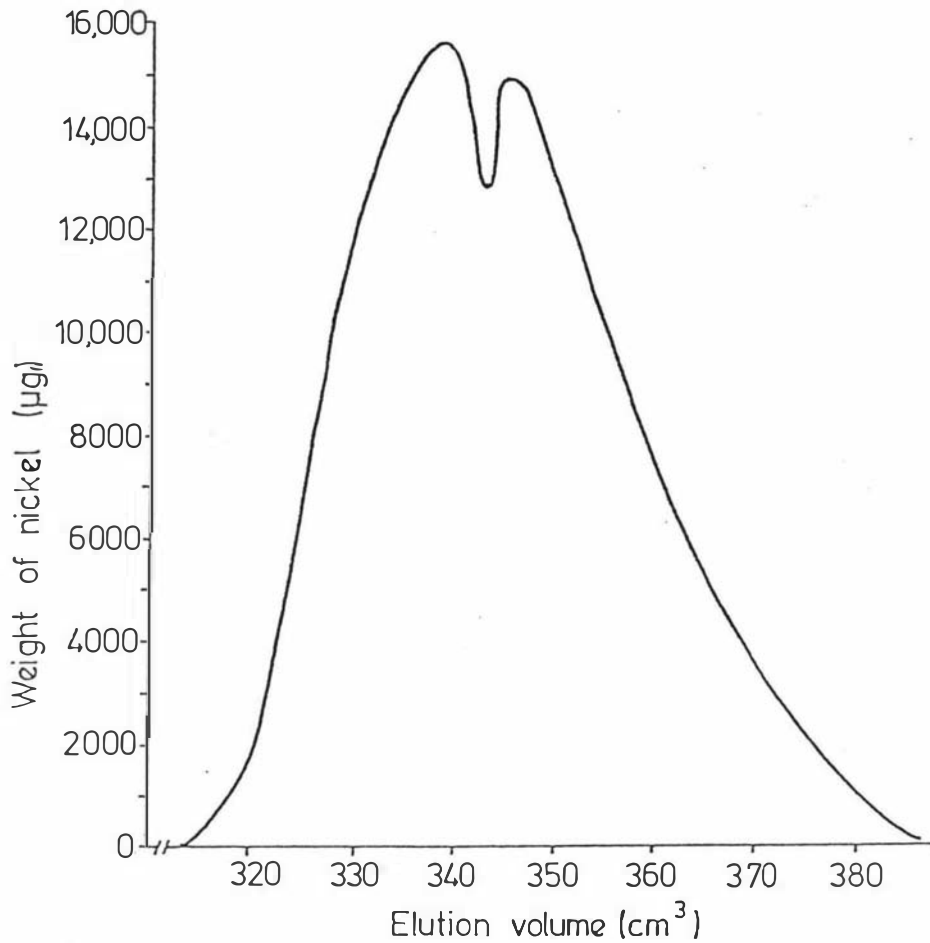


Fig. II.2. Elution profile of Ni-species in crude concentrated extract of D. gelonioides subsp.tuberculatum from Sephadex G-10 column.

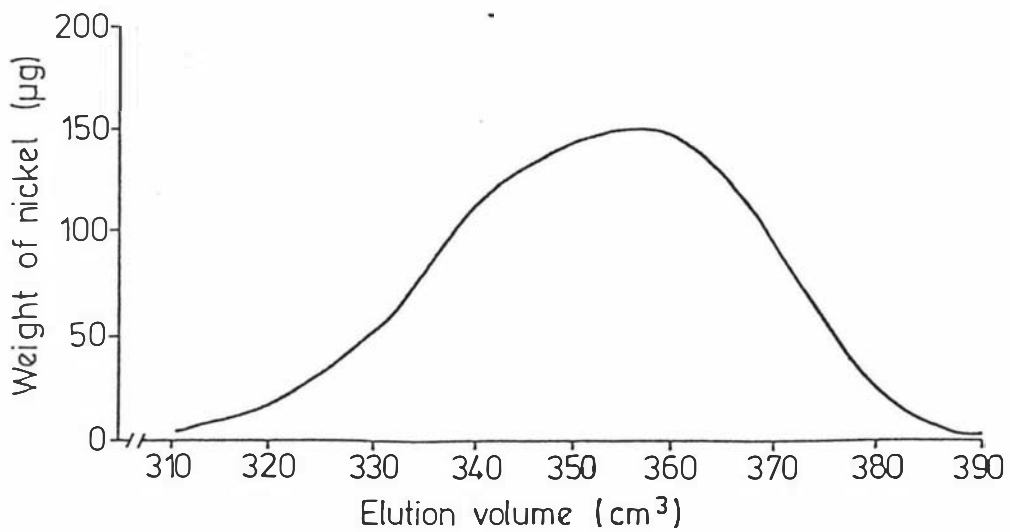


Fig. II.3. Elution profile of Ni species in crude diluted (1/10) extract of D. gelonioides subsp. tuberculatum from Sepjadex G-10 column.

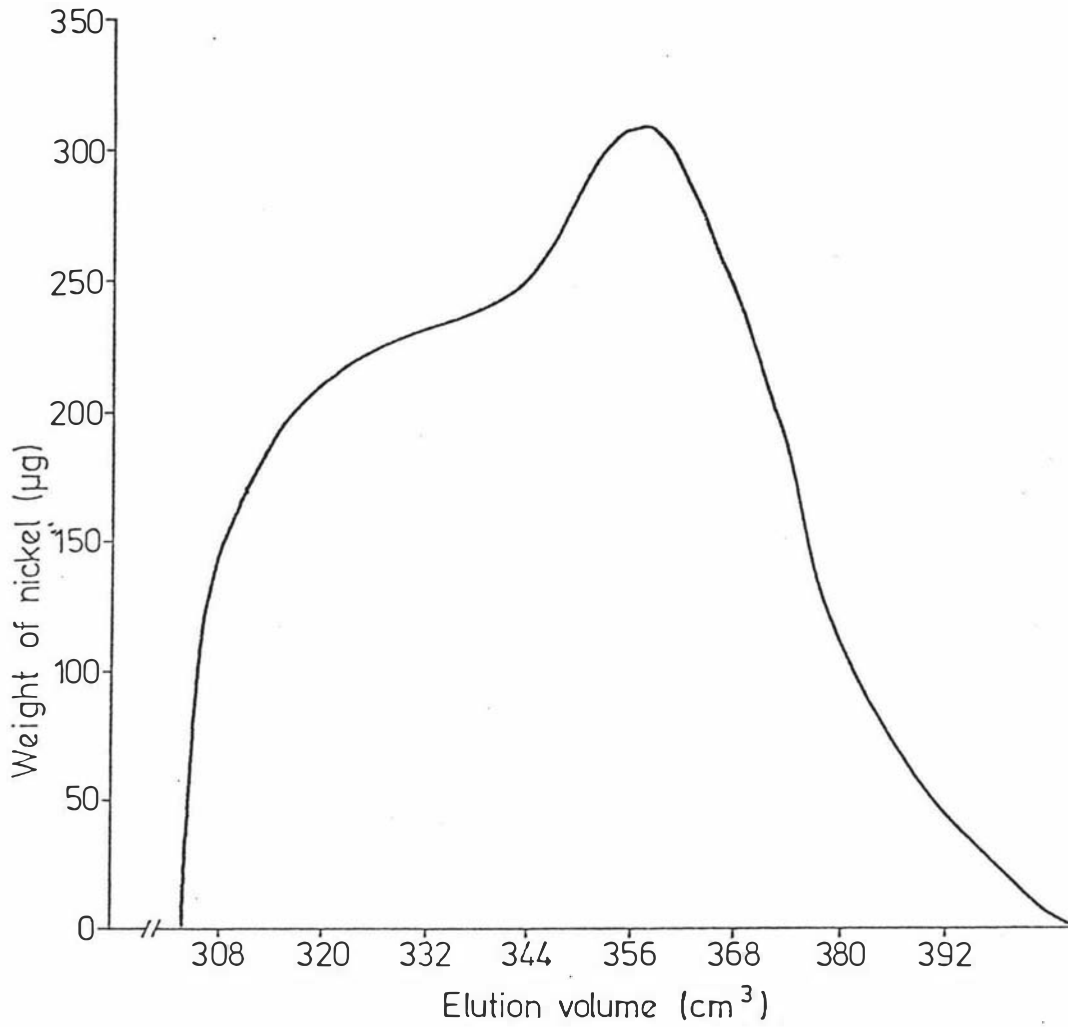


Fig. II.4. Elution profile of Ni species in crude concentrated extract of P.'palawanensis' from Sephadex G-10 column.

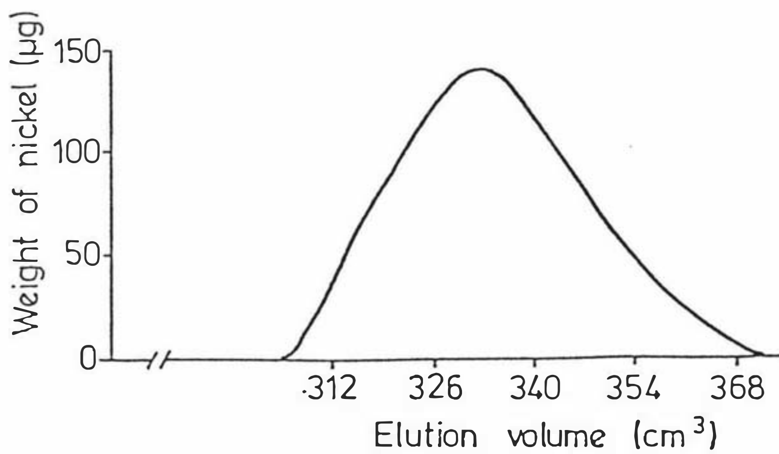


Fig. II.5. Elution profile of Ni species in crude diluted (1/5) extract of P. 'palawanensis' from Sephadex G-10 column.

discussed in subsequent chapters relating to the ionic nature of the Ni species in the two plants, the identification of possible Ni-binding ligands and the chromatographic behaviour of some synthetic Ni-carboxylic acid complexes.

CHAPTER III

The Ionic Nature of Nickel Complexes Isolated from Dichapetalum subsp. tuberculatum and Phyllanthus 'palawanensis'

III.1. Introduction

In order to account for the excessive uptake of Ni by some taxa growing on nickeliferous soil, some buffer system must be operative to facilitate nickel tolerance. It is known that a preponderance of Ni in its common ionic form - aquo nickel is phytotoxic. High Ni concentrations have been shown to cause severe chlorosis and necrosis in plants, as well as growth abnormalities and anatomical changes. Evidence for this is reviewed by Mishra and Kar (1974). According to Welch (1981), Ni in plants at levels >50 $\mu g\ g^{-1}$ on a dry weight basis is toxic. Such a system may operate in one of two ways:- (i) active transport in the plant which relies on metabolism to provide energy for the movement of ions against a concentration gradient and (ii) selective absorption via the root system.

Pfeffer (1900) noted that substances might combine with cellular constituents enabling transport across the membrane even in the absence of a concentration gradient. This idea became later known as the carrier concept (Honert, 1963; Jacobson and Overstreet, 1947; Roberts et al., 1949). It was also suggested that in plants, polyvalent cations such as Ca^{2+}, Mg^{2+} and transition metal ions could be bound by chelating compounds such as amino-acids, amino-acid derivatives and organic acids. Rosenberg (1948), however, pointed out that this kind of binding could necessitate regio-specific membrane permeability to facilitate the passage of ions.

It has been known for several years that plants (and microbes) exude significant amounts of inorganic and organic compounds capable of chelating or precipitating metals (Shaw, 1989). As far back as 1963, Stewart recognised that naturally occurring chelates of heavy metals were probably the most likely form in which metals were absorbed and translocated in plants, in addition to being the most important source. Schmid and Gerloff (1961), and Stewart (1963) suggested that in the absence of chelation at physiological pH, some metals could be precipitated as insoluble phosphates and hydroxides. Precipitation has actually been postulated to play a role in metal resistance in microbes but evidence of such in higher plants is lacking (Ashida,1965). Metals are normally present in their ionic forms in aqueous solution. For many divalent transition metals, the

hydrated forms are usually present in this medium (Tiffin, 1967).

Carriers or chelators have been described as intermediate metabolic products or closely related compounds which show some degree of stability in vitro and possess the ability to undergo chemical alteration during ion transport (Overstreet and Jacobson, 1952). In addition to translocation, Stewart (1963) noted that in plant metabolism, chelates are also involved in storage, oxidation-reduction reactions and transmission of energy. A wide variety of ligands known to complex cations can be found in biological systems e.g porphyrins, purines, riboflavins, nucleotides, nucleic acids, phenolics and sugars as well as amino-acids, proteins and organic acids (Vancura and Hovadik, 1965; Rovira, 1969; Lee, 1977). To date, no general order of affinity of a series of ligands for all cations appears to have been formulated. However, at a given pH, an excess of ligand favours association between certain metals and the ligand in question (Williams, 1959). Metals have been categorised into three classes based on ligand preferences (Nieboer and Richardson, 1980). Class A metals, which includes Li, Na, K, Ca, Mg, Ba and Al, tend to form the most stable complexes with O-containing ligands. Class B metals [Cu (I), Ag, Au, Tl (I &III), Hg, Pt, Pd, Pb (IV) and Bi (III)] form the most stable complexes with ligands rich in S and N atoms. Class C consists of the transition metals such as Cu (II), Ni, Co and Fe. These tend to show a preference for ligands containing both O and N, and are referred to as "borderline metals". It should be noted that many stable complexes, some of which are important in plants (e.g chlorophyll) contain metals that cross the "class boundaries" of Nieboer and Richardson (1980).The classification of Nieboer and Richardson (1980) is related to atomic properties and solution chemistry of the metal ions, but a review of the roles of these ions in biological systems shows that their scheme has potential in interpreting the biochemical basis for metal ion toxicity. It could also be useful in rationalising the selection of metal ions in toxicity studies. It is of interest to note that the stability of organometallic complexes as given by the Irving-Williams Rule of Ni > Cu > Co> Cr > Zn > Mo > Mn (Irving and Williams, 1953), is in agreement with the order of metal-ion activity resulting in toxicity reported by Hunter and Vergnano (1953) in their work on oat plants.

III.2. Forms of Metal in Non-tolerant Plants

With respect to the nature of compounds in which metals occur, some work has been carried out on plants that are non-tolerant to elevated levels of metal. There however appears to be a dearth of information regarding the forms in which metals are translocated. There is general agreement that major cations are essentiality free of organic moieties during translocation. However, consideration of the stability constants of microelement chelates of organic and amino acids suggests some association (Sillén and Martell, 1964). Most of these studies have been carried out using gel filtration chromatography, ion-exchange chromatography, electrophoresis and gas chromatography - mass spectrometry.

Using gel filtration, Schmid and Gerloff (1961) reported that Fe in xylem exudates from tobacco plants was in the form of a high molar mass complex. Initially, using anion-exchange chromatography, these workers also demonstrated the presence of an anionic complex. Based on electrophoretic studies, Tiffin and Brown (1962) showed Fe in soybean exudate to be chelated by malic and malonic acids despite administration of the metal in nutrient solution as a synthetic chelate. Anionic Fe (III) - citrate complexes have been reported in several plant exudates by Tiffin (1970,1972). Bremner and Knight (1970), reported the existence of soluble Cu and Zn in anionic organic forms in ryegrass extracts. Unidentified anionic complexes of Ni and Cu have been found in xylem exudates of tomato, cucmber,corn, carrot and peanut (Tiffin, 1971). Amino-acids were considered as carriers for Cu^{2+} and Ni^{2+} as these show an affinity for O- and N- containing ligands (Tiffin, 1972; Thompson and Tiffin, 1974). Van Goor and Wiersma (1976) observed that a large portion of Zn in extracts of ryegrass was bound as an anionic organic complex possibly containing phosphorus.

Of all the ligands previously mentioned, organic acids appear to be the most common type involved in metal absorption and translocation. Bennett-Clark (1933) reported large amounts of Al-malate in some succulent plants. Al-Rais et al., (1971) isolated calcium oxalate crystals from plants of at least seven different families grown under normal conditions. They also observed Mg in the crystals at a level of 2 %, and noted that in culture solutions, Mg, Ba and Sr could replace Ca to some degree. This is probably not surprising in view of the chemical similarity of the metals.

Fifty per cent of Ca in xylem sap of apple shoots was reported to be complexed by malic and citric acids (Bradfield, 1976). The probable role of B in sugar translocation and the existence of Co as vitamin B-12 in nodules of the N_2-fixing leguminous plant, Trifolium subterraneum are also noteworthy (Gauch and Dugger, 1953; Wilson and Hallsworth, 1965).

III.3. Forms of Metal in Tolerant Accumulating Plants

The role of organic chelates in metal tolerant plants was probably first noted by Gregory and Bradshaw (1965) during studies of Zn-resistant populations of the grass Agrostis tenuis. Working with the same plant, Peterson (1969) reported that Zn tolerance was mediated by cationic binding to cell wall sites. In another study, a Zn-binding complex, enzymatically released from the cell wall, was shown to contain sugar and amino-acids (Wyn-Jones et al., 1971). These workers suggested that tolerance was genetically controlled. In 1977, Mathys observed twice as much mustard oil glucosides in Zn-resistant Thlaspi alpestre (= T. caerulescens J.& C. Presl.) (Ingrouille and Smirnoff, 1986) compared to that found in metal-sensitive types. Great differences were also observed in the malic acid content. He proposed that malate might act as chelator for Zn within the plasma, whereas oxalate, whose production was stimulated in two other Zn resistant taxa, and glucosinolate, may function as "internal acceptors" of large quantities of Zn. It has also been suggested that in Deschampsia caespitosa, Zn resistance might depend on the plant's ability to detoxify Zn by storage as Zn-phytate (inositol hexaphosphate) (Van Stevenick et al., 1987).

In Cu-accumulating species, Reilly (1969) and Reilly et al.(1970) proposed the existence of Cu-protein complexes. Lyon et al.(1969) reported three anionic complexes in the Cr accumulator Leptospermum scoparium, the major one being trioxalato Cr (III). The ability of various plants to tolerate elevated levels of Fe does not appear to be well documented. It has however been reported that Fe tolerance might depend on increased capacity of the phytoferritin (an Fe protein) storage system, iron reductants such as phenolic acids, and increased activity of membrane-bound iron-reducing systems (Brown, 1978; Bienfait and van der Mark, 1981; Bienfait et al., 1983).

In 1975, Farago et al. carried out paper chromatographic tests on ammonium oxalate extracts of Hybanthus floribundus and observed a Ni compound with a R_f similar to that of Ni pectinate.Pectinates are derived from pectic acids which are polysaccharides containing partially methoxylated galacturonic acid residues (Farago and Mahmoud, 1983).They suggested that Ni could be bound to an amino-acid, but this is unlikely in view of earlier findings of Jursik (1968). He reported that unlike amino-acid complexes of Cu, Ni complexes dissociated under normal paper chromatographic conditions. No such results have been reported by Lee (1977) and Kelly et al. (1975) for the Hybanthus species from New Caledonia. Pelosi et al. (1974,1976) compiled evidence to show that Ni associated with malic and malonic acids in aqueous extracts from Alyssum bertolonii. In 1976, Jaffré et al. reported that close to 100 % of the Ni in aqueous extracts from Sebertia acuminata was bound as an anionic citrato complex. The association between Ni and citrate has been reported for other New Caledonian Ni hyperaccumulating plants (Lee et al., 1977; Kersten et al., 1980). A highly significant correlation between the levels of Ni and citric acid was observed among Ni hyperaccumulators belonging to the genera Homalium and Hybanthus (Lee et al., 1978). Kersten et al. (1980) reported the possible role of citric acid in Ni complexation in the New Caledonian plants Casearia silvanae, Lasiochlamys peltata, Phyllanthus serpentinus, Psychotria douarrei, Rinorea bengalensis and Xylosma vincentii. The aqueous extracts of Psychotria douarrei and Phyllanthus serpentinus were also shown to contain malic acid by these workers. In Phyllanthus serpentinus, Ni appeared to be present as anionic Ni-citrate and neutral Ni-malate, while a substantial portion of the metal in Psychotria douarrei seemed to be bound by anionic malate. Anionic Ni-malate is unlikely as the hydroxyl proton will only be lost under alkaline conditions. Working with a Ni-citrate complex, Still and Wikberg (1980), observed that the hydroxyl proton could only be removed at pH >8. Lee et al. (1978) reported that an increase of greater than 100-fold in plant Ni concentration only leads to a 10-fold increase in citrate concentration in some New Caledonian plant species which showed a wide range of Ni-accumulating ability. Hence, the complexation of Ni by citrate alone cannot explain the apparent non-toxic nature of Ni in Ni-hyperaccumulating plants.

III.4. Metal-binding Polypeptides

Recently, the metallothionein and phytochelatin polypeptides have attracted great interest as chelators. Metallothioneins are S-rich proteins reputed to be involved in metal homeostasis in animals, some fungi and some algae (Lerch,1980; Hamer, 1986). These have not yet been reported in higher plants. The phytochelatins, which are poly (γ-glutamylcysteine) glycines are produced by some algae and fungi, and higher plants (Grill et al., 1985; 1986; 1987; Lue-Kim and Rauser, 1986; Robinson and Jackson, 1986; Reese and Wagner, 1987; Reese et al., 1988; Gekeler et al., 1988). Like the metallothioneins, they contain a large number of sulphydryl and carboxyl groups which predispose them to complexation of a wide range of metal ions. To date, in vivo binding of only Cd and Cu has been demonstrated (Reese et al., 1988). However, there is evidence of de novo synthesis of the polypeptides following exposure to a few other metals (Grill et al., 1985,1986, 1987; Gekeler et al., 1988). Grill et al. (1987) and Reese and Wagner (1987), have provided indirect evidence of the binding of Hg and Ag by these compounds. Metal-binding polypeptides reduce cytotoxic concentrations of free metal ions by complexing them in the cytoplasm. Shaw (1989) postulated that modification of polypeptide metabolism could result in an alternative strategy for endowing prokaryotes and eukaryotes with tolerance to abnormally high concentrations of metal ions.

III.5. Phytochelatins and Nickel-hyperaccumulating Plants

Phytochelatins do not appear to play a role in toxicity amelioration in Ni-hyperaccumulating plants. Based on studies of Cd and Cu tolerance in cell cultures of Lyperscion esculentum (tomato) and Datura innoxia, there seems to be limited evidence of quantitative differences in the capacity of phytochelatin production between tolerant and sensitive cultures (Scheller et al., 1987; Jackson et al., 1987). No such observation has yet been reported for intact plants. Grill et al. (1987) have also demonstrated that increased phytochelatin synthesis can be induced by almost any metal. This suggests that phytochelatins could not be suitable chelators in plants known to demonstrate single-metal tolerançe, owing to the high degree of apparent specificity. However, these compounds may be suitable in plants that demonstrate multiple tolerance.

Generally, addition of synthetic or natural chelators to culture solutions results in reduced metal uptake and subsequent reduction in metal toxicity. Using a number of synthetic metal chelates, Sunda and Huntsman (1985) amassed evidence to show that toxicity in the unicellular alga Chlamydomonas sp. was governed by free ion concentration as opposed to the concentration of the chelated metal. Earlier , Coombes et al. (1977) suggested that chelations resulting in positively charged or neutral metal-ligand complexes do not reduce metal uptake as much as predicted from their calculations or measured effect on free ion activity. Perhaps, it can be argued that this may not apply to metalliferous soil owing to the large amounts of freely available metal ions. Nonetheless, in algae where metal resistance is effected by exclusion, resistant ecotypes not only exude more chelators into the medium but also more effective chelators (Butler et al., 1980). There seems to be no evidence of this in higher plants.

III.6. Aim and Rationale

Having isolated Ni complexes in extracts from D. gelonioides subsp. tuberculatum and P. 'palawanensis', the next logical step was to ascertain their ionic nature. This would then be followed by ligand characterisation using preparatory amounts of the complexes. Ion-exchange chromatography and high voltage electrophoresis were used as these proved useful in previous research. Gel filtration chromatography was used for molar mass determination and further purification where necessary.

III.7. Analytical Techniques

III.7.1 Ion-exchange Chromatography

In ion-exchange chromatography, sample components are attracted to exchange sites on a stationary phase. Ionic groups in the sample are separated through differences in their attraction to the exchange sites. Ion -exchange chromatography differs from other forms of liquid chromatography, in that ions rather than neutral molecules are separated. Various stationary phases

(resins) are commercially available. Each consists of a synthetic cross-linked polymer with attached, ionisable functional groups. These are covalently bonded to the insoluble, inert polymeric structure. Anionic groups are used in cation-exchange resins and cationic groups are used in anion-exchange resins. The counter-ion (i.e. the oppositely charged ion) of the bonded group is relatively free to dissolve in the aqueous mobile phase as it flows through the column. Sample ions which have been applied to the column are attracted to the oppositely charged, stationary ionic sites in the resin. If the attraction between sample ions and stationary phase ions is greater than that between counter ions and the stationary phase, sample ions will be adsorbed by the ion-exchange resin, replacing the counter ions. Ion-exchange resins may be strongly (or weakly) basic [i.e. anionic] or strongly (or weakly) acidic [i.e. cationic], depending on the strength of the acidic or basic functional groups bonded to the polymer. Pelosi et al. (1976), Lee et al. (1979) and Kersten et al. (1980) used ion-exchange chromatography to determine the ionic nature of Ni in extracts from several Ni hyperaccumulating plants. The most suitable resins were found to be the weak cation-exchanger Amberlite IRC50 which contains weakly acidic carboxyl groups bonded to a methacrylate-divinylbenzene polymeric support and the weak anion-exchanger Amberlite IR45. This contained weakly basic polyamine groups bonded to a styrene-divinylbenzene polymeric support. Lee (1977) reported the decompostion of Ni complexes from Ni hyperaccumulating plants when a strong cation-exchange resin is used.

III.7.II. Electrophoresis

Electrophoresis involves the use of an electric potential to separate relatively large ions according to their charge-to-mass ratio. It is commonly carried out on a paper or gel support permeated with an electrolyte solution, which is typically a buffer. The sample is spotted on, or applied in band, to the inert support and an electrical potential is applied. Positive ions in the sample are attracted to the negative electrode (cathode), and negative ions are attracted to the positive electrode (anode). The migration rate of ions through the electrolyte solution on the support increases as the charge on the ion increases, but decreases as the size or mass of the ion increases, hence separation being effected according to the charge-to-mass ratio. Following separation, the support is removed from the electrolyte and the sample components are located as in paper chromatography. Although some power supplies can provide more than 2kV, electrophoresis can

easily be performed from 0-500V. Faster separations are achieved at higher applied voltage, current and power.The magnitude of the power used is dependent on the amount of heat generated by the current flow through the support. Excessive heat can damage the support and effect undesirable changes in the ions being separated.

Gross (1956,1959) achieved good separation of organic acids in complex mixtures within reasonably short periods of time at high voltage (5-10kV) using appropriate buffer systems. Tiffin (1966,1967,1971) extensively utilized low voltage (360-450V) to determine the forms of different metals in a number of plants. Lee et al. (1977) used high-voltage electrophoresis (3kV) to study the migration patterns of Ni in extracts from several New Caledonian hyperaccumulating plants. They were able to show that anionic and cationic froms of Ni in Homalium guillianii and Sebertia acuminata behaved similarly to Ni in a 1:2 nickel - citrate soultion, and an aquo nickel solution respectively. Under the same conditions, Kersten (1979) observed identical electrophoretic behaviour of a 1:1 nickel - citrate (anionic) complex in solution and Ni in a purified extract from Psychotria douarrei. He also observed that a 1:1 nickel - malate complex dissociated during electrophoresis.

In this study, electrophoresis was performed using Whatman #1 (Chromatography) paper at pH 6.5 with a 25:1:225 (v/v/v) pyridine / glacial acetic acid / water buffer. Both pyridine and glacial acetic acid were reagent grade. The pH of the buffer was within physiological range. A Savant type apparatus was used, incorporating water and high flash point petroleum spirit for cooling. Run time was 30 minutes at 3kV and 80mA. In all cases $[Ni(H_2O)_6]^{2+}$ served as reference. Nickel and citrate were visualised by spraying with 1 % ethanolic dimethylglyoxime and bromocresol green indicator respectively.

III.8. Separation of Nickel Complexes from D. gelonioides subsp. tuberculatum by Ion-exchange Chromatography and High-voltage Electrophoresis

III.8.I. Cation-exchange Chromatography

Twenty cm^3 of fully hydrated Amberlite IRC50-H resin was transferred to a 25 cm^3 burette and equilibrated with distilled deionised water for 30 minutes. The column was then eluted with ~5 cm^3 of 1.5 M HCl (Analar) to ensure protonation of all the carboxyl groups. Excess acid was removed by passing distilled deionised water through continuously until a pH of 5 was achieved. One cm^3 of concentrated extract containing the Ni complexes (1.50 mg Ni) was then applied to the column. Thirty 2 cm^3 fractions were collected at a rate of 1 cm^3 min^{-1} using distilled deionised water as eluant. Another fifty 2 cm^3 fractions were collected using 1.5 M HCl as eluant. A 0.5 cm^3 portion (6.75 mg Ni) of the crude extract (6.75 mg Ni) was subjected to the same procedure except that fractions 1-25 were eluted with distilled deionised water while fractions 26-50 were eluted with 1.5 M HCl.

Since dissociation of the Ni complexes is expected to be reduced or minimal in the presence of a weak acid, cation-exchange chromatography was also carried out with 1 M CH_3COOH (Analar) as eluant instead of 1.5 M HCl. Carboxyl groups were fully protonated by elution with 10 cm^3 of 1 M CH_3COOH. Elution with distilled deionised water served to remove excess acid. One cm^3 (1.50 mg Ni) was adsorbed onto the column. Using water as eluant, seven 2 cm^3 fractions were collected at the same flow rate as previously. The column was then eluted with 1 M CH_3COOH and forty fractions were collected. This procedure was repeated using 1 cm^3 of extract containing 0.17 mg nickel.

III.8.II. Anion-exchange Chromatography

Twenty cm^3 of fully hydrated IR45-OH resin was transferred to a 25 cm^3 burette and then equilibrated with distilled deionised water for 30 minutes. Hydroxyl counter-ions were then replaced by chloride ions by elution with 4.5 % HCl (75 cm^3). This was the form of the resin used by Kersten (1979). Excess HCl was removed by elution with 0.1 M NaCl (Analar). Water was not suitable for this purpose as it would have caused reconversion of the resin into its OH-form. Distilled deionised water was however used to remove excess NaCl until the effluent exhibited a pH of 7-8. One cm^3 (1.50 mg Ni) of extract was applied to the column which was then eluted with distilled deionised water. Twenty-five 2 cm^3 fractions were collected.

In order to ascertain whether the elution of Ni from the column was influenced by the nature of the counter ion, anion-exchange chromatography was also also performed using the hydroxyl form of the resin. The column was eluted with 70 cm^3 of 1 M NH_3 solution (Analar) to replace the chloride counter ions. After excess NH_3 solution was removed by elution with distilled deionised water, 1 cm^3 of sample containing 1.50 mg Ni was adsorbed onto the column. Seven 2 cm^3 fractions were collected. This procedure was repeated once using the same amount of sample in an attempt to monitor reproduciblity.

The Ni content of all fractions obtained from ion-exchange chromatography was determined by AAS and elution profiles were constructed. Fractions were pooled where appropriate and stored at 4° C for further studies.

III.8.III. High-voltage Electrophoresis

High-voltage electrophoresis was carried out on the samples shown in Table III.1. These were applied in a line along the centre (i.e the origin) of the sheet of Whatman #1 (46 x 57 cm) paper in 200 µL aliquots. A blow-dryer was intermittently used to prevent spreading and to assist in concentration of the sample. A 100 µL aliquot of 200 µg cm^{-3} $NiCl_2$ (Analar) solution was

applied as reference, in a similar manner. Using a glass rod and a 10 cm^3 graduated pipette, the paper was saturated with buffer beginning at the edge. This procedure was repeated at the opposite edge. When saturation was achieved to within ~1.5 cm of the origin on either side, the rods were slowly moved towards the origin in order to saturate it and further concentrate the sample. Excess buffer was removed using blotting paper. The support was then placed in a vertical glass tank so that both its top and base were in contact with the buffer solution. After the paper had been properly secured, the tank was covered and electrophoresis was allowed to progress for 30 minutes. The paper was then secured to a glass rod, removed from the tank and left to dry for two hours. Nickel was detected as pink areas after spraying with dimethylglyoxime and the yellow areas that appeared after spraying with bromocresol green indicator (pH <4) were attributed to citrate and other organic acids present. Once this preliminary distribution of Ni (and citrate) had been made, the Ni distribution was more accurately determined by eluting appropriate strips of paper with dilute acid and analysing the eluates for Ni. For each of the four samples, Ni-rich areas were cut into strips of 2.0 x 6.5 cm (for the reference, strip dimensions were 2.0 x 3.5 cm). These were transferred to test-tubes to which 2 cm^3 of 2 M HCl was added. All tubes were shaken for 10 min. using a Jank-Kunkel Electrical Shaker. The eluates were then analysed for Ni by AAS. The results were used to produce distribution profiles.

Table III.1 Samples Subjected to High-voltage Electrophoresis

SAMPLE NO.	NATURE OF SAMPLE	NICKEL CONCENTRATION ($\mu g\ cm^{-3}$)
1	Isolated Ni complexes from D.gelonioides subsp. tuberculatum (isolated by gel filtration chromatography)	195
2	Aqueous effluent obtained via cation-exchange chromatography of crude extract from D.gelonioides subsp. tuberculatum	146
3	Crude (aqueous) extract from P. 'palawanensis'	250
4	Crude (aqueous/acetone) extract from P.'palawanensis'	1350

III.8.IV Results and Discussion

The results of ion-exchange chromatography are presented in Table III.2. Some 90-94 % of the Ni in the plant extract was retained in a cationic form by the carboxylated resin when sample containing 0.17-1.50 mg Ni were applied (Runs 1,3 & 4). When a sample containing a larger amount of Ni was used, more of the Ni was still retained in its cationic form. However, the ratio of the amounts of anionic to cationic Ni was lower. Despite this, the higher percentage of cationic Ni indicates a stronger interaction between the resin and Ni than exists between Ni and the complexing ligand in the sample. This is suggestive of decomposition of an anionic or neutral complex. The lower percentage of cationic Ni obtained from the sample containing 6.75 mg Ni could be attributed to less decompostion of the Ni complex. HCl (1.5 M) and CH_3COOH (1 M) appear to be equally suitable as eluants for the removal of cationic nickel. Elution profiles are presented in Fig.III.1a-d.

With the weakly basic exchanger IR45-Cl, only 79 % of the Ni appeared to be cationic indicating that the remaining 21 % was anionic. It seems as though the passage of the Ni complexes through the cation exchanger resulted in greater decompostion compared to passage through the the anion exchanger. When the hydroxyl form of the resin was used, 59 % of the Ni was retained. A repeat experiment yielded a similar result. The elution profiles (Fig.III.1e) show a close association between anionic Ni and the resin. Perhaps the pH was sufficiently alkaline to adversely affect the complex. In both cation-exchange and anion-exchange chromatography, 80-90 % of the Ni applied to the columns was recovered in the fractions collected. The presence of cationic and anionic Ni is in agreement with the findings of Lee et al. (1977) and Kersten et al. (1980).

At pH 6.5, Ni in the two extracts from D.gelonioides subsp.tuberculatun showed exclusively cathodic migration during high voltage electrophoresis (Figs. III.2 and III.3). Citrate appeared to migrate anodically showing that the Ni-citrate had dissociated. In the P.'palawanensis' extracts, both cationic and anionic Ni were detected, but all the citrate migrated towards the anode.

Table III.2 Results of Ion-exchange Chromatographic Separation of Nickel Complexes in Extracts from D.gelonioides subsp. tuberculatum

RUN NO.	NATURE OF SAMPLE*	RESIN	ELUANT	% NICKEL ELUTED	IONIC NATURE OF FRACTION
1	Concentrated extract containing isolated Ni complexes [1.50 mg]	IRC50-H	H_2O	6	anionic/neutral
			HCl (1.5 M)	94	cationic
2	Crude extract [6.75 mg]	"	H_2O	40	anionic/neutral
			HCl (1.5 M)	60	cationic
3	Concentrated extract containing isolated Ni complexes [1.50 mg]	"	H_2O	8	anionic/neutral
			CH_3COOH (1.0 M)	92	cationic
4	" [0.17 mg]	"	H_2O	Nil	-
			CH_3COOH	88	cationic
5	" [1.50 mg]	IR45-Cl	H_2O	79	cationic/neutral
6	" [1.50 mg]	"	"	41	cationic/neutral
			NH_4OH (1.0 M)	59	anionic
7	" [1.50 mg]	"	H_2O	45	cationic/neutral
			NH_4OH (1.0 M)	55	anionic

* - Weight of Ni applied to the column is shown in square brackets.

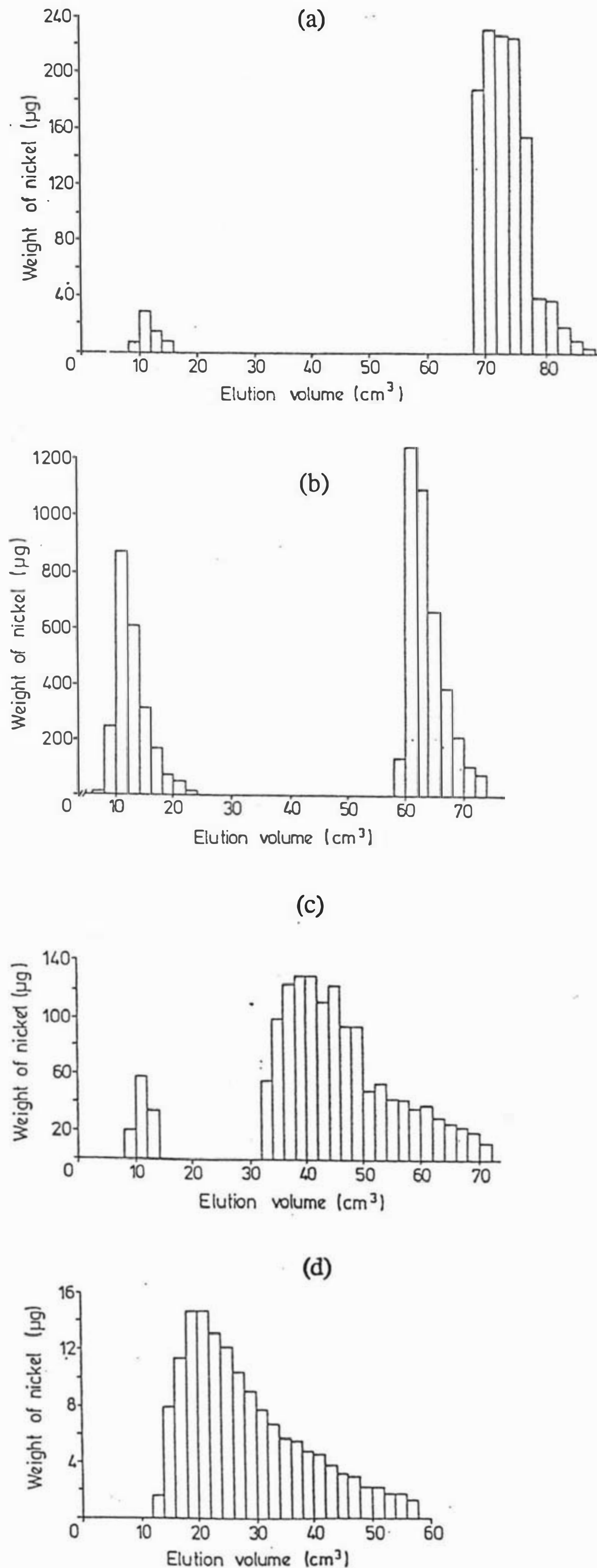


Fig. III.1(a)-(d).Elution of Ni species in D. gelonioides subsp. tuberculatum from IRC50-H cation-exchange column.Profiles correspond to Runs 1to 4 in Table III.2.

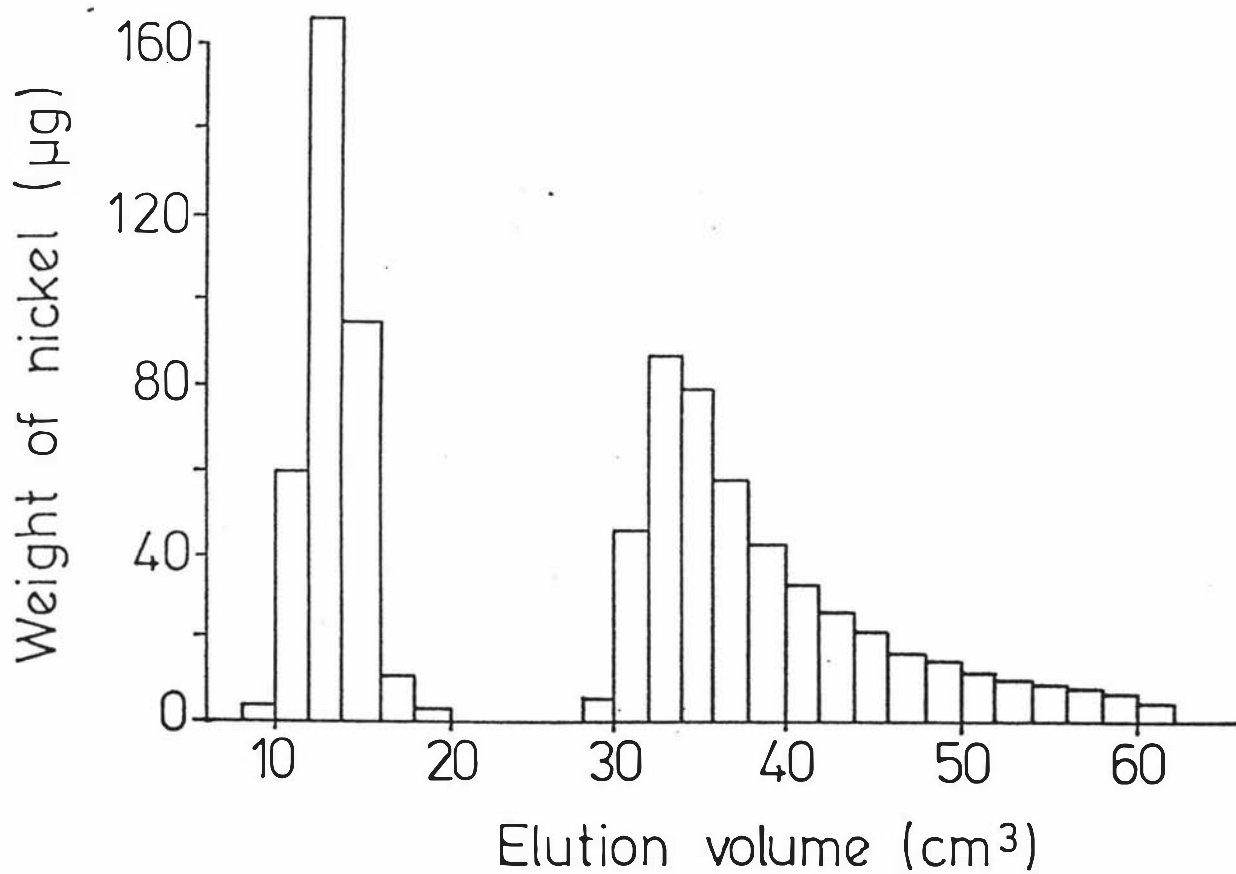


Fig. III.1(e) Elution of Ni species in D.gelonioides subsp. tuberculatum from IR45-OH anion-exchange column .Profile corresponds to Run 6 in Table III.2.

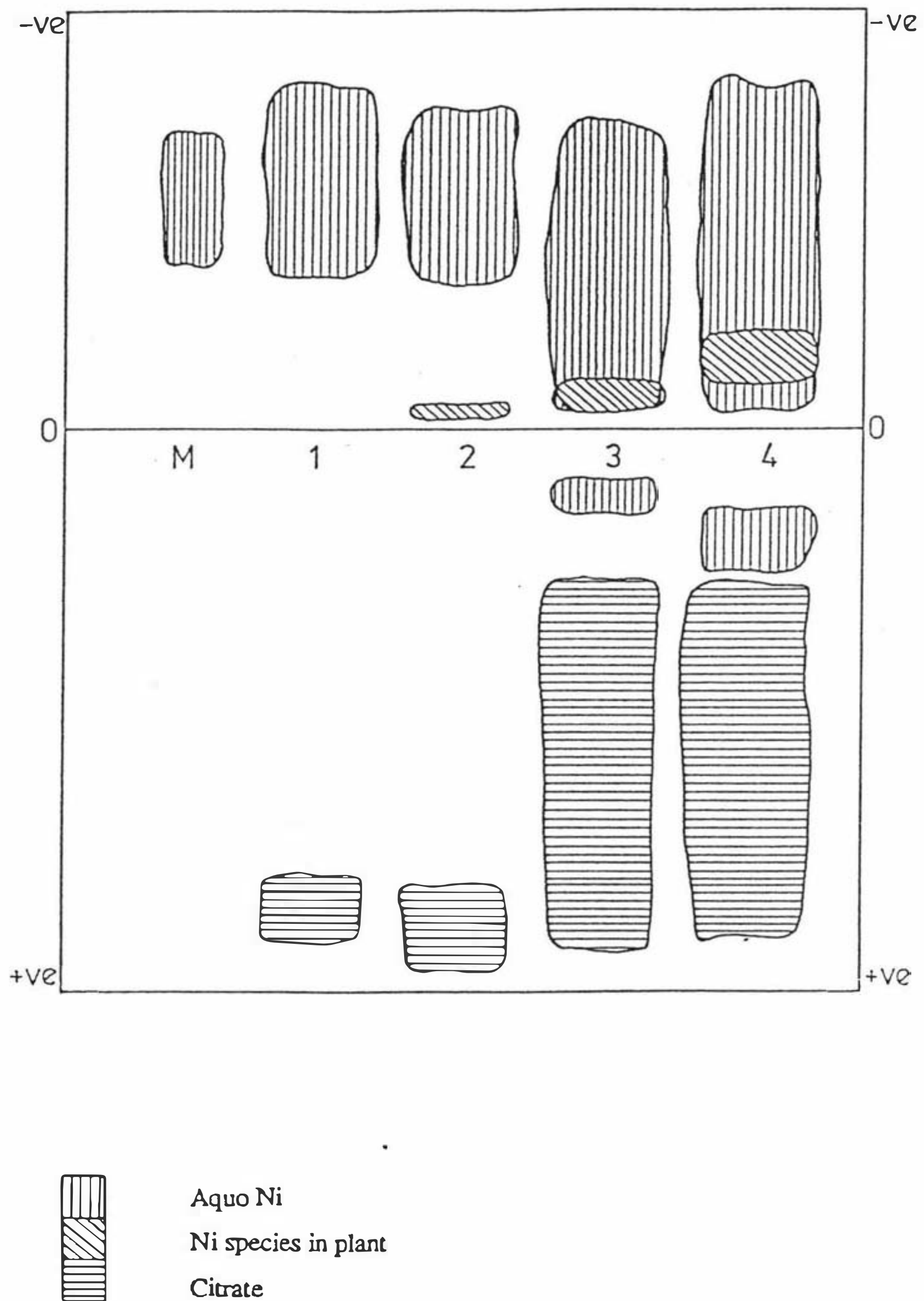


Fig. III.2 Electrophoretic separation of Ni species in D.gelonioides subsp. tuberculatum and P. 'palawanensis' at pH 6.5. (M) Hexa-aquo Ni, (1) D.gelonioides subsp. tuberculatum crude, concentrated extract passed through Sephadex G-10 column, (2) D.gelonioides subsp. tuberculatum crude, concentrated extract passed through IRC50-H column, (3) P. 'palawanensis' crude concentrated aqueous extract, (4) P. 'palawanensis' crude concentrated acetone/water extract.

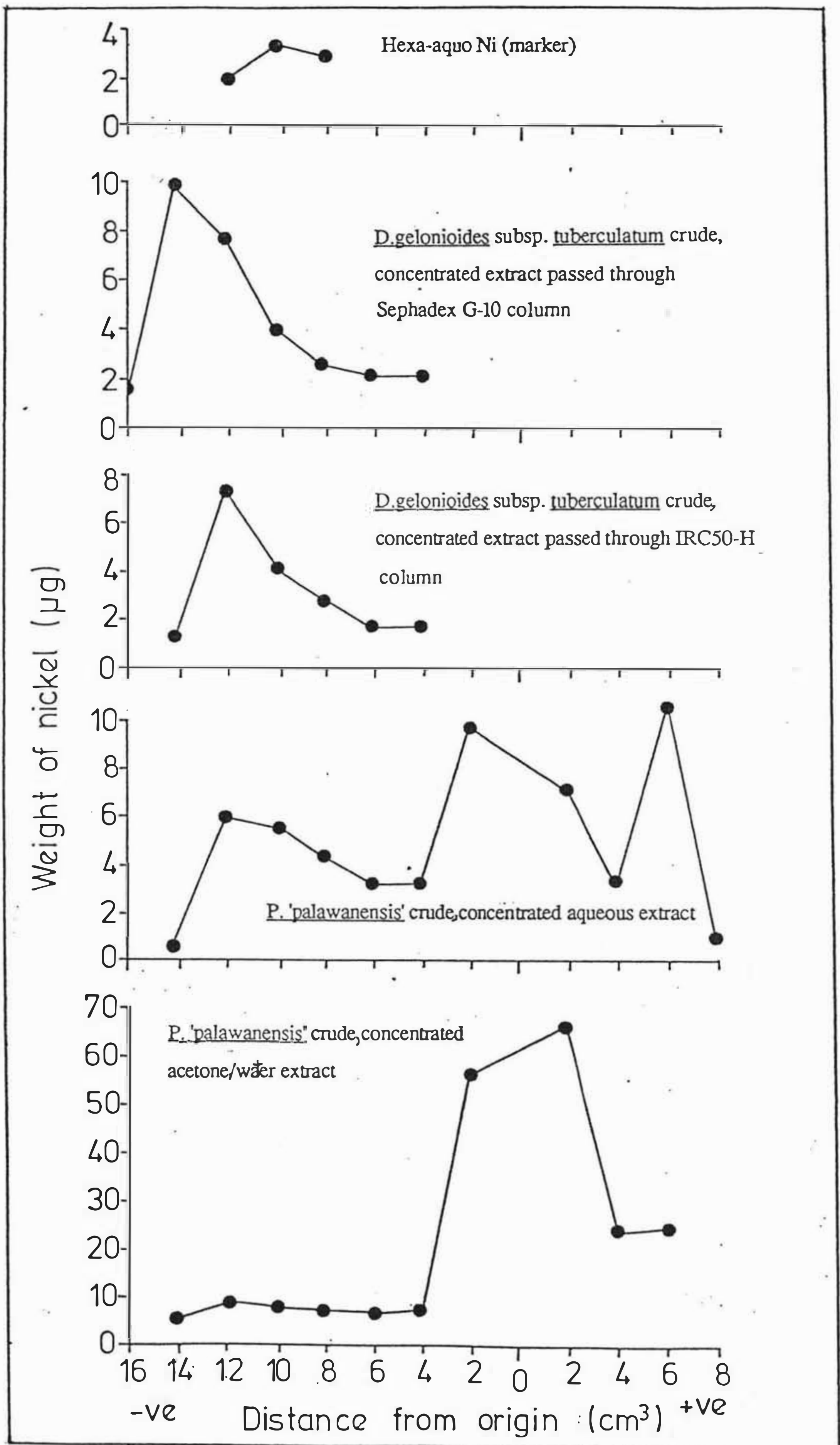


Fig. III.3. Electrophoretic Distribution of Ni species in D.gelonioides subsp. tuberculatum and P. 'palawanensis'at pH 6.5.

A slight difference in migratory behaviour was observed between anionic Ni and cationic Ni in the two extracts (Fig.III.3). The difference may be attributed to the nature of the extractant used.Electrophoresis was originally carried out on these extracts at the time out of curiosity and for expediency. Extensive ion-exchange studies were subsequently carried out on various extracts from the plant. The blue-coloured areas observed in the electrophoretogram of samples 2,3 and 4 may have been due to one or more of the following interactions :- (i) Ni and pyridine, (ii) Ni-citrate and pyridine, (iii) an unknown Ni complex and pyridine. This view is supported by the observation that both the pyridine containing buffer (pH 6.5) and trisodium citrate solution turned the bromocresol green indicator blue. It is recognised that in addition to citrate, malate and malonate, anions of other organic acids may be present.Further, the stabilities of Ni-malate and Ni-malonate complexes are lower than that of anionic Ni-citrate (Sillén and Martell, 1964).

III.9. Separation of Nickel Complexes in Extracts from *P.'palawanensis'* by Ion-exchange Chromatography

III.9.I. Anion-exchange Chromatography

Nickel-rich extracts from *P.'palawanensis'* were subjected to anion-exchange chromatography on Amberlite IR45-OH resin. Table III.3. provides information relevant to methodology and the results obtained for the samples used.

III.9.II. Cation-exchange Chromatography

A crude extract from *P.'palawanensis'* was also subjected to cation-exchange chromatography along with effluents from the previously used anion-exchange columns (Section III.9.I). The weakly acidic Amberlite IRC50-H resin was used in each case. Relevant information on methodology and the results of the experiments are presented in Table III.3.

Table III.3 Results of Ion-exchange Chromatographic Separation of Nickel Complexes in Extracts from P. 'palawanensis'

RUN NO.	NATURE OF SAMPLE*	RESIN	ELUANT	% NICKEL ELUTED	IONIC NATURE OF FRACTION# §
8	Acetone/water extract (crude)	IR45-OH	H_2O	47	cationic[1]/neutral
	[1.28 mg]		NH_4OH	53	anionic[2]
			(1.0 M)		
9	"	"	H_2O	51	cationic[3]/neutral
			NH_4OH	49	anionic[4]
			(1.0 M)		
10	Aqueous extract (crude)	"	H_2O	49	cationic[5]/neutral
	[1.07 mg]		NH_4OH	51	anionic[6]
11	" [0.43 mg]	"	H_2O	18	cationic[7]/neutral
			HCl	82	anionic (2)[8]
			(4.5%)		
12	Combined cationic	IRC50-H	H_2O	Nil	-
	fraction (1 + 3 + 5)		CH_3COOH	77	cationic[a]
	[0.20 mg]		(1.0 M)		
13	Combined anionic	"	H_2O	Nil	-
	fraction (2 + 4 + 6)		CH_3COOH	75	cationic[b]
	[0.20 mg]		(1.0 M)		
14	Combined crude extract	"	H_2O	43	anionic (2)[c]/neutral
	(acetone/water and water,		CH_3COOH	57	cationic[d]
	as in Runs 8 & 10)		(1.0 M)		
	[3.16 mg]				

* - Weight of Ni applied to the coulmn is shown in square brackets

\# - Number of peaks in elution profile is shown in curved brackets

§ - Superscripts in letters represent fraction reference code

Fig. III.4(a)-(c) Elution of Ni species in P. 'palawanensis' from IR45-OH anion-exchange column. Profiles (a) and (c) correspond to Runs 8 and 11 in Table III.3 respectively, profile (b) removal of adhering Ni by elution with 4.5 % HCl.

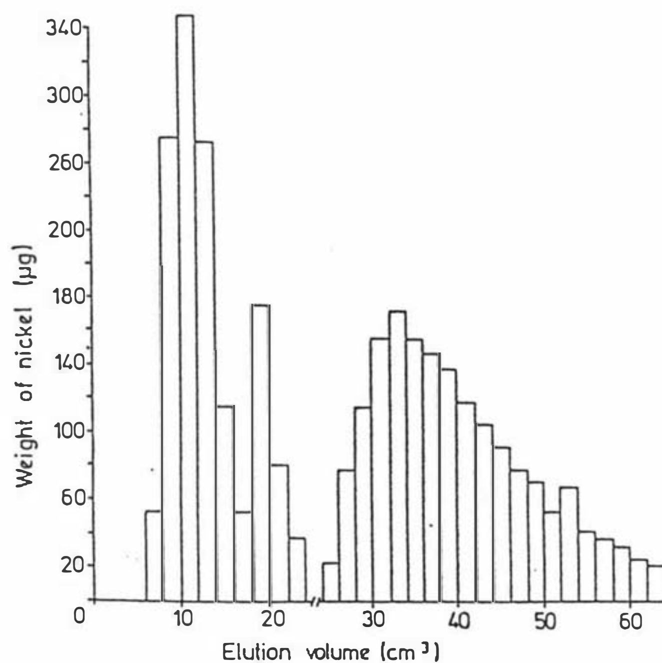
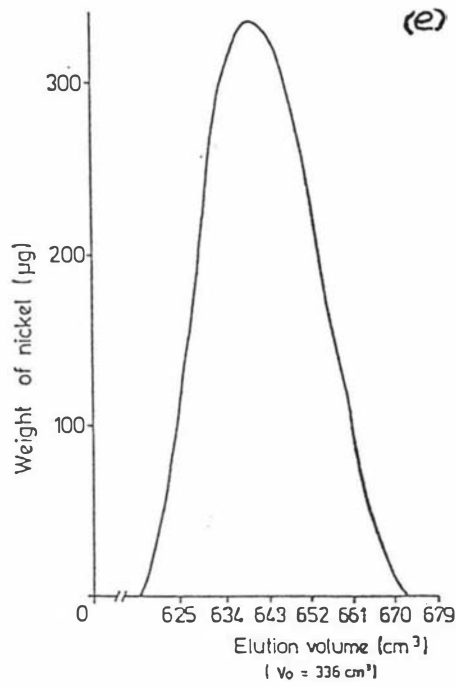


Fig III.4(d) Elution of Ni species in P. 'palawanensis' from IRC50-H cation-exchange column. Profile corresponds to Run 14 in Table III.3



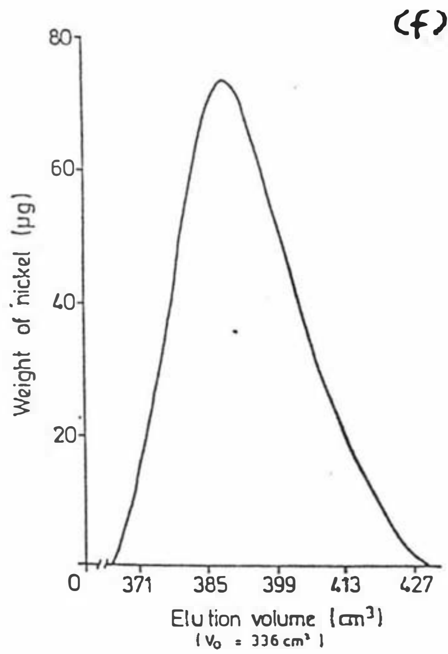


Fig.III.4 Elution of (e) cationic and (f) anionic Ni species in P.'palawanenesis' from Sephadex G-10 following ion-exchange chromatography.

III.9.III. Results and Discussion

Typical elution profiles are presented in Fig. III.4a,c & d. The percentage of Ni recovered from Runs 8, 9 and 10 was in the range of 50-66 %, thus prior to Run 11, Ni adhering to the column was removed using 4.5 % HCl. The elution profile (Fig. III.4b) showed the presence of two poorly resolved components. The column was then reconverted into its OH-form following elution with distilled deionised water to remove excess acid. Hydrochloric acid (4.5 %) was used as the second eluant in Run 11 instead of 1 M NH_3 solution. The percentage of recovered Ni rose to 86 % despite the lower weight of Ni applied to the column. Nickel was distributed among three peaks (Fig. III.4c), two of which corresponded to anionic fractions. The other was cationic. These results suggest that use of acid as the second eluant may lead to less interaction between the resin and Ni complexes present. The chloride ion is a weaker base than the hydroxyl ion, and would therefore be expected to replace anionic Ni on the resin more readily than the hydroxyl ion. The volatility of ammonia solution may also lead to changes in pH during the separation process. The unsuitablility of ammonia solution as an eluant was alluded to earlier in Section III.8.IV.

Prior to cation-exchange chromatography, the pH of fractions 1-6 was determined using an Orion Research 501 Digital Ionalyser equipped with a combination type electrode. The values obtained were compared to those of the concentrated crude (aqueous) extract and the solution containing complexes isolated from the crude acetone/water extract. As shown in Table III.4, the six fractions all had pH values higher than the samples which had not been subjected to anion-exchange chromatography. Except for Run 10, from which fractions 5 and 6 were derived, the pH of fractions obtained by elution with distilled deionised water was slightly higher than those obtained by eluting with ammonia solution. Obviously, traces of ammonia still remained on the column despite elution with water after the column was converted to the OH-form. The use of water as eluant in subsequent experiments therefore served to remove traces of the base. This is manifested as a progressive decline in the magnitude of the difference between the pH values of fractions derived from aqueous and ammonia solution, from Run 8 through to 10. Further evidence of the unsuitability of ammonia solution (1 M) as eluant has therefore been provided.

Table III.4 The pH of Nickel-rich Solutions from P. 'palawanensis'

SAMPLE	pH
Concentrated crude (aqueous extract)	4.95
Ni complexes isolated from acetone/water extract	3.35
Fraction 1	9.75
Fraction 2	8.20
Fraction 3	9.50
Fraction 4	8.10
Fraction 5	9.65
Fraction 6	10.9

Solvent was initially removed from each fraction by rotary evaporation at 33-35° C, and finally by evaporation in vacuo using liquid air. Two cm^3 of distilled deionised water was then added to each of the six residues. Each flask was then swirled to expedite dissolution. Anionic (odd-numbered) and cationic (even-numbered) fractions were combined separately.

When the combined cationic fraction (Run 12) was subjected to cation-exchange chromatography, the results confirmed the cationic nature of Ni complexes present. Similar findings were not obtained for the combined anionic fraction (Run 13). Perhaps the removal of NH_3 solution and subsequent addition of water, coupled with the small amount of Ni present, resulted in the dissociation of the original anionic complex to form cationic aquo Ni. The percentages of Ni recovered during both runs was 75-77. Between runs, adhering Ni was removed from the column by elution with 1.5 M HCl. Nickel in the combined crude extract was shown to be both anionic and cationic (Run 14). Similar results were earlier obtained using electrophoretic

separation. It was observed that elution with distilled deionised water removed the brown colouration from the extract. The resin therefore acted as an effective filter. The brown colouration was thought to be due to the presence of pigments. Obviously, treatment of the extract with chloroform/butanol reagent did not remove these.

Fractions a and d were combined and the Ni complexes present were then purified by gel filtration on Sephadex G-10 using distilled deionised water as eluant. Fraction c was subjected to the same procedure. Nickel from the two fractions eluted as a single peak (Fig.III.4e & f). The Ni-rich column effluents were retained and are henceforth referred to as Cat I and An I.

III.10. Molar Mass Determination of Nickel Complexes Isolated from P.'palawanensis'

In view of the conflicting results obtained using ion-exchange chromatography, a molar mass determination was carried out on the supposedly cationic and anionic Ni complexes in an attempt to ascertain whether the complexes were in fact different. The procedure was not applied to the ionic Ni fractions derived from D.gelonioides subsp. tuberculatum as the results of ion-exchange chromatography were less ambiguous.

III.10.I. The Use of Gel Filtration Chromatography for Molar Mass Determination

In gel filtration chromatography, the molar mass dependent elution volume of a given substance is easily reproduced on the same column. One may thus determine the molar mass of an unknown substance from the elution volume (Determann, 1968). This necessitates calibration of the column with a suitable selection of molecules. In order to preserve the relationship between (molecular) size and molar mass, the standards used for calibration should be closely related to the substance to be studied. Unfortunately, compounds suitable for calibration are not readily available, vinyl polymers and proteins being the exceptions. A wide range of synthetic polystyrenes of narrow molar mass distribution is available for molar mass determinations. The fact that proteins are found naturally, and have exactly defined molar masses, makes them extremely useful as calibrants. Both the vinyl

polymers and proteins are usually available commercially as "Molecular Weight (molar mass) Marker Kits". Each consists of a set of standards for various molar mass ranges.

The elution behaviour of a substance can be described by different variables, all of which are derived from the elution volume (V_e). Since these parameters are independent of the geometry of the column, a comparison of the two experiments can easily be made. To this end, several relationships have been derived. These are either empirical or based on theoretical considerations of the dependence of V_e on molar mass (M). A comprehensive listing of these relationships is provided by Determann (1968). The most frequently used relationship is that of linear dependence of elution volume on the logarithm of the molar mass (Determann and Michel, 1966).

Over the past twenty years, gel filtration has been used to determine the molar mass of macromolecules such as proteins (Locascio et al.,1969; Andrews, 1970). According to Andrews (1970), the accuracy of the determination is within ± 10 per cent. Its application to small molecules is less common.Generally information regarding the choice of suitable markers for such applications is therefore understandably, not readily available. However, the technique has been applied to Ni complexes isolated from Ni hyperaccumulating plants (Kersten, 1979; Freeman, 1989). Using the relationship derived by Porath (1963), Kersten (1979) was able to determine the molar masses of Ni complexes isolated from Psychotria douarrei to be 360 and 220 g mol^{-1} on Sephadex G-10. Although the organic dyes (along with aquo Ni) used as markers suffered severe tailing on the column, a significant portion of each eluted within a 20 cm^3 volume, and characteristic peaks were readily identified. All markers possessed molar mass < 700. Freeman (1989) showed the molar mass of a Ni complex obtained from Walsura monophylla (Philippines) to be within the range 292-357 g mol^{-1}, using a Bio-Gel P_2 (polyacrylamide) column. Ammonium acetate (Analar - 0.5 M) was used as eluant owing to its mild buffering capacity (pH ~7). In his application, Freeman used Whitaker's (1963) relationship: $V_e/V_o = k \log M$, where V_e = elution volume; Vo = void volume and M = molar mass. Several biochemical compounds and an Fe(III) - complex served as markers. The method selected for determining the molar mass of Ni complexes isolated from P. 'palawanensis' by ion-exchange chromatography and gel filtration chromatography was that used by Freeman (1989).

III.10.II. Methodology

About 300 cm^3 of swollen Pharmacia Fine Bio-Gel P_2 (200-400 mesh) was transferred to a 67 x 2.5 cm glass column. The column was then equilibrated with 0.05 M ammonium acetate for 1 hour. A Re Cy Chrom Peristaltic Pump [type 4912 A] was used to force the eluant through the column at a rate of 0.53 cm^3/min^{-1}. The void volume was determined using 3 cm^3 of aqueous (2mg cm^{-3}) Blue Dextran and an LKB Ultrorac Fraction collector as in Section II.9. Ammonium acetate (0.05 M) served as reference. The void volume was calculated as 72 cm^3. The Bio-Gel column was suitable for the determination of molar masses over the range 200-1800 g mol^{-1}.

The following Sigma biochemicals were used as calibrants. AMP (365.4 g mol^{-1}), cyclic-AMP (329.2 g mol^{-1}), reduced glutathione (307 g mol^{-1}), guanosine (283 g mol^{-1}),adenosine (267.2 g mol^{-1}) and uridine (244 g mol^{-1}). Individual standards of concentration 0.7-0.9 mg cm^{-3} were prepared in 0.05 M CH_3COONH_4 except for guanosine which was insoluble. Acetic acid (0.05 M) was used instead for preparing this standard solution. Samples were weighed on a Mettler AE 163 Electronic Balance with a precision of ±0.01mg. A multistandard was then prepared in 0.05 M CH_3COONH_4, with the concentration of the constituents ranging from 74-90 μg cm^3. All standard solutions were stored in a freezer when not in use. Three cm^3 of multistandard was applied to the P_2 column. One hundred cm^3 of effluent was discarded after which 200 - 2 cm^3fractions were collected. The absorbance of each fraction was measured against 0.05 M CH_3COONH_4 in 0.6 cm^3 quartz cuvettes using a Shimadzu 160 UV-VIS Recording Spectrophotometer at 260 nm (Freeman, 1989). The elution profile is shown in Fig.III.5a.

Compared to cyclic-AMP, reduced glutathione, guanosine, adenosine, AMP and uridine eluted as rather small peaks. This was attributed to poor choice of wavelength. In order to further investigate, the spectrum of each standard was obtained over the wavelength range 1100-200 nm. The wavelength yielding the highest absorbance was recorded in each case (Table III.5). Groups of

fractions of each standard that gave the highest absorbances at 260 nm were then reanalysed at the appropriate wavelength maximum. It was observed that the fractions that exhibited the highest absorbances at the wavelength maxima were the same as those that showed maximum absorbance at 260 nm. In view of the spread of the wavelength maxima, and having recognised that solute-solute interactions can alter absorbance characteristics, the entire batch of fractions was then analysed at 245 nm as a better compromise wavelength. While the sensitivity to AMP improved only slightly, there was substantial improvement in the detection of uridine (Fig.III.5b). Based on these findings, the wavelength of 245 nm was selected for future use.

To determine whether potassium hexacyanoferrate was suitable as a calibrant, 3 cm^3 of sample comprising 50 mg of Analar trihydrate salt and 1 cm^3 of 0.8 mg cm^{-3} uridine made up to 10 cm^3 in 0.05 M CH_3COONH_4, was loaded onto the column. Elution and fraction collection were carried out as before. The absorbances of the 200 fractions collected were obtained at 245 nm. As a check, the identified Fe-rich fractions were then analysed by AAS at 248.3 nm. Gel filtration was repeated using a 3 cm^3 sample comprising 50 mg of the Fe salt and 1 cm^3 each of standard AMP,reduced glutathione, guanosine and adenosine diluted to 10 cm^3 with 0.05 M CH_3COONH_4. Fractions were analysed in like manner.

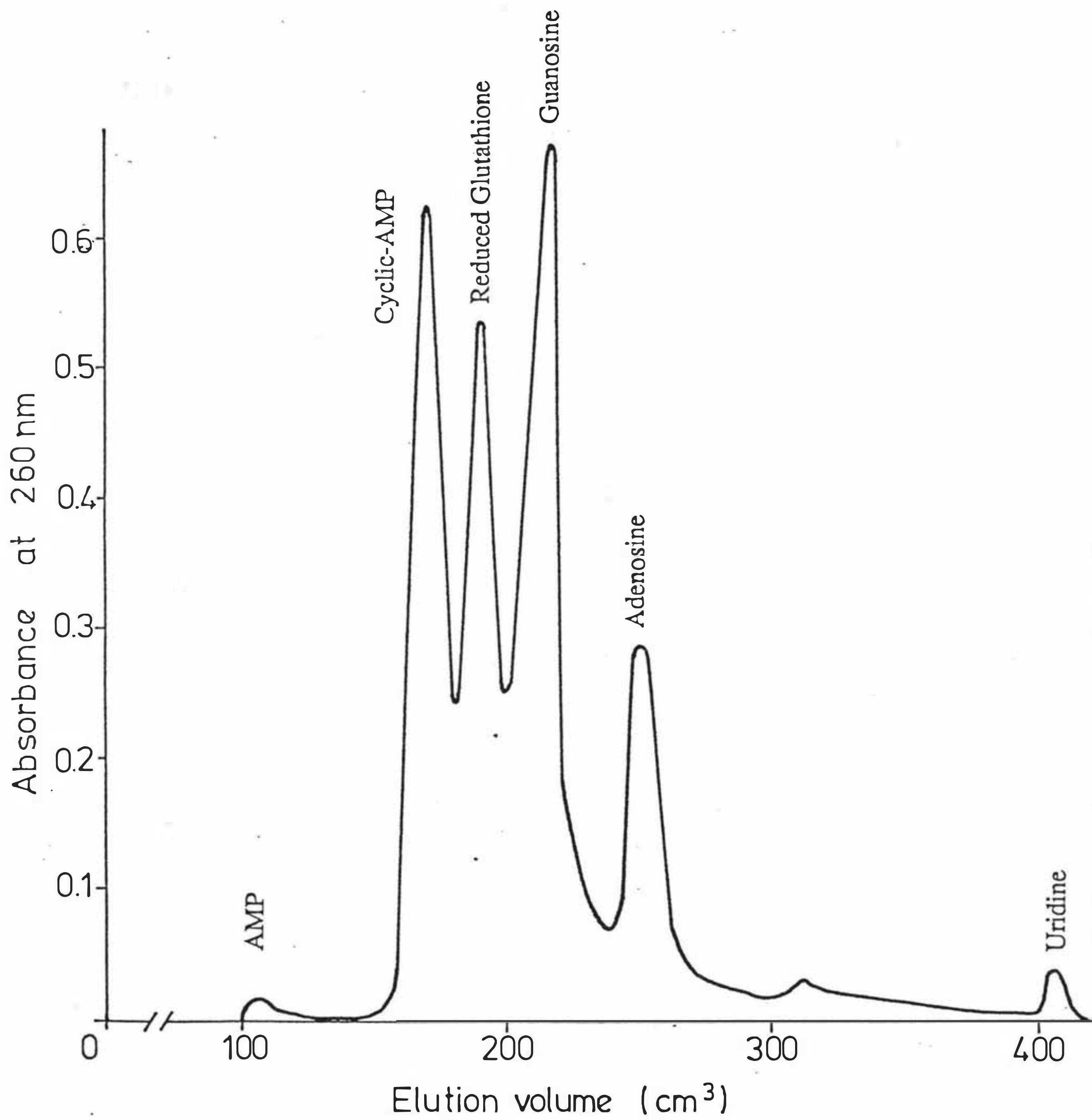


Fig. III.5(a) Elution of molar mass markers from Biogel P_2 column at 260 nm.

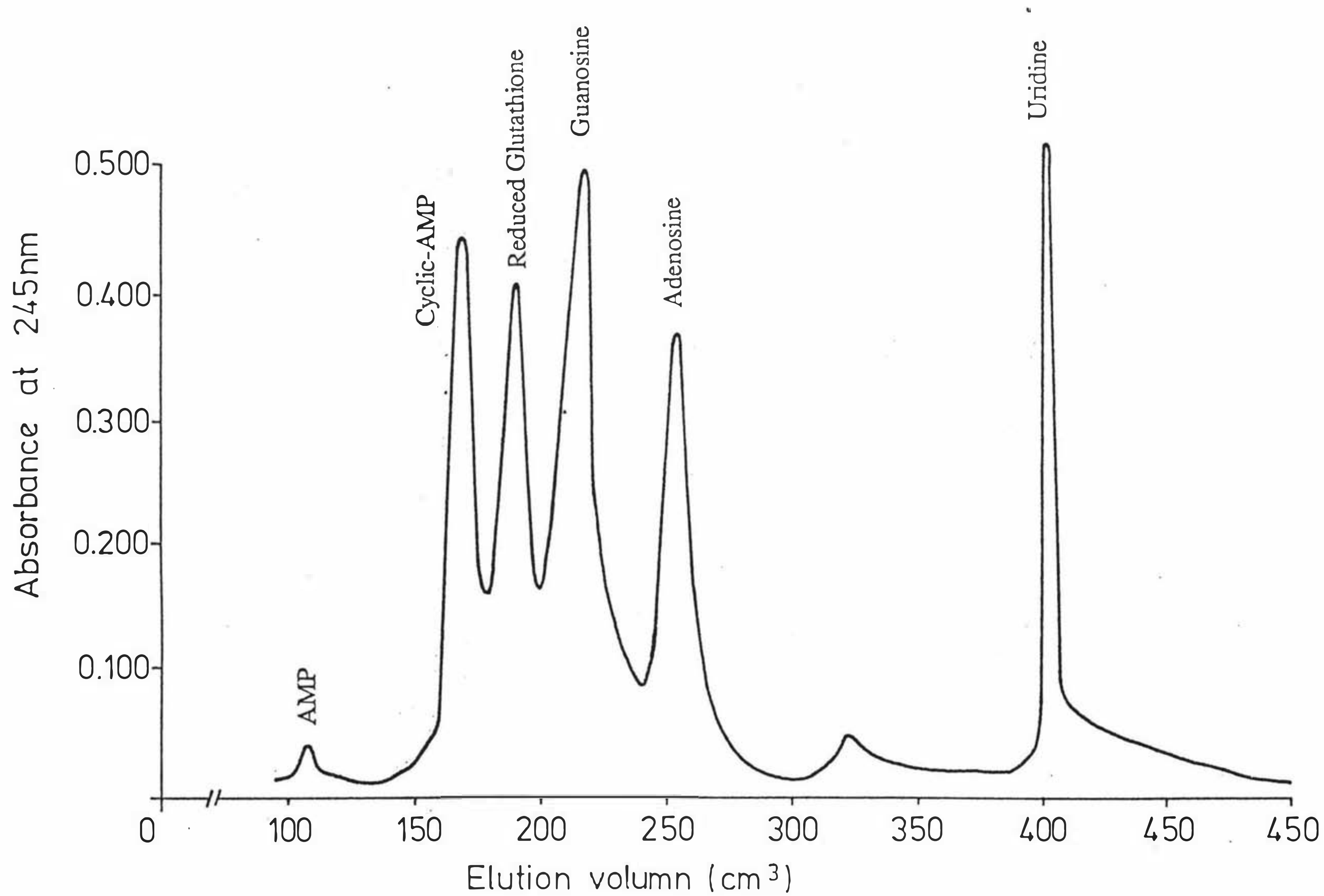


Fig. III.5(b) Elution of molar mass markers from Biogel P_2 column at 245 nm.

Table III.5 Absorbance Maxima [λ max] of Biochemicals Used in Molar Mass Determination

BIOCHEMICAL	THIS WORK λ max (nm)	FREEMAN (1989) λ max (nm)
AMP	245	246
Cyclic-AMP	249	282
Reduced Glutathione	217	225
Guanosine	251	290
Adenosine	248	283
Uridine	262	287

A similar procedure was applied to a solution of (1:1) Fe(III)-EDTA complex; the preparation based on the method of Bassett et al., (1978). A 5 cm^3 aliquot of 250 $\mu g\ cm^{-3}$ Fe (prepared from a 1000 $\mu g\ cm^3$ BDH-AAS stock standard) was transferred to a 25 cm^3 volumetric flask. Eight mg of Analar EDTA-disodium dihydrate salt, dissolved in a minimum amount of distilled deionised water was quantitatively transferred to the flask. The solution was diluted to the mark with distilled deionised water and thoroughly mixed. Ten cm^3 of composite solution of reduced glutathione, guanosine and adenosine, each of concentration ~0.08 mg cm^{-3} was prepared. Three cm^3 of a mixture consisting of 2.5 cm^3 each of Fe-EDTA and the composite solution was then applied to the column. AMP and cyclic-AMP were not included because of poor resolution owing to the proximity of their molar masses to that of the Fe-EDTA complex. One hundred and forty fractions were collected. Iron-rich fractions were analysed by both UV/VIS and AA spectrometry. The entire procedure was repeated to determine whether the elution behaviour of the complex was reproducible.

About 33 cm^3 of fraction Cat I (Section III.9.III) was concentrated down to ~1.6 cm^3 at 33-35 ° C in vacuo. One and a half cm^3 of this solution containing 375 μg Ni was mixed with 1.5 cm^3 of a solution containing reduced glutathione, guanosine and adenosine at a comparable concentration level. Two and a half cm^3 of the resultant solution was adsorbed onto the column. Ninety-seven fractions were collected. An elution profile was obtained using UV/VIS and AA spectrometry (Fig.III.5c). Since only 71 % Ni was recovered from the column, the column was eluted with 0.1M HCl to remove adhering Ni. This was followed by elution with distilled deionised water to remove excess acid and subsequently, equilibration with 0.05 M CH_3COONH_4. Fraction An I (Section III.9.III) was similarly concentrated down to ~0.8 cm^3. This volume contained ~546 μg Ni. The concentrate was mixed with 2.5 cm^3 of the composite solution containing AMP, cyclic-AMP, reduced glutathione, guanosine and adenosine at a concentration of ~0.3 mg cm^{-3}. The resultant solution was applied to the Bio-Gel column. An elution profile was obtained using the same techniques as before (Fig.III.5c). Only 25 % of the Ni adsorbed onto the column was eluted. The Ni-rich fractions were separately combined and refrigerated. The combined cationic and anionic effluents were henceforth referred to as Cat I' and An I' respectively. The column was eluted with 0.1 M HCl to remove adhering nickel.

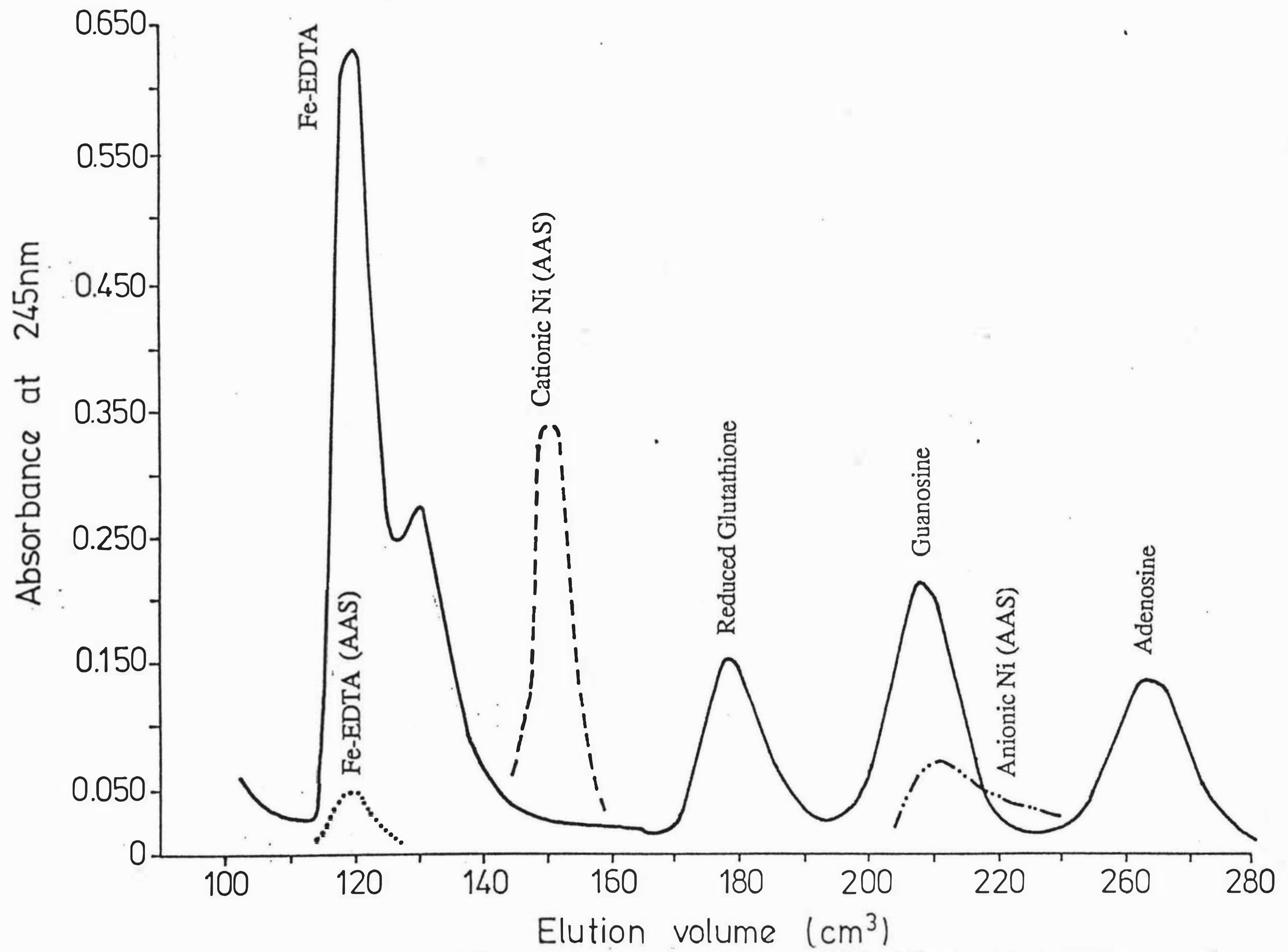


Fig. III.5(c) Elution of molar mass markers and Ni species in P. 'palawanensis' from Biogel P_2 column.

The molar masses of the cationic (Cat I) and anionic (An I) Ni complexes were extrapolated from a plot of V_e/V_o versus log M which showed good linearity (Fig.III.6). Relevant data are presented in Table III.6.

Fig. III.6. Calibration curve for molar mass determination.

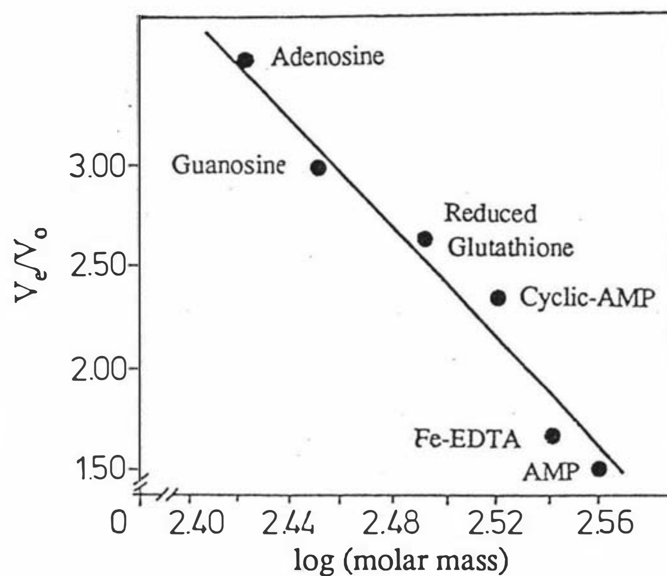


Table III.6 Data for Molar Mass Determination

STANDARD	MOLAR MASS	$\log_{10}$ (MOLAR MASS)	V_e¶ (cm^3)	V_e/V_0
AMP	365.2	2.56	106	1.47
Fe-EDTA	344	2.54	125	1.74
Cyclic-AMP	329.2	2.52	169	2.35
Reduced Glutathione	307.2	2.49	183	2.54
Guanosine	283.2	2.45	212	3.00
Adenosine	267.2	2.42	252	3.50

¶ - mean values of V_e

III.10.III. Results And Discussion

The consistent elution of potassium hexacyanoferrate (V_e = 163 cm^3) close to cyclic-AMP (V_e = 156 cm^3) and before uridine (V_e = 420 cm^3), suggests that Fe did not elute as the neutral salt - $K_4 Fe(CN)_6$. The molar mass of the neutral salt is ~380 g mol^{-1}, therefore, if Fe had eluted in this form, it would have been expected to elute before AMP. Elution of Fe as the anion $Fe(CN)_6^{4-}$ can also be discredited on the basis of molar mass; since a V_e in excess of that obtained for uridine would have been expected. Because of this anomalous behaviour which was probably related to the stability of the complex under the experimental conditions, potassium hexacyanoferrate was considered unsuitable as a marker. During both experiments, the Fe-EDTA complex eluted at ~125 cm^3 (Fig.III.5c). There was close agreement between the elution volumes obtained via UV/VIS and AA spectrometry.

The ionic Ni complexes from P. 'palawanensis' eluted as single peaks with different elution volumes, confirming the presence of two distinct complexes (Fig.III.5c). The cationic complex eluted at 150 cm^3 between Fe(III)-EDTA and cyclic -AMP, while the anionic complex eluted at 210 cm^3 just ahead of guanosine. The lower percentage of Ni recovered from the column when An I was applied could be an indication of lability. Using Andrew's (1970) estimation of ±10 % accuracy, the molar mass of the cationic complex was in the range 298.6-398.2, and that of the anionic complex was in the range 263.2-321.6 (Fig.III.6). The mid-points of the ranges corresponded to V_e/V_0 values of 2.08 and 2.92 for Cat I and An I respectively. Based on these results, Ni did not therefore elute as the hexa-aquo complex. While the calibrants were not closely related to the complexes of interest, this did not appear to adversely affect the results, as the molar masses obtained were similar to those reported by Kersten (1979) & Freeman (1989)

III.11. Improved Extraction of Nickel from P. 'palawanensis' and Separation of Resulting Nickel Complexes.

III.11.I. Methodology

Because of the apparent association of Ni with pectates and proteins in leaves of P. 'palawanensis' (Section II.7), ~10 g of ground leaf material was mixed with a minimum (25 cm^3) of 95 % Analar ethanol prior to extraction with distilled deionised water (50 cm^3) as per usual (Section II.8). This procedure was based on the reported solubility of pectate in 95 % ethanol (Merck, 1989). Ethanol was also used in between repeat extractions, these numbering three in all. The initial solubilization appeared to facilitate suction filtration thereby resulting in more expedient removal of nickel. A higher percentage of Ni was extracted under these conditions compared to extraction using the 3:1 acetone/water mixture (Section II.10). The exact extraction procedure was repeated using another 10 g of dried ground leaf material. It was observed that the two extracts exhibited different colours despite being obtained under identical conditions. The first extract was brown while the second was greenish/brown. High molar mass, low polarity compounds were removed from each using the chloroform/butanol reagent. The resulting crude extracts were not combined. Traces of chloroform/butanol reagent and much of the water and ethanol present were removed by rotary evaporation at 33-35 $^{\circ}$ C. The extracts were concentrated down to 21 and 9 cm^3 respectively. The brown extract (2A) was shown to contain 112 mg Ni (i.e in 21 cm^3) and the 9 cm^3 of greenish/brown extract (2B) contained 18.1 mg Ni. Any precipitate observed during storage at 4 $^{\circ}$ C was removed after centrifugation using a Dupont Sorvall RC-5B Refrigerated Centrifuge at 18000 r.p.m. for 30 minutes. Centrifugation was carried out at 22-24 $^{\circ}$ C.

III.11.II. Ion-exchange Chromatography and High-voltage Electrophoresis

Extracts 2A and 2B were subjected to both cation and anion exchange chromatography. Less then 5 cm^3 of extract was applied to the resin in each instance. Operating conditions and results of the experiments are presented in Table III.7. High-voltage electrophoresis was carried out on the following samples:-

(i) Brown cationic effluent from the anion-exchange column (2A')

(ii) Greenish/brown cationic effluent from the anion-exchange column (2B')

(iii) Cationic Ni eluted from the P_2 acrylamide column (Cat I')

(iv) Anionic Ni eluted from the P_2 acrylamide column (An I')

(v) Cationic Ni separated via cation-exchange chromatography and purified by gel filtraion on Sephadex G-10 (Cat I)

(vi) Anionic Ni separated via cation-exchange chromatography and purified by gel filtration on Sephadex G-10 (An I)

(vii) Cationic and anionic Ni isolated from crude extract by gel filtration on Sephadex G-10 (in duplicate)

The experiment was carried out as in Section III.8.III. All samples were concentrated in vacuo at 33-35 ° C to ensure that Ni was readily detected by 1 % ethanolic dimethylglyoxime. Two Ni solutions (M_1 and M_2) were used as references. The hexa-aquo Ni complex (M_1) was prepared by suitably diluting the BDH-AAS standard of concentration 1000 $\mu g\ cm^{-3}$. Anionic nickel-citrate (M_2) was prepared according to Liddle (1979) who reported the stability of the complex to be optimum in the pH range of 5-8. Equal volumes of 9.554×10^{-4} M Analar nickel chloride hexahydrate and 9.556×10^{-4} M Analar citric acid monohydrate were mixed and the pH of the resulting solution was adjusted to ~6.28 with 1M NaOH (Analar). Samples and reference solutions

were applied to the support at a loading of 100 μL per 20 mm. Bromocresol green indicator was used to locate areas rich in citrate.

Table III.7 Results of Ion-exchange Chromatographic Spearation of Nickel Complexes in Ethanol/Water Extracts from P. 'palawanensis'.

RUN NO.	NATURE OF SAMPLE*	RESIN	ELUANT	% NICKEL ELUTED	IONIC NATURE OF FRACTION
15	2A [8.64 mg]	IRC50-H	H_2O	72	anionic/neutral
			CH_3COOH (1.0 M)	28	cationic
16	" [8.27 mg]	"	H_2O	78	anionic/neutral
			CH_3COOH (1.0 M)	22	cationic
17	" [23.0 mg]	"	H_2O	84	anionic/neutral
			CH_3COOH (1.0 M)	16	cationic
18	2A' [32.0 mg]$^{\Delta}$	"	H_2O	86	anionic/neutral
			CH_3COOH (1.0 M)	14	cationic
19	" [26.2 mg]	"	H_2O	88	anionic/neutral
			CH_3COOH (1.0 M)	12	cationic
20	2B [8.07 mg]	"	H_2O	52	anionic/neutral
			CH_3COOH (1.0 M)	48	cationic
21	2A [8.27 mg]	IR45-OH	H_2O	77	cationic/neutral
			HCl (4.5%)	23	anionic
22	"	"	H_2O	77	cationic/neutral
			HCl (4.5 %)	23	anionic
23	2B [3.28 mg]	"	H_2O	53	cationic/neutral
			HCl (4.5 %)	47	anionic

* - Weight of Ni applied to the column is shown in square brackets.
Δ - Freshly prepared extract.

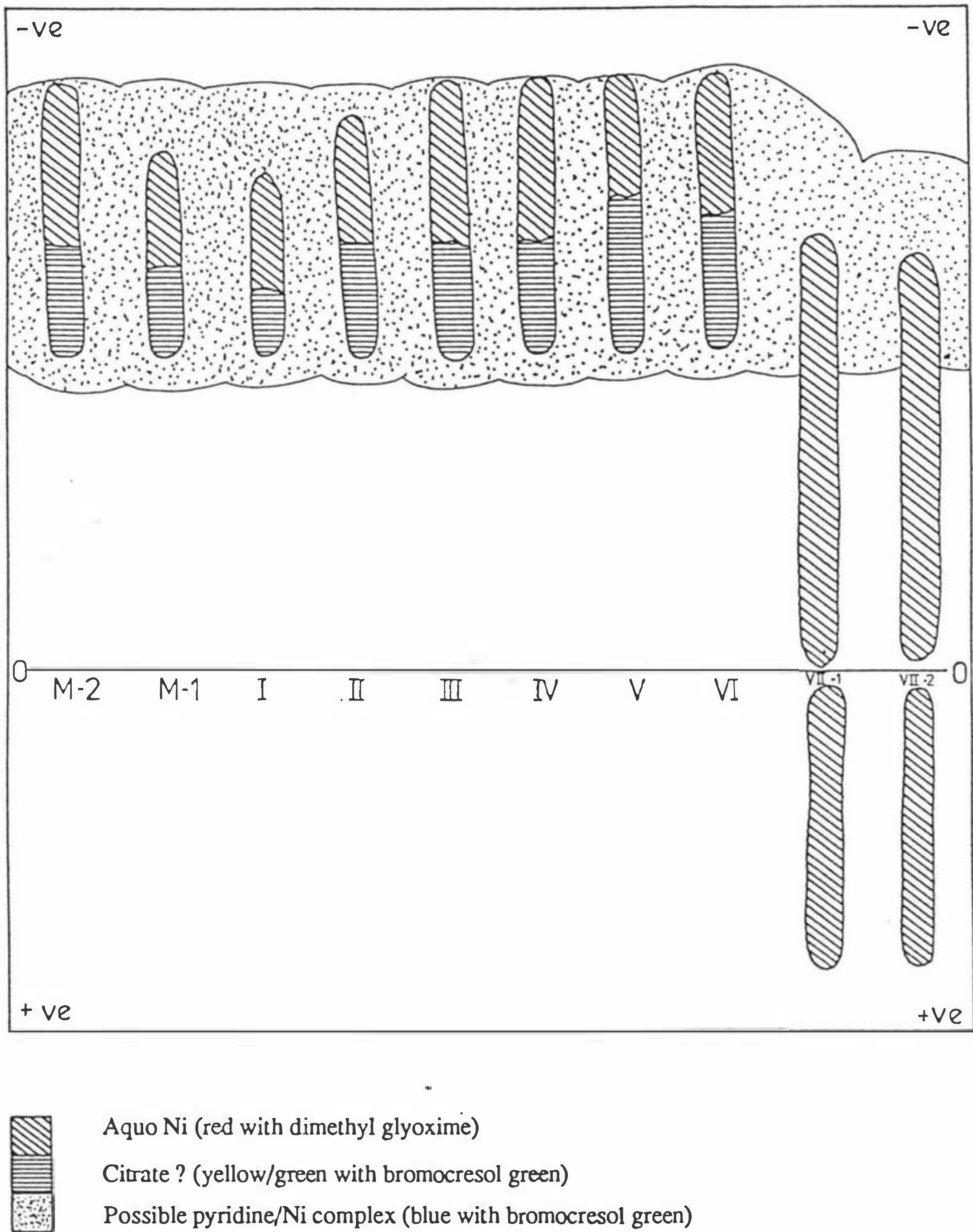


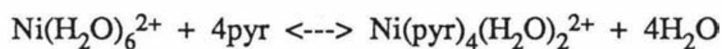
Fig. III.7. Electrophoretic separation of Ni species in P. 'palawanensis' at pH 6.5. (M_2) anionic Ni-citrate complex, (M_1) aquo Ni, (I) anionic fraction from P_2 column, (II) cationic fraction from P_2 column, (III) crude extract passed through Sephadex G-10 column, (IV) a repeat of (III), (V) anionic fraction passed through Sephadex G-10 column, (VI) cationic fraction passed through Sephadex G-10 column, (VII-1) cationic fraction not subjected to gel filtration chromatography, (VII-2) a repeat of (VII-1)

III.11.III Results and Discussion

The results of ion-exchange separation (Table III.7) are similar to those obtained earlier; both cationic and anionic Ni complexes were present in the aqueous (or part aqueous) extract. The distribution of Ni between the two complexes was obviously influenced by the amount of Ni applied to the column. The relative amounts of ionic Ni in the brown extract differed from those observed in the greenish/brown extract. The ratio of anionic to cationic Ni was > 1 in the brown extract (2A), but close to 1 in the greenish/brown extract (2B). It is possible that the original ground leaf material was not completely homogeneous. Overall, the results were highly reproducible.

Except for the Ni in samples (i) and (ii) which appeared to be cationic and anionic, Ni in all the other samples migrated towards the cathode displaying cationic behaviour (Fig.III.7). While the electrophoresis results confirmed the presence of cationic Ni, the question of lability still arises with respect to anionic Ni. It was observed that in the absence of ion-exchange separation only cationic Ni was detected in duplicates of sample (vii). Upon spraying with bromocresol green, most of the paper in the vicinity of the cationic Ni exhibited a blue colour. Yellow areas were observed immediately beneath all Ni-rich areas except those pertaining to samples (i) and (ii). The remainder of the paper was also yellow. There was therefore no conclusive evidence of citrate being present . It was appreciated that bromocresol green was simply an acid-base indicator, thus at this stage in the research, the use of this indicator was abandoned.An alternative means of visualisation was not sought as the presence of citric acid and/or other organic acids in purified Ni rich extracts could easily be identified using gas chromatography-mass spectrometry.

The observation of the blue area near where Ni was detected was somewhat unusual. As mentioned earlier (Section III.8.IV), the electrophoretic buffer turned bromocresol green indicator blue. It was therefore felt that one or more of the buffer components had interacted chemically with Ni. It was possible that this interaction may have influenced the migratory behaviour of the nickel. The literature revealed that of all the buffer components, pyridine was the most likely to exert an influence (Sillén and Martell, 1964). Relevant equilibrium data are presented in Table III.8. Ni could interact with pyridine according to the following equation.



$$Ni(H_2O)_6^{2+} + 4pyr <---> Ni(pyr)_4(H_2O)_2^{2+} + 4H_2O$$

Judging from the magnitude of log K for the Ni-pyridine and Ni- acetate systems, interference from acetate would be minimum. Other Ni complexes such as Ni-malate and Ni-malonate could be affected by pyridine to a greater extent in view of their lower stability. An attempt to investigate the nature of the effect of pyridine on Ni complexation was made using UV/VIS spectrophotometry.

III.12. UV/VIS Spectrophotometric Study of the Effect of Pyridine on Aquo Nickel and Nickel-citrate Systems

When ligands binding a metal are displaced by competing ligands, a change in UV/VIS absorption characteristics occurs. These are manifested as shifts in absorption bands with increased or decreased intensity, and the splitting of peaks. Based on an experimental study of spectra of a large number of complexes containing various metals and ligands, it was found that ligands may be ranked according to their ability to cause shifts. The series that evolved is referred to as the spectrochemical series, and for the more common ligands it is $I^- < Br^- < Cl^- < F^- < OH^- < C_2O_4^{2-} \sim H_2O < NCS^- <$ pyridine $\sim NH_3 <$ ethylenediamine < o-phenanthroline $< NO_2^- < CN^-$. The idea behind the series is that the nature of the shift (or other changes) in absorption bands for complexes having the same metal but different ligands, can be predicted irrespective of what the metal might be. However, in applying the series cognisance must be taken of two qualifications:- a) the series is based on data for metal ions in common oxidation states and b) inversions of rank of adjacent or nearly adjacent members sometimes occurs (Cotton and Wilkinson, 1988). Generally, replacement by strong ligands tends to result in shifts to shorter wavelengths (hypsochromic shifts) while replacement by weak ligands tends to result in shifts to longer wavelength (bathochromic shifts).

The following solutions were used in this study:-

(i) Aquo Ni (200 μg cm^{-3})

(ii) 1:1 (v/v) Aquo Ni / electrophoretic buffer

(iii)1:1 (0.004 M) Ni-citrate

(iv)1:1 (v/v) Ni-citrate / electrophoretic buffer

The concentrations used were approximately equal to that of pyridine in the buffer. Solutions were scanned over the wavelength range of 800-200 nm using distilled deionised water as reference. The spectra (Fig.III.8) show a hypsochromic shift of ~15 nm as H_2O molecules (395 nm) were replaced by pyridine molecules (380 nm). A hypsochromic shift of the same order of magnitude was observed as citrate bound to Ni (390 nm) was replaced by pyridine (374 nm). These results are in accordance with the positions of water and pyridine in the spectrochemical series. Very little change was observed in the absorption bands when citrate (390 nm) replaced H_2O molecules. This was expected in view of the positions of the organic acid anion - oxalate and H_2O in the series.

Having established thst pyridine does interfere with aquo Ni and Ni-citrate systems, an alternative buffer was sought. A phosphate buffer (pH 6.89) comprising 0.025 M Na_2HPO_4 and KH_2PO_4 was selected (Bassett et al., 1978). Analar salts were dried at 110 ° C for 1 hr and a composite buffer solution was prepared using CO_2-free distilled deionised water. A similar study was then undertaken. No shifts were observed. Henceforth, the phosphate buffer was used in all electrophoretic separations.

Table III.8 Equilibrium Constants for Nickel Complexation by Pyridine, Citric Acid and Acetic Acid. (Source: Sillén and Martell, 1964)

METHOD	TEMPERATURE (° C)	MEDIUM	LOG EQUILIBRIUM CONSTANT¥
Spectrophotometric	25	0.5 M C_5H_5N, HNO_3	$K_1 = 1.92$, $ß_2 = 2.77$, $ß_3 = 3.37$, $ß_4 = 3.50$ $K_1 = 1.17$, $ß_2 = 1.96$, $ß_3 = 3.48$, $ß_6 = 9.80$
Glass electrode	25	"	$K_1 = 1.78$, $K_2 = 1.05$, $K_3 = 0.31$
Distribution between two phases	20	1 M NH_4NO_3 and C_5H_5N	$K_1 = 1.98$, $ß_2 = 3.02$, $ß_3 = 3.42$, $ß_4 = 3.44$
Glass electrode	32.5	citric acid (0.025 ionic strength)	K ($Ni^{2+} + HL^{3-}$ <---> Ni HL^-) = 5.10
Glass electrode	25	citric acid (0.015 ionic strength)	K ($Ni^{2+} + HL^{3-}$ <---> $NiHL^-$) = 5.11
Glass electrode	25	0.1 M acetic acid	$K_1 = 1.0$
Ion-exchange	20	acetic acid and $NaClO_4$	$K_1 = 0.67$, $K_2 = 0.58$
Spectrophotometric	20	acetic acid in various	$K_1 = 1.65$, $K_2 = 1.31$

¥ - K and ß are log equilibrium constants for combination of ligands with metals

(i) K = consecutive or stepwise constant.

M + L <--> ML

K = [ML] / [M][L]

(ii) ß = cumulative or gross constant. This is normally used if it is the only parameter which can be determined or if the sequence of stepwise constants is incomplete.

M + nL <--> ML_n

$$ß_n = [ML_n] / [M][L]^n = \prod_{i=1}^{n} K_i$$

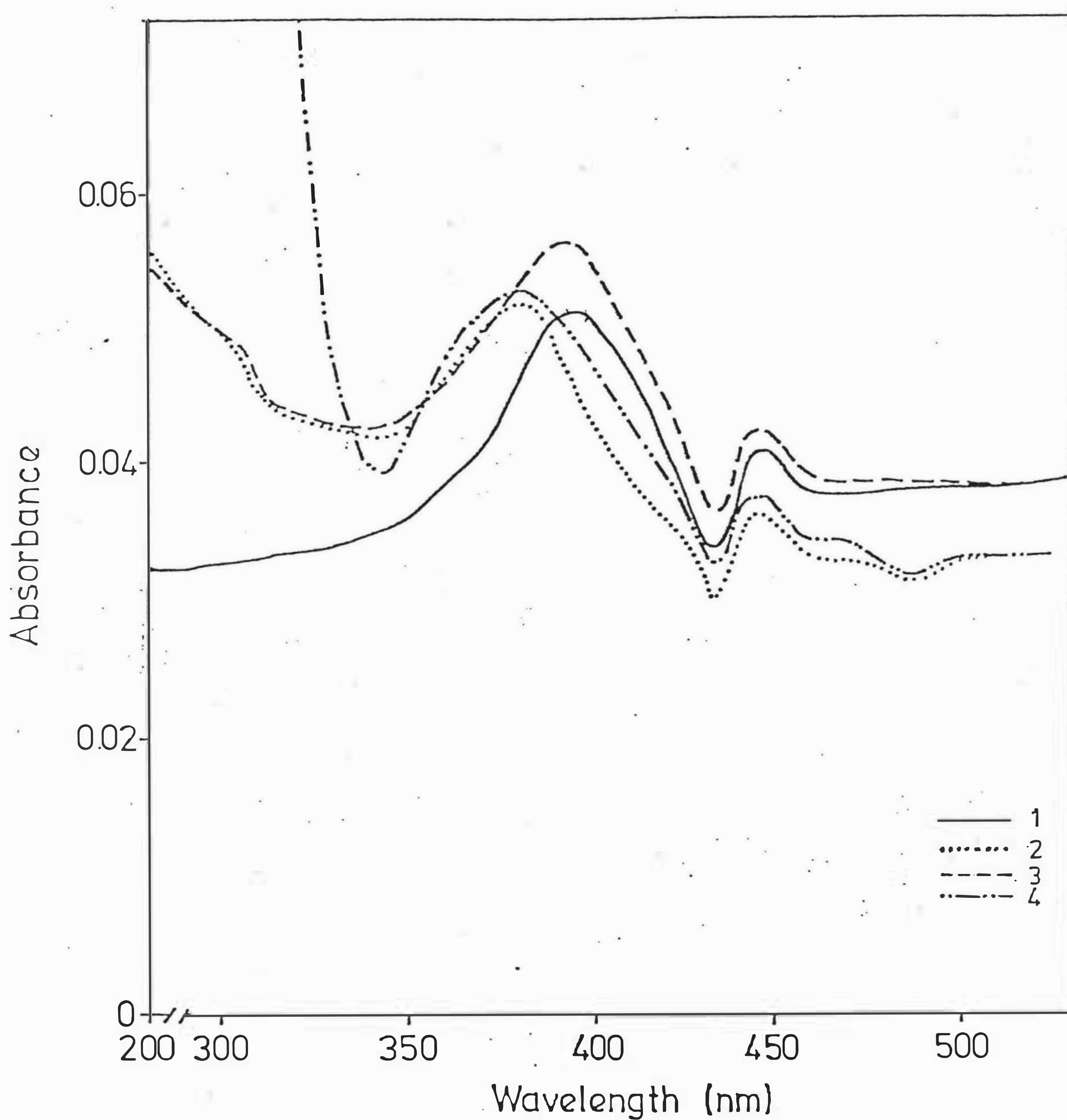


Fig. III.8. Absorbance spectra of Ni in different environments. (1) Aquo Ni, (2) 1:1(v/v) Aquo Ni/electrophoretic buffer, (3) anionic [1:1]Ni-citrate complex, (4) 1:1 (v/v) anionic Ni-citrate complex/electrophoretic buffer.

III.13. High-voltage Electrophoresis Using Phosphate Buffer

Various Ni-rich solutions obtained from D.gelonioides subsp.tuberculatum and P. 'palawanensis' were subjected to high voltage electrophoresis using phosphate buffer (pH 6.89). Most of the samples upon which electrophoretic separations were previously carried out were included in this study. A horizontal type apparatus, cooled with refrigerated methanol, was utilized. The separation was performed over 75 minutes at 1.3 kV and 55 mA. Aquo nickel served as reference and Ni was visualised as above. The electrophoretogram is presented in Fig.III.9. The ionic nature of the Ni in the solutions is compared with that obtained earlier from ion-exchange chromatography and electrophoresis in Table III.9. It would appear that the effect of pyridine on electrophoretic separations was minimal based on the close agreement between results obtained using the pyridine/ glacial acetic acid/ water and phosphate buffers. It may be concluded that the passage of Ni-rich extracts of these two plants through ion-exchange resins under the prevailing experimental conditions can result in changes in the ionic nature of whatever complexes are present.However, complexes may also be adversely affected during electrophoretic separation due to the non-equilibrium conditions that can be created.

III.14. Additional Cation-exchange Chromatography of Nickel Complexes in D.gelonioides subsp. tuberculatum and P. 'palawanensis'

III.14.I Background

In view of the somewhat conflicting nature of the results of cation-exchange chromatography and high voltage electrophoresis, the suitability of cation-exchange chromatography as a technique for separating Ni complexes in extracts from Ni hyperaccumulating plants, was readdressed. Factors considered were cation-exchange capacity, sample loading and column length.

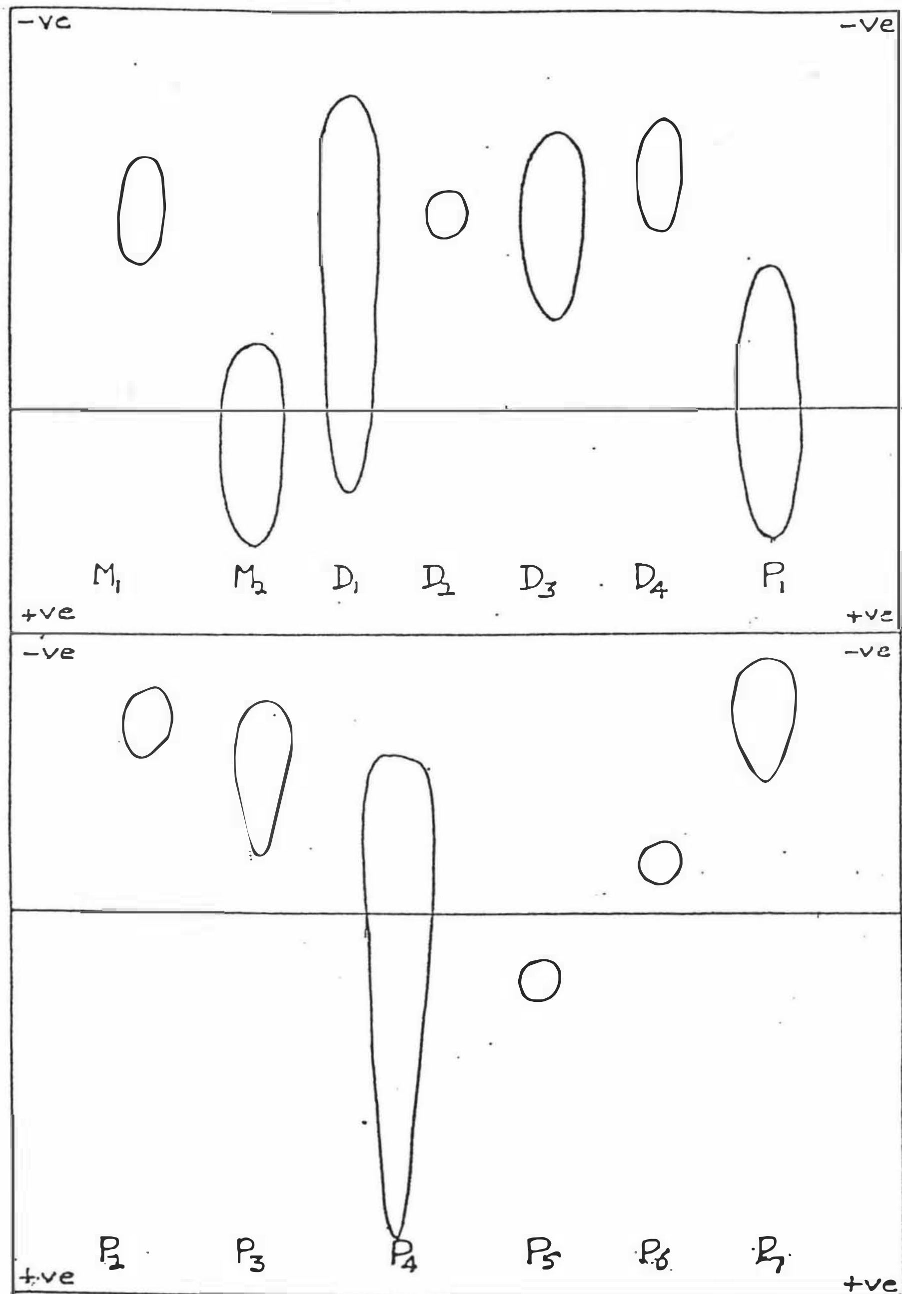


Fig. III.9 Electrophoretogram of Ni-rich fractions from D.gelonioides subsp. tuberculatum and P. 'palawanensis'. (M_1) Aquo-Ni, (M_2) anionic Ni-citrate complex [0.1 M]. D - Fractions from D.gelonioides subsp. tuberculatum; (1) crude aqueous extract, crude aqueous extract passed through (2) IRC50-H column and (3) Sephadex G-10 column, (4) isolated purified extract [18 % Ni]. P - Fractions from P. 'palawanensis'; (1) crude ethanolic/water extract, crude ethanolic/water extract passed through (2) Sephadex G-10, (3) IRC50-H and Sephadex G-10 columns consecutively, (4) IR45-Cl [anionic], (5) IR45-Cl and Bio-Gel P_2 columns consecutively [anionic] and (6) IR45-Cl and Bio-Gel P_2 columns consecutively [cationic], (7) isolated purified extract [5 % Ni]

Table III.9 Comparison of Results of Ion-exchange Chromatography and High-voltage Electrophoresis of Nickel-rich Extracts from D. gelonioides subsp. tuberculatum and P. 'palawanensis'.

NATURE OF SAMPLE	IONIC NATURE OF NICKEL		
	ION-EXCHANGE CHROMATOGRAPHY	HIGH VOLTAGE ELECTROPHORESIS pH = 6.5	pH = 6.89
D.gelonioides subsp. tuberculatum			
- crude aqueous extract	cationic	-	cationic
- crude passed through IRC50-H resin	anionic	cationic	cationic
- crude passed through Sephadex G-10	cationic	cationic	cationic
P. 'palawanensis'			
- crude ethanol/water extract	cationic and anionic (various ratios)	cationic and anionic (1:1)	cationic and anionic (1:1)
- crude passed through Sephadex G-10	cationic and anionic	cationic	cationic
- crude passed through IRC50-H resin and Sephadex G-10	anionic	cationic	cationic
"	cationic	cationic	cationic
-crude passed through IR45-Cl	cationic	cationic and anionic (cationic : anionic >> 1)	cationic and anionic (cationic : anionic << 1)
- crude passed through IR45-Cl and Bio-Gel P_2	anionic	cationic	cationic
- crude passed through IR45-Cl and Bio-Gel P_2	cationic	cationic	cationic

A series of separations was performed on IRC50-H resin using a 50 cm^3 burette in place of the 25 cm^3 burette used in previous studies. Column preparation and elution were carried out as before. Fifty 2 cm^3 fractions were collected using distilled deionised water as eluant. Acetic acid (1 M) was then used to elute the remainder of the Ni. Samples applied to the column were as follows:-

(i) anionic Ni from D.gelonioides subsp.tuberculatum (crude aqueous extract)

(ii) isolated Ni complexes from the same plant

(iii) cationic Ni from P.'palawanensis' (eluted from the IR45-Cl resin)

(iv) 1:1 anionic Ni-citrate complex

(v) aquo Ni

No more than 1 cm^3 of each sample was applied to the column. Except for the concentrated solution of Ni-citrate, close to 80 % of the Ni applied was eluted. Hydrochloric acid (1.5 M) was therefore passed through the column in between runs to remove adhering nickel. The results of these experiments along with pertinent information are shown in Table II.10.

III.14.II. The Effect of Concentration and IRC50-H Column Height on the Adsorption of Nickel

Approximately 0.400 g of swollen resin was weighed into each of fourteen test-tubes. This weight was chosen after consideration had been given to the cation-exchange capacity of the resin as quoted by the manufacturer, and the maximum concentration of cations in the samples to be used. Besides Ni, the only other cation was K^+, since KOH was used for pH adjustment during complex preparation (Baker et al., 1983). Five cm^3 each of 0.001 M Ni-citrate was then added to 2 test-tubes. The same was done for the remaining six pairs of tubes using Ni-citrate solutions of concentration 0.002, 0.004, 0.02, 0.04 and 0.1 M. The tubes were shaken for ten min. using a Jank-Kunkel Electrical Shaker, then centrifuged for 5 min. using a Gallenkamp Junior Centrifuge. Supernatants were then subjected to Ni determination by AAS along with reference solutions. These were solutions in which there was no resin. Results are shown in Table III.11.

Table III.10 Results of Cation-exchange Chromatography of Nickel-rich Samples on a Longer Column.

RUN NO.	NATURE OF SAMPLE*	ELUANT	% NICKEL ELUTED	IONIC NATURE OF FRACTION
24	Anionic Ni from D.gelonioides subsp. tuberculatum (crude aqueous extract) [0.146 mg]	H_2O	Nil	-
		CH_3COOH (1.0 M)	78	cationic
25	Isolated Complexes from D.gelonioides subsp. tuberculatum (crude aqueous extract) [0.142 mg]	H_2O	Nil	-
		CH_3COOH (1.0 M)	80	cationic
26	Aquo Ni [0.200 mg]	CH_3COOH (1.0 M)	74	cationic
27	Cationic Ni from P. 'palawanensis' (crude ethanol/water extract passed IR45-Cl resin [0.164 mg]	H_2O	Nil	-
		CH_3COOH (1.0 M)	87	cationic
28	"	H_2O	Nil	-
		CH_3COOH (1.0 M)	80	cationic
29	1:1 Anionic Ni-citrate [0.111 mg]	H_2O	Nil	-
		HCl (1.5 M)	80	cationic
30	" [18.8 mg]	H_2O	70	anionic/neutral
		HCl (1.5 M)	30	cationic

* - Weight of Ni applied to the column is shown in square brackets.

Table III.11 Variation of Nickel Adsorption with Concentration.

NICKEL CONCENTRATION			% NICKEL IN
REFERENCE SOLUTIONS (~M)	REFERENCE SOLUTIONS (µg cm^{-3})	SUPERNATANT (µg cm^{-3})	SUPERNATANT
0.001	11.9	2.50	21.0
0.002	24.4	8.75	35.9
0.004	49.0	24.6	50.2
0.01	126	88.0	69.8
0.02	262	214	81.7
0.04	521	445	85.4
0.10	1262	1148	91.0

Table III.12.Variation of Nickel Adsorption with Column Height.

WEIGHT OF RESIN (g)	CONCENTRATION OF NICKEL IN SUPERNATANT† (µg cm^{-3})	% NICKEL IN SUPERNATANT
0.6	1009	87.9
0.8	983	85.6
1.0	956	83.3
1.5	869	75.7
2.0	835	72.7

† - Concentration of Ni in reference solution = 1148 µg cm^{-3}

Five cm^3 of 0.1 M Ni-citrate was added to each of 5 test-tubes containing 0.6, 0.8, 1.0, 1.5 and 2.0 g of swollen resin representing various column heights. The rest of the procedure was identical to that used earlier. The results are presented in Table III.12.

III.14.III Discussion and Conclusions

In the main, the results in Tables III.9, 10, 11 and 12 confirm that the ionic nature of Ni changes during cation-exchange chromatography. No doubt, similar observations could be made with respect to anion-exchange chromatography and gel filtration chromatography. There appeared to be a continuous change in the degree of dissociation of the complex as the diluted solutions interacted with the resin. An equilibrium shift may have occurred between free and complexed Ni, such that free Ni was favoured. This could explain the reduction in the number of peaks when Ni-rich extracts from D.gelonioides subsp. tuberculatum containing substantially different levels of Ni, were subjected to gel filtration chromatography (Section II.13). It should be recalled that a reduction in the number of peaks was also observed for extracts from P. 'palawanensis'. Majumdar and Chakrabatty (1957, 1958a, 1958b) noted similar chromatographic behaviour of some Pt, Pd, Ir Os and Rh complexes. This may also be true of the Ni complexes in D.gelonioides subsp.tuberculatum and P. 'palawanensis' which have not yet been identified (Table III.9). The behaviour of Ni on the column seems to be influenced by the amount of Ni applied. For Ni-citrate, the tendency for free Ni to predominate increased with dilution (Table III.11). The amount of free Ni increased with increased column height (Table III.12). This set of experiments therefore provided some explanation of the results obtained in earlier cation-exchange studies.

CHAPTER IV

Characterisation of Nickel-binding Ligands in Dichapetalum gelonioides subsp. tuberculatum and Phyllanthus 'palawanensis'

IV.1. Introduction

Over the past 15 years, several workers have reported the potential of malic, malonic and citric acids as Ni-binding ligands in Ni-hyperaccumulating plants. In 1976, Pelosi et al. reported the association of malic and malonic acids with Ni in aqueous extracts from Alyssum bertolonii. Pancaro et al. (1978b) confirmed these observations and reported the same association in another European species, Alyssum pintodasilvae T. R Dudley. The malonic acid content however, was low. Shaw (1980) confirmed the findings of Pancaro et al. with respect to A.pintodasilvae, and extended her research to A. serpyllifolium subsp. malacitanum. In this plant, malonic acid appeared to be the dominant complexing agent. During the same study, minute amounts of citric acid were also found. Shaw (1980) and Morrison (1980), were the first to report the association of citric acid with Ni in Ni-rich extracts from Alyssum species. Morrison (1980) observed noticeably higher concentrations of citric and iso-citric acids in the non-accumulator A. serpyllifolium subsp. serpyllifolium, compared to hyperaccumulators A. pintodasilvae and A. murale. Several unidentified organic acids were also present in Ni-rich extracts from the three species.

Lee (1977) and Kersten (1979) observed citric and/or malic acid in Ni-rich extracts from Ni-hyperaccumulating plants from New Caledonia. Stockley (1980) reported the association of citric acid with Ni in extracts from the Zimbabwean Ni-hyperaccumulating plant Pearsonia metallifera. His results also indicated the possible involvement of homocitric acid in Ni complexation. Both citric and malic acid were identified in similar extracts obtained from the Philippine hyperaccumulating plant Walsura monophylla (Freeman, 1989). The high incidence of involvement of organic acids in Ni complexation may not be surprising as it has been reported that organic acid production can be stimulated by Ni (Dekock and Morrison, 1958). It has been suggested that increased organic acid synthesis may be a response to excess cations absorbed by plants (Pierce and Appelman, 1943; Torii and Laties, 1966).

IV.2. Aim and Rationale

Despite the lack of evidence of the presence of citric acid in Ni-rich extracts from D. gelonioides subsp. tuberculatum and P. 'palawanensis' (Chapter II), an attempt was made to identify the Ni-binding ligands present using gas chromatography-mass spectrometry. Previous use of this technique resulted in the identification of citric, malic and malonic acids in Ni-rich extracts from several Ni-hyperaccumulating plants (Pelosi et al., 1976; Lee, 1977; Pancaro et al.,1978b; Kersten, 1979; Shaw, 1980; Morrison, 1980; Stockley, 1980; Freeman, 1989). There appeared to be no reason why one or more of the three acids should not be present in purified Ni-rich material obtained from the two Ni-hyperaccumulating plants under consideration. Gas chromatography-mass spectrometry studies were carried out on purified extracts derived from preparatory amounts of crude extract. Attempts were also made to elucidate the structure of a synthetic Ni-rich species simulating the isolated, purified Ni-rich extract obtained from D. gelonioides subsp. tuberculatum. Preliminary studies on the possible role of amino acids in the binding of Ni were also carried out since the metal is known to preferentially bind ligands containing oxygen and nitrogen (Tiffin, 1972; Thompson and Tiffin, 1974; Nieboer and Richardson, 1980).

IV.3. Preparation of Purified Nickel Complexes from D. gelonioides subsp. tuberculatum

IV.3.I. Extraction and Isolation

Fifteen gram of dried leaf material was extracted with two-75 cm^3 aliquots of distilled deionised water as in Section II.8. The final residue was then washed with 35 cm^3 of distilled deionised water. The slurry was filtered, and the filtrate retained. The combined extract was freed of high molar mass, low polarity compounds in the usual manner. The final volume of purified extract was ~ 19 cm^3, and was shown by AAS to contain 249 mg nickel. This extract was combined with the remaining extract earlier produced (Section II.8) to give a volume of 23 cm^3 containing 309 mg nickel. The Ni complexes were isolated from a 2 cm^3 sample of the extract (~ 27 mg Ni) using gel filtration chromatography on Sephadex G-10. Distilled deionised water was used

as eluant. Nickel eluted in two poorly resolved peaks as before (Section II.12) (Fig.IV.1a). Ninety-one percent of the Ni applied to the column was eluted. The Ni-rich fractions were retained, combined and the volume measured (17 cm^3). The mass of Ni present was determined by AAS (~ 10 mg). Four cm^3 of this effluent (~2 mg Ni) was then passed through the Sephadex G-10 column to effect further purification of the complexes. Only 29 % of the Ni applied to the column was recovered, and this eluted as a single peak (Fig. IV.1b). Repetition of the procedure yielded a similar result. These observations were in agreement with those made earlier (Section II.11) which were later explained in Section III.14.III. More evidence of Ni-stationary phase interaction at low Ni concentrations is therefore provided. The remaining 21 cm^3 of aqueous crude extract (282 mg Ni) was concentrated by rotary evaporation at 33-35 °C down to ~ 5 cm^3 and then applied to the column. Nickel-rich effluent was retained as previously, and the 70 cm^3 collected was concentrated down to ~ 9 cm^3 (214 mg Ni) as above. The effluent was divided into two portions and each was subjected to further purification by another chromatographic separation on Sephadex G-10. Close to 90 % of the Ni was recovered from the column as a single peak in each case. The resulting two sets of Ni-rich effluent were combined along with material obtained earlier during the initial isolation procedure. These were thoroughly mixed and concentrated down to 6 cm^3 in vacuo at 33-35 ° C. This green solution was shown to contain 108 mg nickel.

IV.3.II. Crystallisation

Attempts were made to obtain Ni complexes of higher purity via crystallization. Lee (1977) reported the precipitation of Ni complexes from aqueous solution as a pale green powder using ethanol, methanol or acetone. He found that slow evaporation of a 1 : 1 (v/v) mixture of aqueous Ni-rich solution and methanol at a temperature not exceeding 35 ° C gave the best result. The use of boiling solvent was not considered an alternative since a temperature in excess of 35° C might adversely affect the Ni complexes present. In this study, methanol was therefore used for crystallization at a temperature of 33-35° C.

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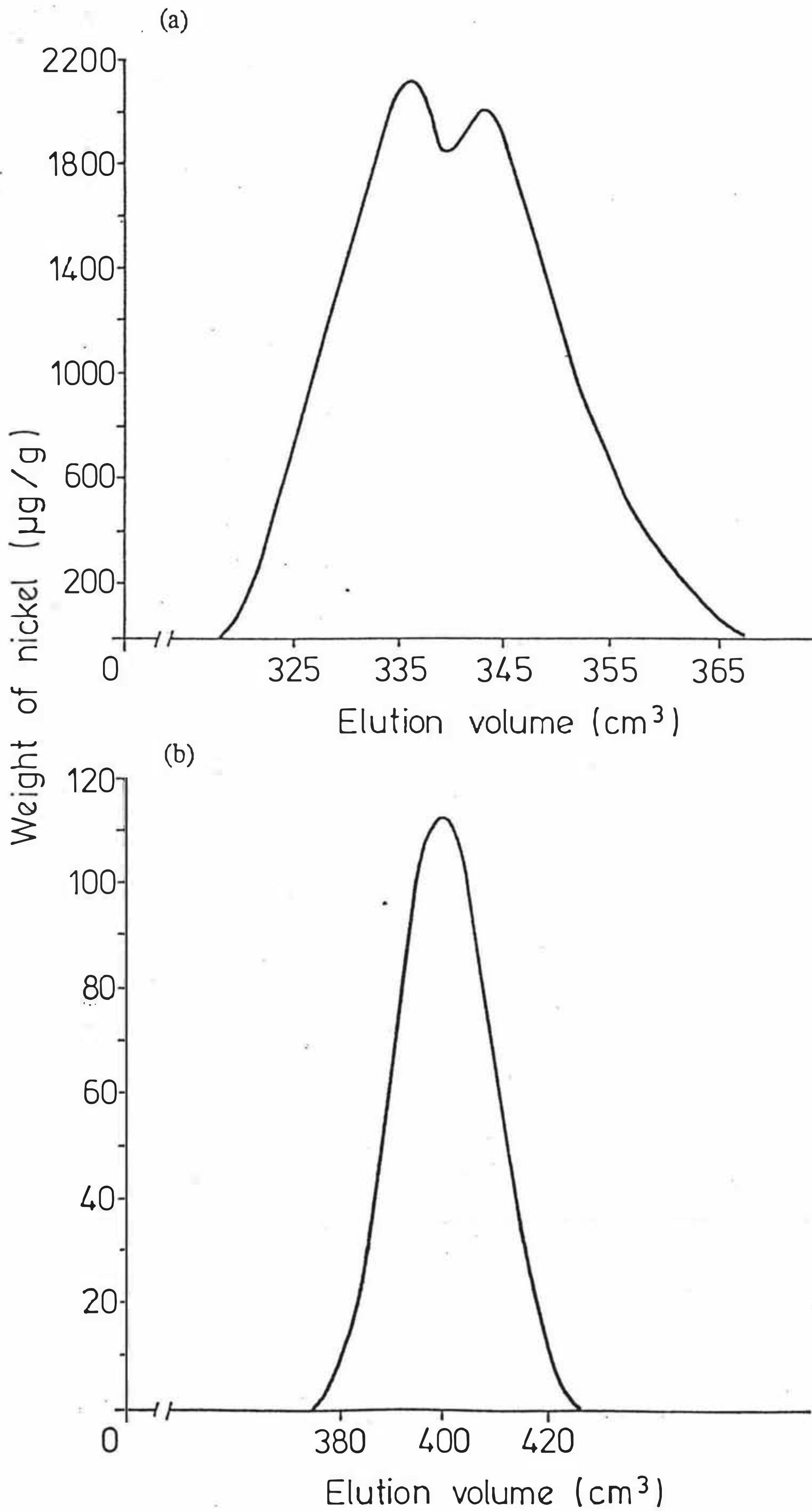


Fig. IV.1. Elution of Ni species in D.gelonioides subsp. tuberculatum. (a) one pass through Sephadex G-10 column (b) two passes through Sephadex G-10 column.

The 6 cm^3 extract obtained via gel filtration (Section IV.3.I) was concentrated down to 1.4 cm^3 by rotary evaporation at 33-35 ° C. The concentrate was quantitatively transferred to a graduated glass centrifuge tube using a minimum amount of distilled deionised water. An equal volume of Analar methanol was then added. The contents of the tubes were thoroughly mixed using a Vortex mixer, then centrifuged for 10 minutes. A cream-coloured precipitate (P_1) and pale green supernatant (N_1) were observed. Both phases were retained. An equal volume of methanol was added to the precipitate and the procedure was repeated. A negligible amount precipitate (P_2) was observed. The resulting supernatant (N_2) was treated with an equal volume of methanol. The mixture was then transferred to a Quickfit flask, and evaporated to dryness at 33-35 ° C. A pale green powder remained (Sample 1). Supernatant N_1 was subjected to the same procedure. This resulted in a pale green precipitate (P_3) and pale green supernatant (N_3). As above, both phases were retained. Precipitate $P_3$was treated in the same manner as precipitate P_1. However, no green powder was observed even after quenching in an ice-bath. Supernatant N_3 was divided in half. Each half was subjected to the same procedure applied to N_2. An observable but negligible amount of precipitate (P_4) was produced along with a pale green supernatant (N_4). Upon treating supernatant N_4 with methanol, two phases were again observed. The pale green precipitate P_5 was quantitatively transferred to a Quickfit flask using a minimum amount of methanol. The resulting mixture was evaporated to dryness as for N_2 above. After quenching in an ice-bath, a pale green powder remained (Sample 2). The procedure was repeated using the pale green supernatant N_5. A pale green powder (Sample 3) resulted. Successive precipitations were carried out to ensure the availability of an adequate amount of high purity material for further study.

Traces of moisture were removed from the three samples in vacuo using liquid air. The weight of each was obtained using a Mettler AE 163 Analytical Balance with precision of ± 0.01 mg. These were 16,18 and 20 mg for Samples 1, 2 and 3 respectively. Using AAS, the corresponding Ni concentrations were calculated at 11, 18 and 15 % respectively. Samples were stored in plastic vials with screw-caps at 4° C. They were later subjected to gas

chromatography-mass spectrometry.

IV.4. Preparation of Purified Nickel Complexes from P. 'palawanensis'

IV.4.I. Isolation

The anionic Ni fractions obtained from CEC Runs 15-19 (Table III.7) were combined giving a volume of 51 cm^3. The use of these fractions was justified on the basis of the relative ease with which Ni in the crude extract eluted from the cation exchange resin compared to the gel filtration chromatography resin, during initial isolation of the Ni complexes.The composite sample was centrifuged at 13000 r.p.m in a Dupont-Sorvall Refrigerated Supercentrifuge for 60 min., at a temperature of ~ 21° C. This powerful centrifuge was employed owing to the larger amount of precipitate observed during storage. A 25 cm^3 aliquot of sample was then applied to the Sephadex column. This was eluted with distilled deionised water. The remaining 26 cm^3 of composite sample was subjected to the identical procedure. Both sets of Ni-rich fractions collected were combined and thoroughly mixed. The final volume of 125 cm^3 was shown to contain 46 mg nickel. Some 75-90 % of the Ni applied to the column was recovered during elution. The elution profile is presented in Fig IV.2.

IV.4.II. Crystallisation

The crystallization procedure applied to Ni complexes of D.gelonioides subsp. tuberculatum was also applied to those of P. 'palawanensis'. Three pale green solid samples were obtained :- Samples 1´,2´and 3´. The appearance of these samples differed from that of the samples derived from D. gelonioides subsp. tuberculatum. The former appeared as green flakes while the latter appeared as green crystalline powders. Remaining moisture was removed in vacuo using liquid air. Samples 1´,2´and 3´ weighed 123,34 and 8 mg respectively. Sample1´ contained 5 % Ni, while Samples 2´and 3´each contained 4 % nickel.

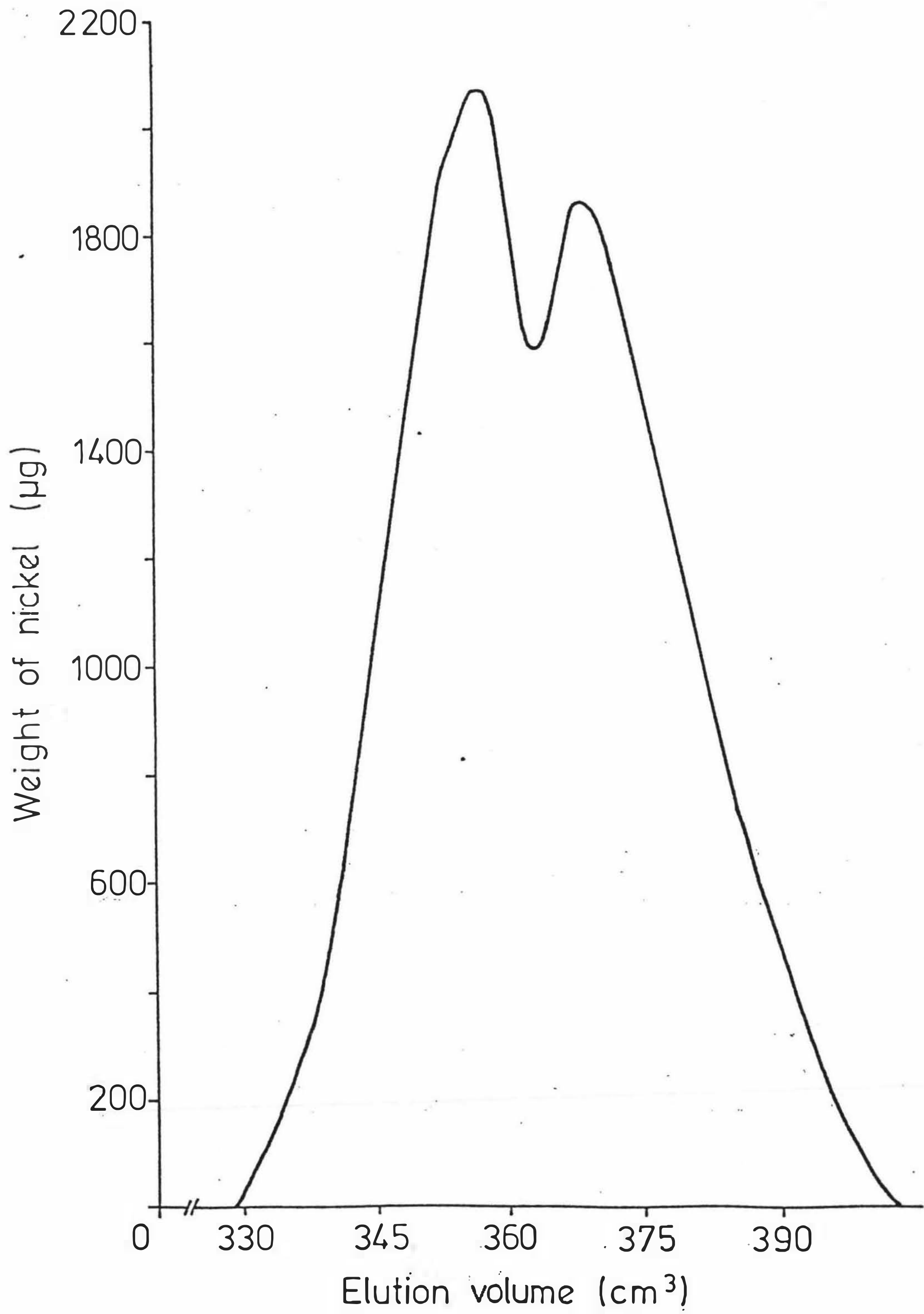


Fig IV.2 Elution of anionic Ni in P. 'palawanensis' from Sephadex G-10 column.

IV.5. Elemental Composition of Nickel-rich Fractions

IV.5.I. Aim

The aim of this experiment was to compare the elemental composition of the purified complexes with that of the dried ground leaf material in order to assess the level of purity and to ascertain which metals (if any) besides Ni are found in association with the complexes.To this end, Sample 2 from D. gelonioides subsp. tuberculatum and Sample 1´ from P. 'palawanensis were analysed for 23 elements, mostly metals, by inductively coupled plasma atomic emission spectroscopy (ICP-AES). Sample 2 was selected owing to its high Ni content, and the relatively large amount of Sample 1´ obtained dictated its selection.

IV.5.II. Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES)

ICP-AES was conceived and developed by Greenfield et al.(1964), and Wendt and Fassel (1965). It is based on the principle that the energy emitted at specific wavelengths by excited atoms upon return to the ground state is proportional to the concentration of the atoms. The energy for atomisation and excitation is provided by a partially ionised stream of argon gas known as an argon plasma. The plasma is electromagnetically formed by the action of a radio-frequency (rf) generator and an induction coil on a stream of argon, and reaches temperatures of 8000-10000 K. The sample solution is aspirated into the centre of the plasma where it is atomised. Emission spectra are observed via a monochromator and photomultiplier tube. Because of the high temperatures reached, many more spectral lines are observed with ICP-AES than with flames (as in flame emission spectroscopy), so a high-resolution monochromator is commonly employed. Using a series of photomultiplier tubes, the simultaneous determination of a large number of elements can be accomplished. Under such circumstances a polychromator is required for effective resolution of spectral lines covering the range of 175-900 nm (Skoog, 1985).

Over the past eight to ten years, the technique has been recognised as an analytical method of outstanding capability (Fassel, 1984). The lowest concentration of analyte which gives rise to a detectable signal (i.e the detection limit) for most elements falls in the range of 1-100 μg dm^{-3}. For AAS, the detection limit is higher by two to three orders of magnitude. ICP-AES has found application in trace element analysis of a wide variety of materials. In this research, the instrument used was the Applied Research Laboratories 34000 Plasma Emission Spectrometer equipped for simultaneous determination of 23 elements. Samples 2 and 1´ were prepared for analysis by first dissolving 0.94 and 6.95 mg respectively in minimum amounts of distilled deionised water then diluting to 4 cm^3. This volume was the minimum amount of solution required for 23 simultaneous determinations.

Table IV.1 Elemental Compositions in Dried Leaf Material and Isolated Purified Nickel Complexes from D. gelonioides subsp. tuberculatum and P. 'palawanensis'

ELEMENT	CONCENTRATION (%, w/w)			
	D.gelonioides subsp. tuberculatum		P. 'palawanensis'	
	Dried Leaf Material*	Isolated Purified Ni Complexes	Dried Leaf Material*	Isolated Purified Ni Complexes
Al	<0.01	<0.04 (<0.001)#	<0.01	<0.01 (<0.001)#
As	<0.01	<0.09 (<0.001)	<0.01	<0.01 (<0.001)
B	<0.01	<0.01 (<0.001)	<0.01	0.01 (0.001)
Ca	1.84	5.79 (0.144)	1.08	5.56 (0.139)
Cd	<0.01	0.01 (<0.001)	<0.01	N.D ¥
Co	<0.01	0.01 (<0.001)	<0.01	N.D
Cr	<0.01	0.01 (<0.001)	<0.01	N.D
Cu	<0.01	0.01 (<0.001)	<0.01	N.D
Fe	<0.01	0.01 (<0.001)	<0.01	N.D
Pb	<0.01	<0.01 (<0.001)	<0.01	0.01 (<0.001)
Mg	0.25	1.25 (0.051)	0.55	1.17 (0.048)
Mn	<0.01	0.01 (<0.001)	<0.01	0.01 (<0.001)
Mo	<0.01	0.01 (<0.001)	<0.01	N.D
Ni	2.58	18.2 (0.310)	1.05	5.61 (0.096)
P	0.04	<0.19 (<0.001)	0.03	0.08 (0.003)
K	0.73	1.35 (0.035)	0.17	0.90 (0.023)
Se	<0.01	<0.09 (0.001)	<0.01	N.D
Si	0.06	0.05 (0.002)	0.04	0.01 (0.004)
Na	0.23	0.60 (0.026)	0.20	1.00 (0.043)
Sr	<0.01	0.01 (<0.001)	<0.01	0.01 (<0.001)
S	0.19	0.83 (0.026)	0.22	2.74 (0.085)
Sn	0.02	<0.01(<0.001)	0.01	N.D
Zn	1.00	0.56 (0.009)	<0.01	0.05 (<0.001)

* - Taken from Baker and Proctor (1988).

¥ - N.D = Not detected.

\# - Number of moles is given in brackets.

IV.5.111. Results and Discussion

The results of the analyses are presented in Table IV.1 The samples showed only slight variation in their elemental composition. Nickel was substantially enriched in both preparations. With the exception of Ca, Mg and K, most of the metals were apparently removed during the isolation and purification processes. These findings are similar to those of Lee (1977) with respect to the New Caledonian Ni-hyperaccumulators Sebertia acuminata and Hybanthus austrocaledonicus. Similar behaviour was also reported for solid Ni-rich material obtained from Psychotria douarrei (New Caledonia) and Walsura monophylla of the Philippines (Kersten, 1979; Freeman, 1989). Lee (1977) found Ni, Ca and Mg to be the only metals present in measurable quantities when purified material was subjected to AAS. In three Ni-rich samples obtained from Psychotria douarrei, Kersten (1979) observed Ca to be present at a level of ~ 0.007 %. The percentages of Ni were 0.07, 0.09 and 0.11. The first two samples, which contained less Ni, were shown to contain ~ 0.006 % sodium. The sodium content of the third sample was 0.002 %. Freeman (1989) found the levels of Ca and Mg in purified Ni-rich material from Walsura monophylla to be 2.92 and 3.60 % respectively. The dried ground leaf material was shown to contain 1.07 and 0.355 % Ca and Mg respectively. There was little difference in the K levels. However, there was about a ten-fold increase in the Ni concentration.

The consistent association of Ca and Mg with purified Ni-rich material obtained from these Ni-hyperaccumulating plants may be attributed to one or both of the following:-

a) Similarity in behaviour during gel filtration and ion-exchange chromatography

b) The ions being an integral part of the complex

Both Ca and Mg play an important role in plant structure and function. It would therefore not be unusual for the ions to form complexes with suitable ligands that may be present. It is possible that such complexes could co-elute with or elute close to the Ni complexes during the chromatographic

procedures employed. This would certainly lead to enrichment of the metal in the purified Ni-rich material. In gel filtration chromatography, the two kinds of complexes would have to possess similar molar masses or interact similarly with the stationary phase. In ion-exchange chromatography, the pre-requisite for such behaviour would have to be similar charge and ionic size. If the ions neutralise the charge on the Ni complexes, and dissociation is minimal during gel filtration, then co-elution will also occur. The high levels of Ca ,Mg and K in the solid material derived from the two Ni-hyperaccumulating plants under study could also be accounted for in this manner. Of the two possible explanations, the latter is favoured in view of the evidence for anionic 1 : 1 Ni-citrate in Ni-hyperaccumulating plants (Lee, 1977). The anionic 1:1 Ca-citrate and Mg-citrate complexes are equally stable (log K ~3.26) (Sillén and Martell, 1964). Both are less stable than the anionic 1:1 Ni-citrate complex (log K = 5.10). The malate complexes of Ca and Mg are cationic and even less stable (log K < 2.0). On account of their charge and lower mass, the possibility of these two complexes eluting with Ni complexes in purified Ni-rich extracts of the two Ni-hyperaccumulating plants can be ignored. A neutral Ni-malate complex has been reported by Campi (1963), but is also of lower stability (log K = 3.30). The number of moles of Ni in the purified extract from D.gelonioides subsp. tuberculatum is higher than that of Ca, Mg and K. In the case of P. 'palawanensis', the number of moles of Ni and Ca are comparable, these being higher than the number of moles of Mg and K. In the light of the above observations, the anionic 1:1 Ni-citrate complex is expected to predominate. It may be concluded that lower stability and molar mass of the other citrate complexes renders them unsuitable for co-elution with the Ni-citrate complex. An anionic 2 : 1 Ni-citrate complex has also been characterised (Liddle, 1979). This complex has however not been reported in Ni-hyperaccumulating plants.

IV.6. Gas Chromatography - Mass Spectrometry

IV.6.I. Instrumentation

Gas-liquid chromatography, frequently referred to as gas chromatography (GC) is a quick, easy and sensitive method for determining the number of components in a mixture, their identities, and the absence or presence of impurities. Gas chromatography is therefore used to

evaluate the effectiveness of purification procedures. Essentially, the sample is injected into the head of a chromatographic column which is maintained at a constant temperature or subjected to a temperature gradient in a thermostatically controlled oven. Elution is effected by the flow of an inert gaseous mobile phase (carrier gas). Separation is achieved as the sample components are retarded according to their affinity for the stationary phase. Packed columns (2-10 mm x 1-4 m) or capillary columns (0.2-0.5 mm x 20-80 m) may be used. A coiled glass tube packed with stationary phase on an inert support constitutes a packed column, while the capillary column is characterised by the confinement of the stationary phase to the inner wall. In contrast to most of the other types of chromatography the mobile phase does not react with the sample molecules. Transporting the molecules through the stationary phase is its only function. The column effluent usually passes into a heated detection system which in conjunction with a signal processor and recording system yields peaks corresponding to the various components in the sample. The most common detector is the flame ionisation detector. The use of standards facilitates component identification by a comparison of retention times (t_r). Peak areas are commonly used in quantitative analysis.

In gas chromatography - mass spectrometry (GC-MS), the mass spectrometer is used as a detection system. A mass spectrum is obtained by converting sample components into rapidly moving, positively-charged gaseous ions, then resolving them on the basis of their mass-to-charge (m/e) ratios. The sample components are ionised by bombardment with a stream of electrons, ions, fast atoms, protons or gaseous molecules (chemical ionisation). Resolution of the ions (fragments) produced is normally effected by suitably disposed electric and/or magnetic fields. When detected, the series of fragments produces a number of lines each of which corresponds to a definite m/e ratio. The height of each line represents the per cent abundance. These lines constitute the mass spectrum. The mass spectrum of a pure compound furnishes information such as molar mass and molecular formula which can be used for identification purposes. In addition, a study of the fragmentation pattern gives some idea of which functional groups are present (or absent). The final identification of the compound can be established by comparison of its mass spectrum with those of known compounds until a close match is obtained.

The suitability of the mass spectrometer as a detection system stems from the fact that the effluent from the GC column frequently contains compounds which have potential to form positive ions. The low flow rate of the capillary column facilitates direct entry of effluent into the ion source of the mass spectrometer. For packed columns which have higher flow rates, a separation procedure must be employed to remove most of the mobile phase prior to the introduction of effluent into the ion source. The interfacing of GC equipment with a mass spectrometer greatly increases the scope of GC for qualitative analysis.

IV.6.II. Application to Plant Organic Acids

The use of GC in the separation of organic acids in plant extracts has been reported by Horii et al., (1965); Canvin, (1965); Atkins and Canvin, (1971); Barta and Osmond, (1973); Pinelli and Colombo (1976); Phillips and Jennings (1976). Similar work was later carried out by Pelosi et al., (1976), Lee (1977), Pancaro et al., (1978b) Kersten (1979), Shaw (1980), Morrison (1980), Stockley (1980) and Freeman (1989) on Ni-rich material derived from Ni-hyperaccumulating plants of Europe, New Caledonia, South Central Africa and the Philippines. A common feature in the preparation of the samples for chromatographic separation was the derivatisation of the organic acids present. Derivatisation increases the volatility of the acids thereby facilitating their elution through the column by the carrier gas. Methylation and silylation are the most common methods of derivatisation. Both procedures have been applied to Ni-rich samples from Ni-hyperaccumulating plants. Derivatisation methods for a wide variety of compound types have been documented by Blau and King (1977). For this investigation, methylation was chosen. The reagents required were readily available and methyl esters are less prone to undesireable changes during storage than silyl derivatives.

IV.6.III. Gas Chromatographic - Mass Spectral Analysis of Isolated Purified Nickel Complexes

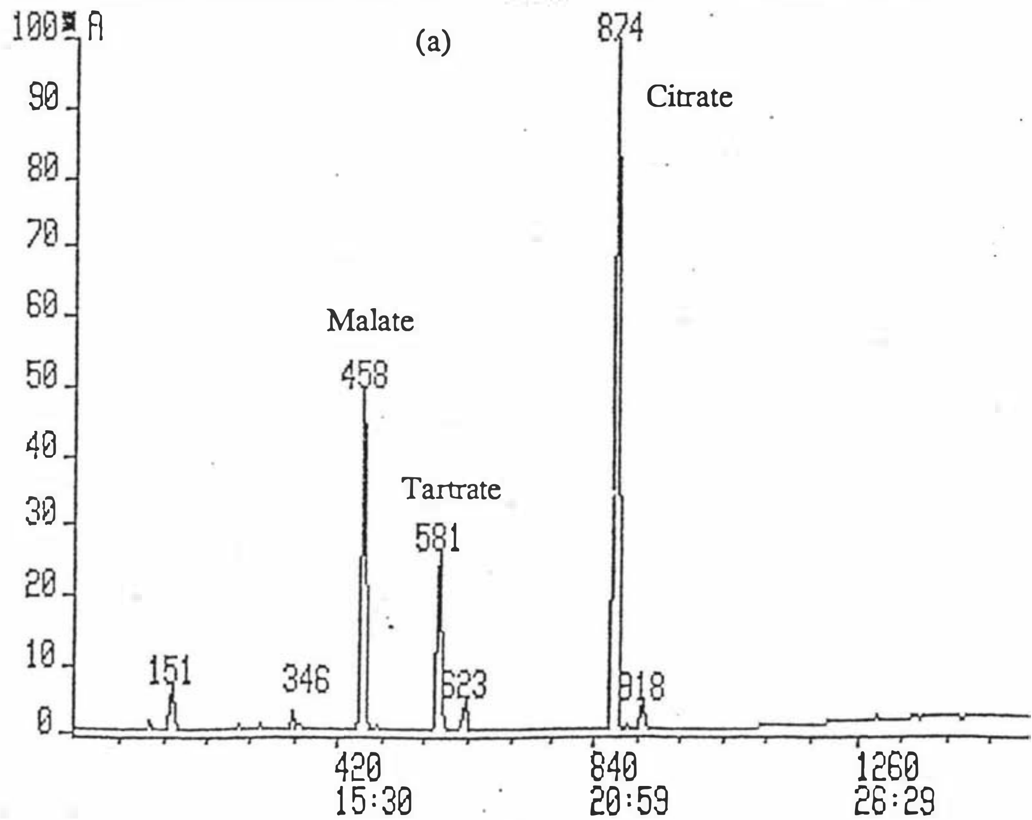
In anticipation of the presence of carboxylic acids, 1.1 and 6.1 mg amounts of the dry Ni-rich material obtained from D.gelonioides subsp.tuberculatum and P. 'palawanensis' respectively were methylated in the following manner. Each sample was treated with 1 cm^3 of 5 %

Analar methanolic HCl and then heated at 80 ± 1° C for four hours in a derivatisation vial. The resulting product was concentrated under reduced pressure and 200 μL of Analar dichloromethane was then added.

The methyl esters were separated on a fused silica WCOT BP 1 capillary column (0.2 mm x 30 m) installed in a Hewlett - Packard 5890 Series 1 Gas Chromatograph. Sample (1 μL) was applied via a splitless injector at 220° C. The column was held at 50° C for 2 min., then programmed to 280° C at 10° C min^{-1}. The pressure of helium (the carrier gas) at the column head was 2 psi and the flow rate was 0.9 cm^3 min^{-1}. The column outlet passed directly into the mass spectrometer ion source via a heated transfer line. Both low and high resolution (5000 rp) electron impact (70 eV) spectra were acquired in a conventional manner at a scan rate of 1.5 s per decade and interscan time of 0.1 s. Low resolution chemical ionisation spectra were acquired at a scan rate of 1 s per decade and an interscan time of 0.5 s using ammonia as reagent gas.

IV.7. Quantitative Gas Chromatography

Methyl esters of Analar citric acid (1.20 mg/2 cm^3) and Analar malic acid (1.47/2 cm^3) were usde as standards for quantitative analysis. In addition to the samples obtained from D. gelonioides subsp. tuberculatum and P. 'palawanensis', a 5 mg sample from Walsura monophylla was also methylated, and its citric and malic acid content determined. The material was provided by Freeman (1989) who reported the acids to be present at the level of only 0.3 %. Separation was achieved under the same conditions as those used in qualititative analysis. The concentrations of citric and malic acids were calculated by comparing standard acid peak areas with sample acid peak areas. The resulting chromatograms and mass spectra are presented in Figs. IV.3 and IV.4.



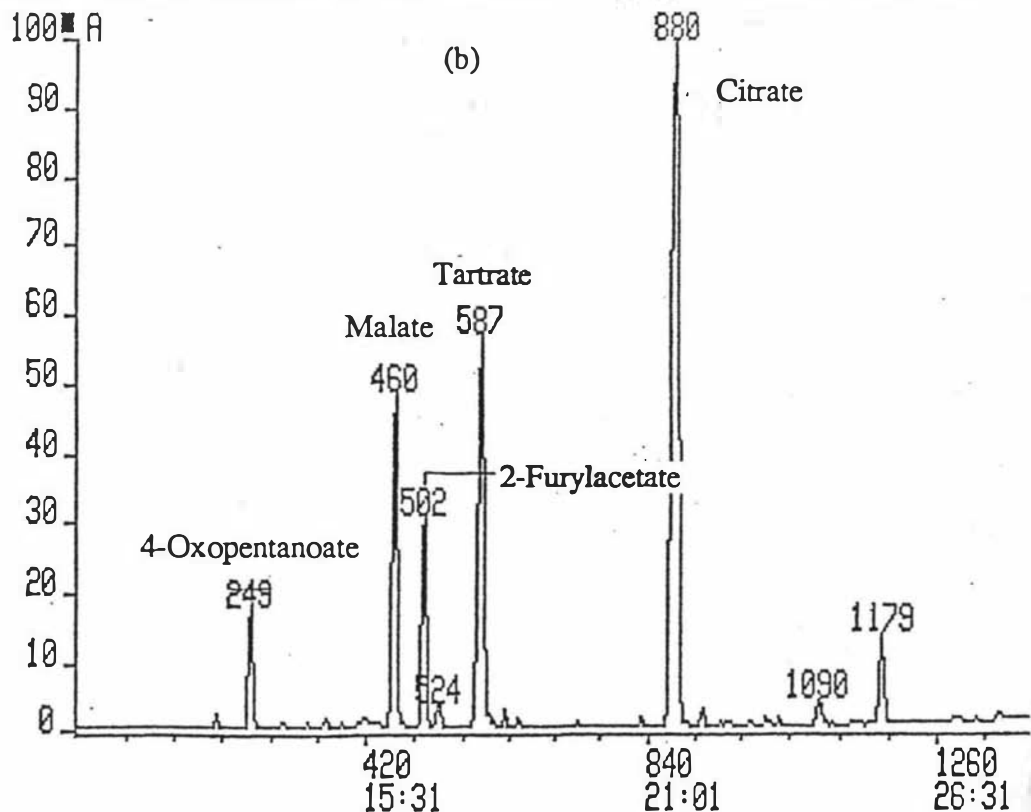


Fig. IV.3.I Total-ion chromatogram of Ni-rich species from (a) D.gelonioides subsp. tuberculatum, (b) P. 'palawanensis'.

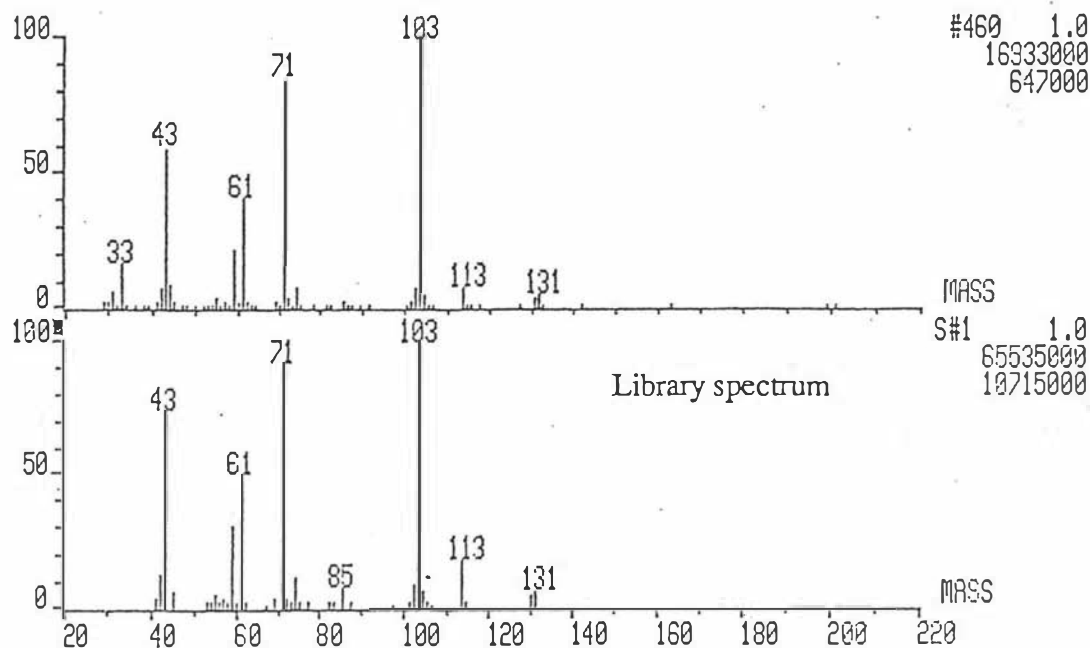


Fig. IV.3.II. Comparison of component spectrum with library spectrum of hydroxy-butanedioic acid methyl ester.

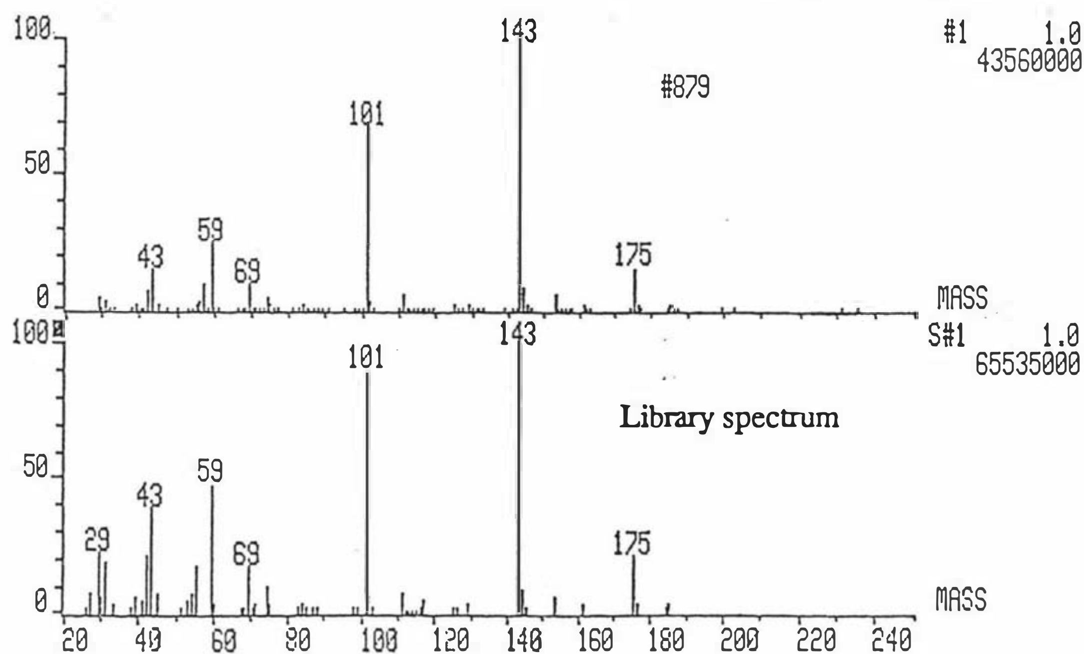


Fig. IV.3.III. Comparison of component spectrum with library spectrum of 2-hydroxy-1,2,3-propanetricarboxylic acid trimethyl ester.

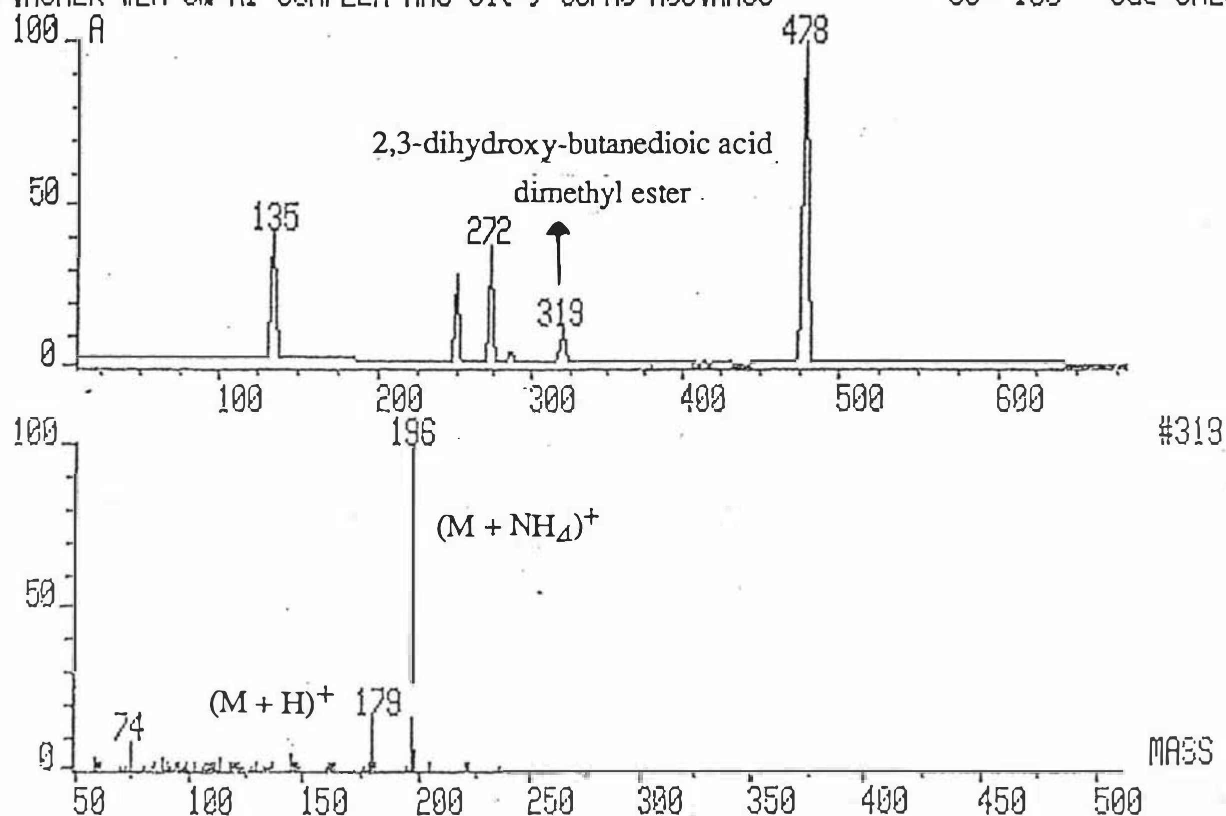


Fig.IV.3.IV. Chemical ionisation spectrum of 2,3-dihydroxy-butanedioic acid dimethyl ester.

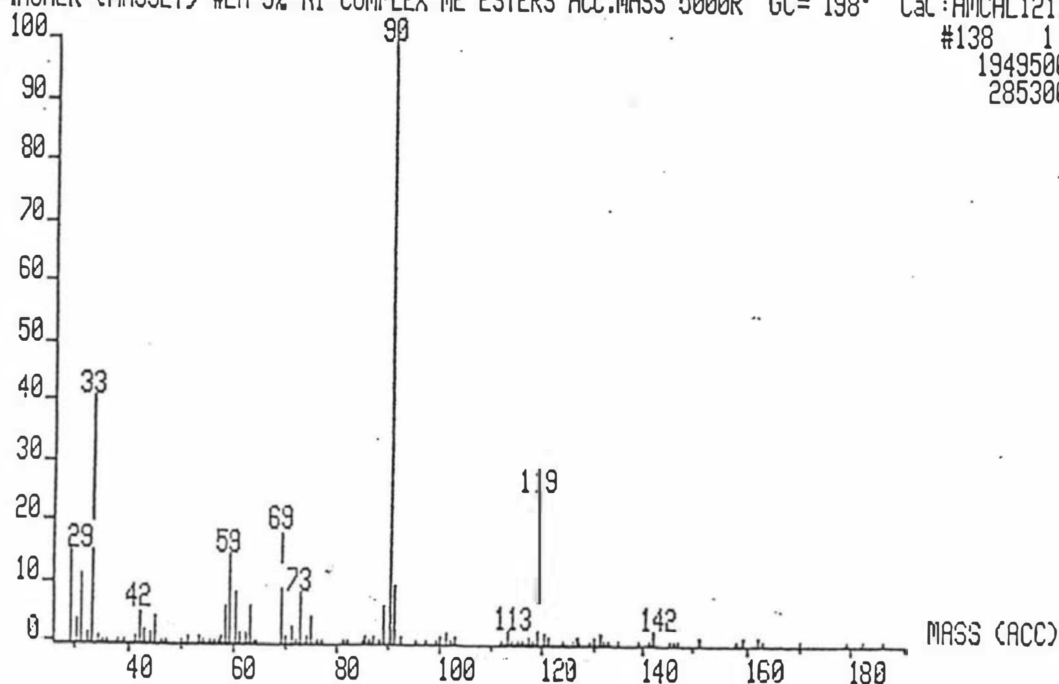


Fig. IV.3.V. Electron impact ionisation spectrum of 2,3-dihydroxy-butanedioic acid dimethyl ester.

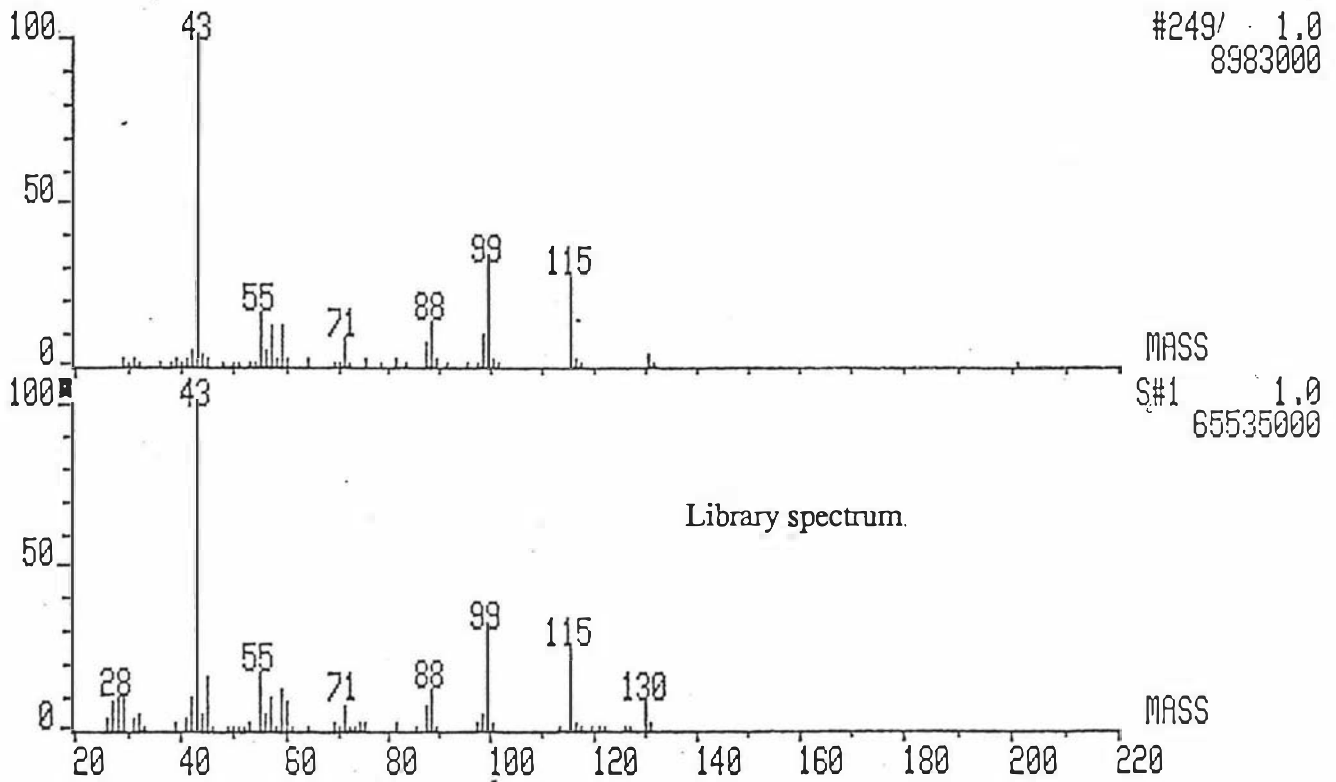
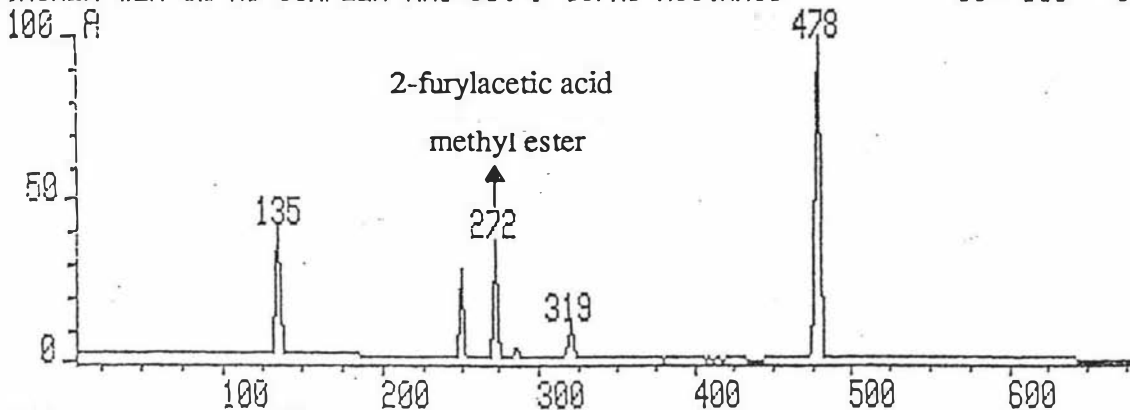


Fig. IV.3.VI. Comparison of component spectrum with library spectrum of 4-oxopentanoic acid methyl ester.

HOMER4#272 x1 Bgd=265 12-OCT-89 15:54+0:16:32 70-250S C2+
BpM=0 I=1.5v Hm=0 TIC=19142000 Acnt:F.HOMER Sys:IBGC2
F.HOMER #2A 5% NI COMPLEX NH3 CI(+) GC/MS ACC.MASS GC= 185⁰ Cal:CALCI121B



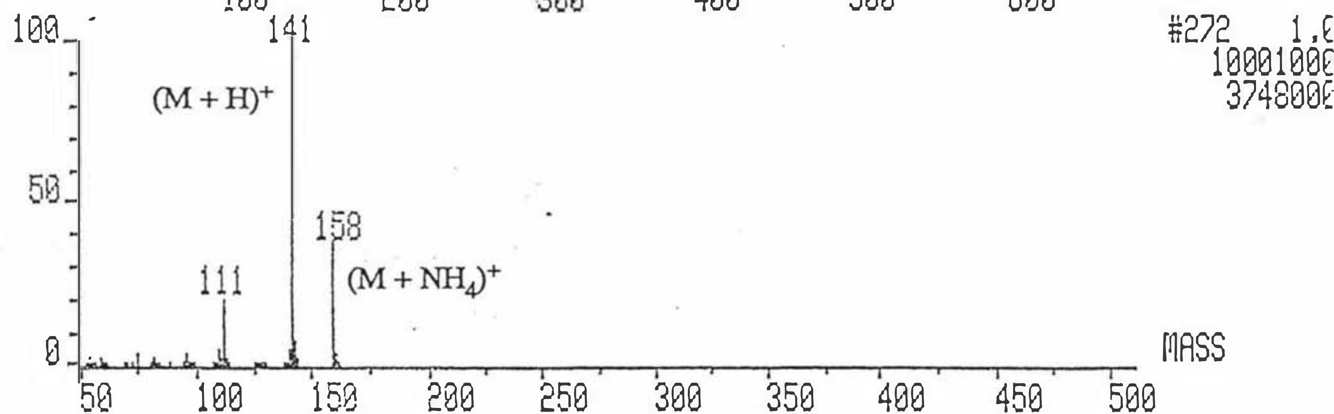


Fig. IV.3.VII. Chemical ionisation spectrum of 2-furylacetic acid methyl ester.

HOMER1#502 x1 Bgd=491 12-OCT-89 09:07+0:16:34 70-250S EI+
BpM=0 I=2.2v Hm=0 TIC=41882000 Acnt:F.HOMER Sys:IBGC2
F.HOMER (MASSEY) 5 % NI COMPLEX METHYLATED 0.5/.2 DB1 30M GC= 187⁰ Cal:CAL1210A

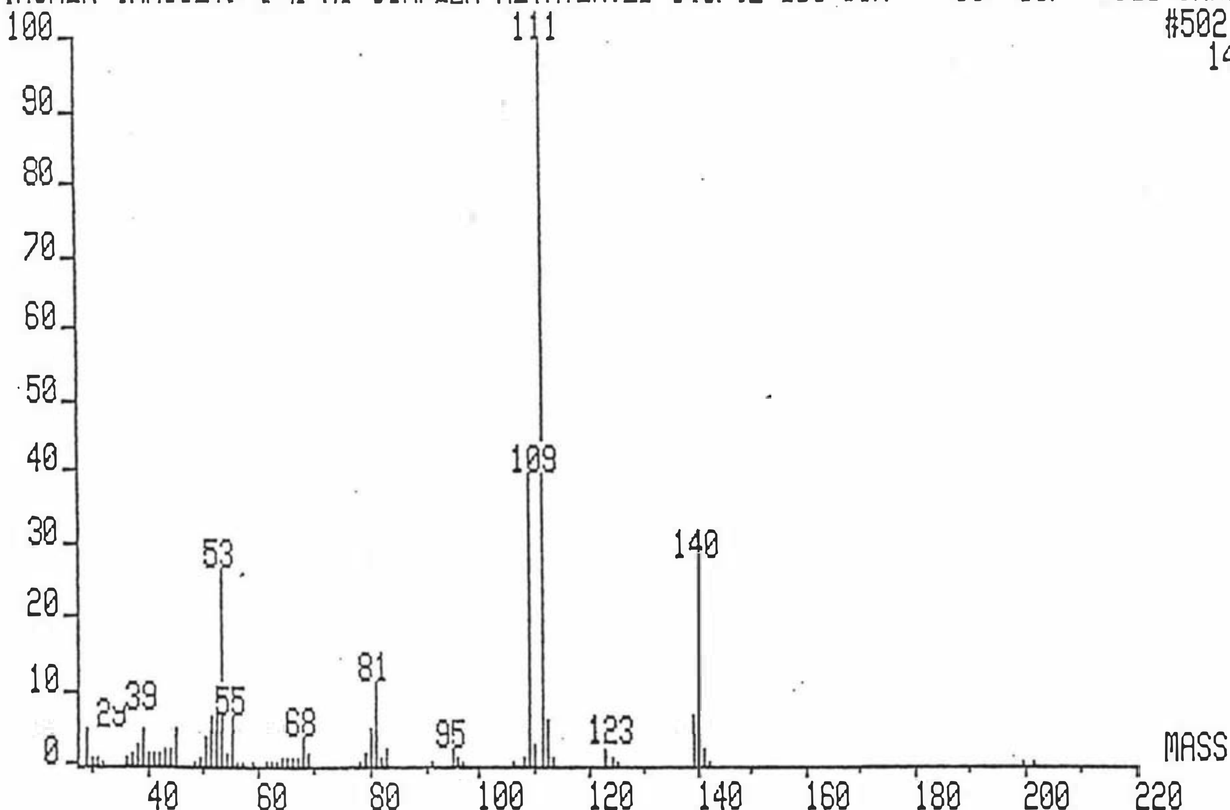
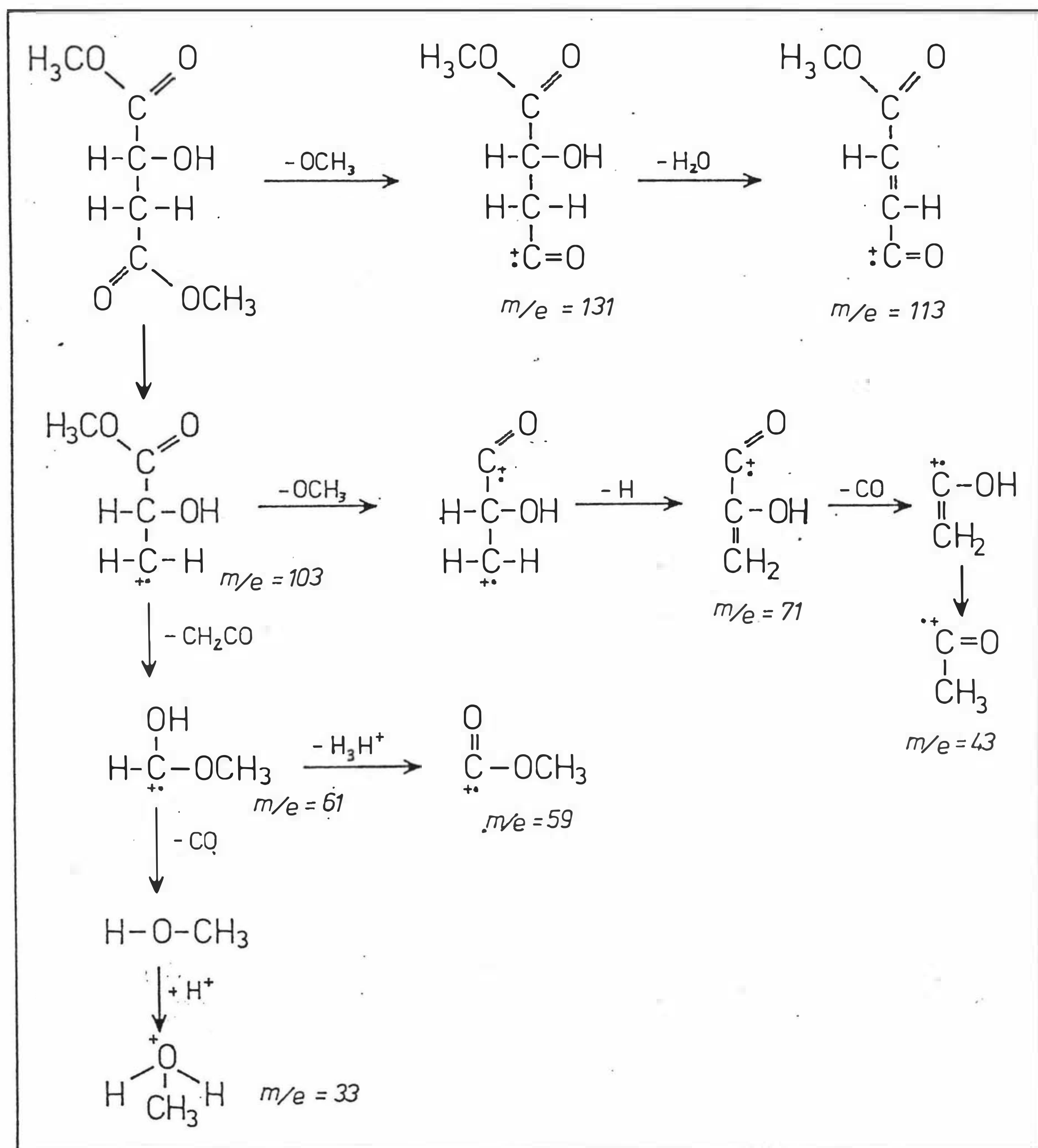


Fig. IV.3.VIII. Electron impact ionisation spectrum of 2-furylacetic acid methyl ester.

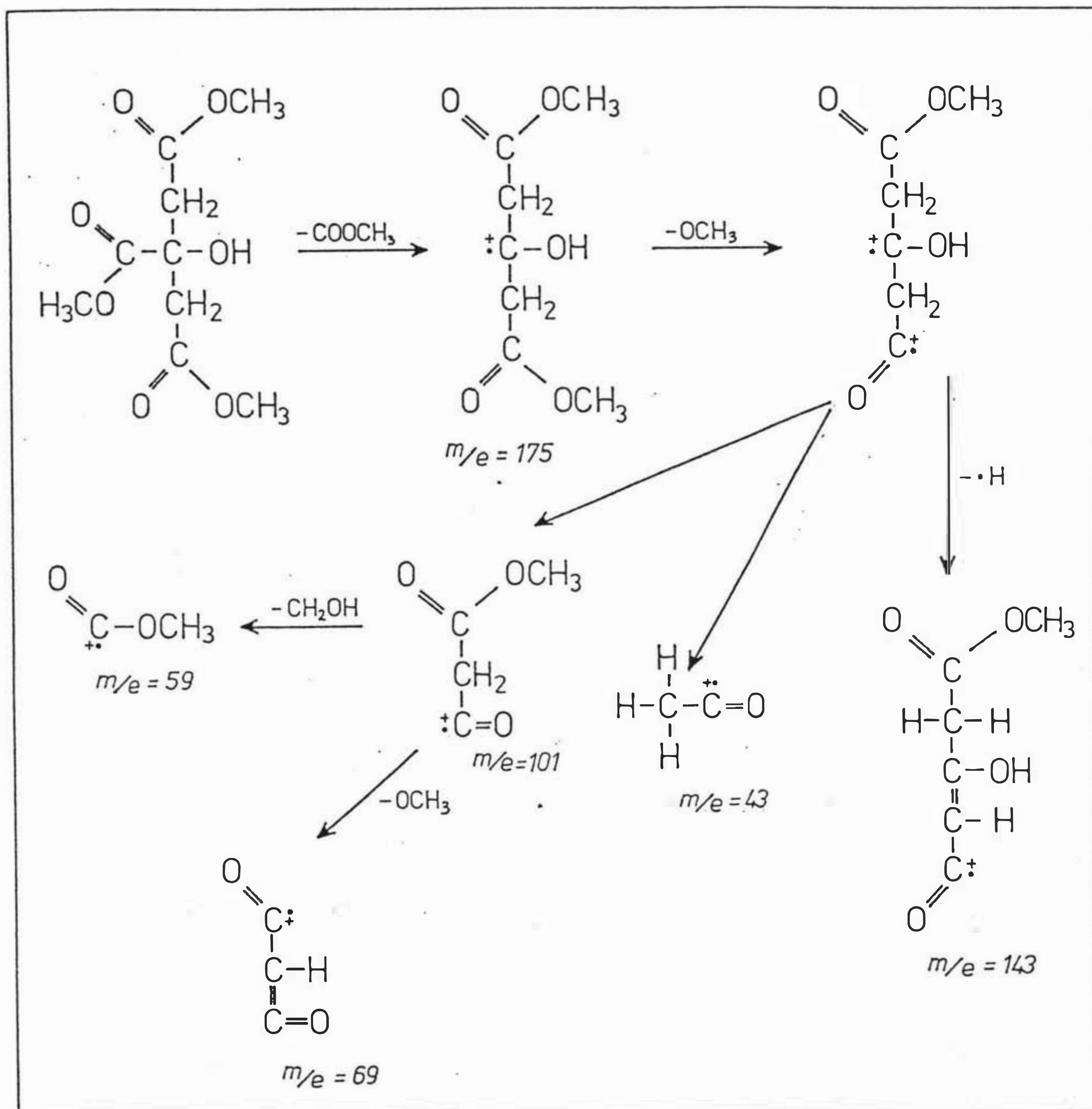
IV.8. Results and Discussion

As shown in Fig IV.3.I, there was a close resemblence between the total ion chromatograms of D.gelonioides subsp. tuberculatum and P. 'palawanensis' obtained via low resolution GC-MS; three peaks were common to both. At high resolution, two of these (scan nos. 458-460 and 874-880) were readily identified as dimethyl malate ester (hydroxybutanedioic acid dimethyl ester) and trimethyl citrate ester (2 -hydroxy -1,2,3-propanetricarboxylic trimethyl ester) respectively by comparing their spectra with library spectra (Figs. IV.3.II and 3.III). Fragmentation patterns are presented in Schemes IV.1 and 2. There was no matching library spectrum for the high resolution spectrum of the component with scan no. 581-587. Since ionisation had occurred via electron impact, the base peak (m/e 90) could not have corresponded to the parent molecular ion (M^+). Extensive fragmentation of ions which is a feature of electron impact ionisation, usually makes compound identification very difficult. Chemical ionisation often leaves the M^+ ion intact thereby providing an estimate of the molar mass. This alternative was pursued using ammonia, and a low resolution spectrum was obtained (Fig .IV.3 IV). The base peak (m/e 179) corresponding to the$(M+ H)^+$ ion indicated a molar mass of 178. This information together with the assignment of structures to many of the fragments represented by spectral lines in Fig. IV.3.V led to the identification of the component as dimethyl tartrate ester (2,3-dihydroxybutanedioic acid dimethyl ester).The fragmentation pattern is shown in Scheme IV.3. The reactions are characteristic of carboxylic acid esters and diols (Budzikiewicz et al., 1965).

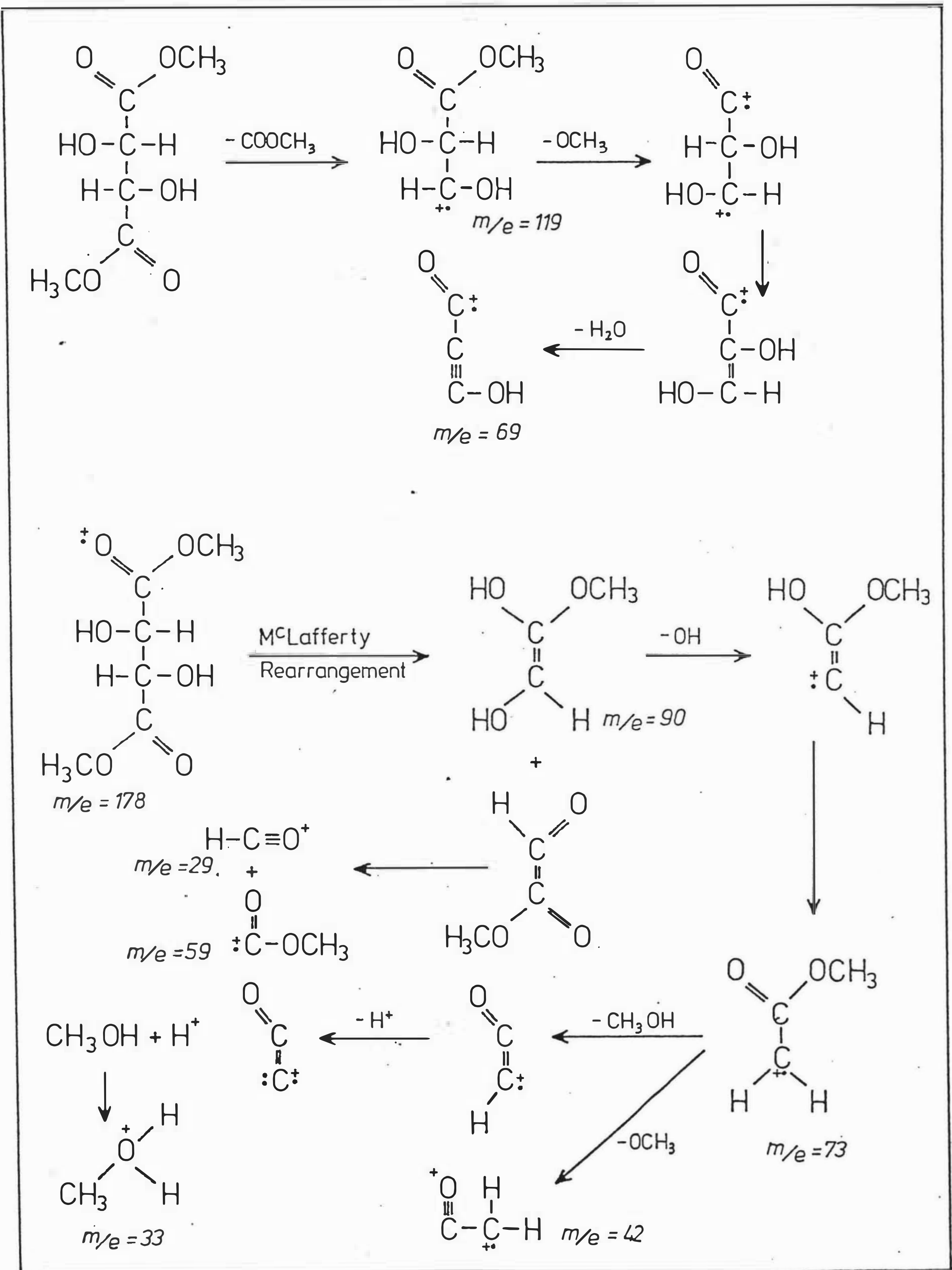
Esters that gave rise to peaks of scan nos. 249 and 502 (Fig. IV.3.Ib) in the sample from P. 'palawanensis' were identified as 4-oxo-pentanoic acid methyl ester and 2-furylacetic acid methyl ester respectively. All peaks in the spectrum of the oxo-acid corresponded to those in the library spectrum (Fig. IV.3 VI). Like the tartaric acid ester, the furylacetic acid ester was identified by examining spectra that had resulted from both chemical and electron impact ionisation (Figs. IV.3.VII and 3.VIII), and by working out fragmentation patterns. The base peak (m/e 141) which corresponded to the $(M+H)^+$ ion indicated a molar mass of 140. Proposed fragmentation patterns for the two esters are presented in Schemes IV.4 and 5. Fragments of m/e 39 and 53 are diagnostic of furans and related compounds (Budzikiewicz et al., 1965; Beynon et al., 1968).



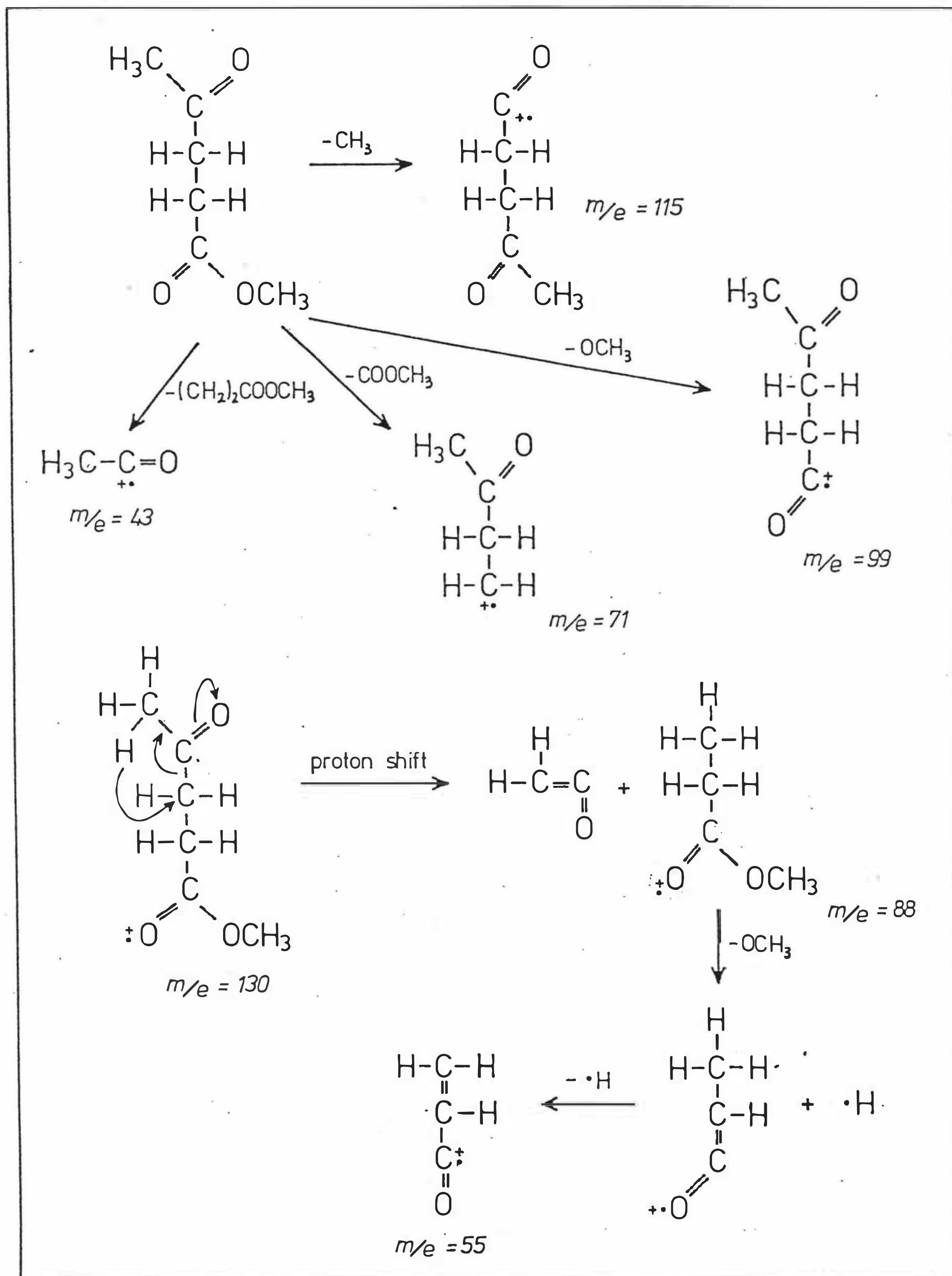
Scheme IV.1. Fragmentation pattern for dimethyl malate ester.



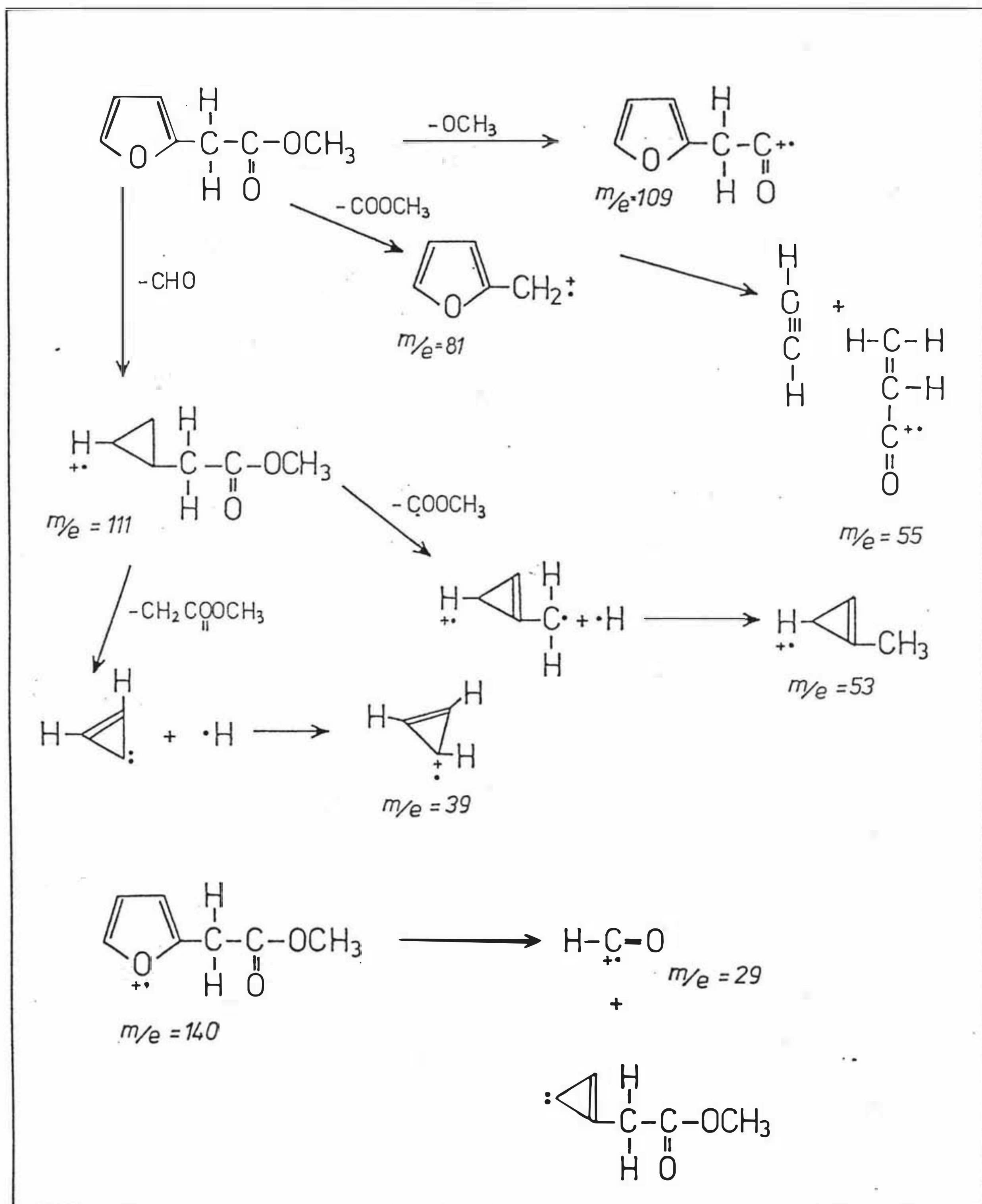
Scheme IV.2. Fragmentation pattern for trimethyl citrate ester.



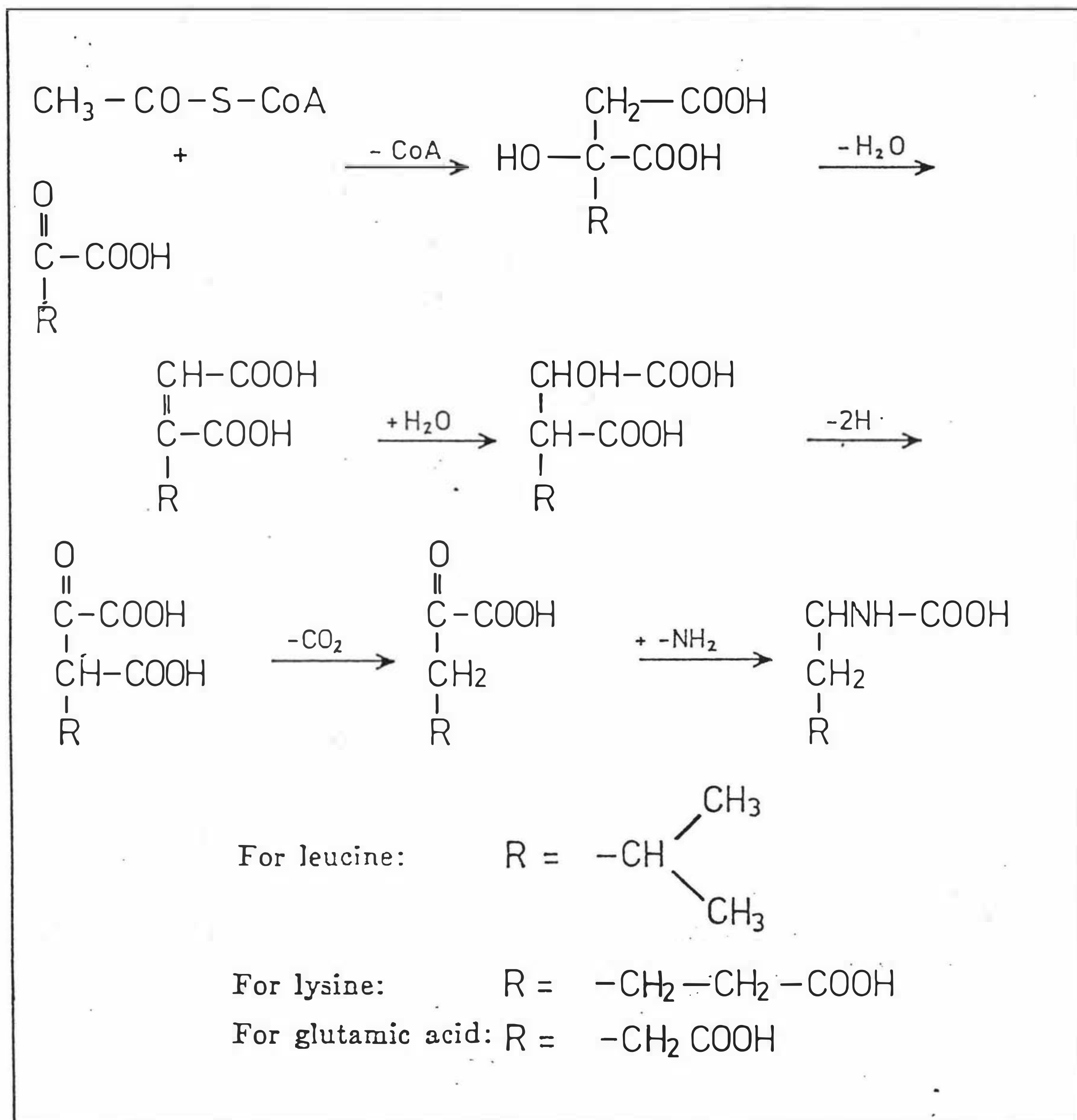
Scheme IV.3. Fragmentation pattern for dimethyl tartrate ester.



Scheme IV.4. Fragmentation pattern for methyl 4-oxopentanoate ester.



Scheme IV.5. Fragmentation pattern for methyl 2-furylacetate ester.



Scheme IV.6. Proposed generalised mechanism for the biosynthesis of the α-keto acid precursors of leucine and lysine (Source: Strassman and Ceci, 1963)

The presence of citric and malic acids in purified material from the two Ni-hyperaccumulating plants provides further evidence for their possible role as Ni chelators. With the exception of tartaric acid, the results are therefore in accordance with those of Pelosi et al. (1976), Pancaro et al. (1977), Lee (1977), Kersten (1979), Stockley (1980), Morrison (1980) and Freeman (1989). Although it has been reported in some plants, notably fruits, both in the free state and as its potassium or calcium salt, tartaric acid appears to be of minor metabolic importance (Vickery and Palmer, 1954; Peynaud and Maurie, 1958). In 1959, Stafford surveyed the tartaric acid distribution in leaves of angiosperms. He reported the acid to be fairly widely distributed in trace amounts, being present in larger amounts only in the Violaceae, Geraniaceae and Leguminosae. None of the plants under investigation is a member of these families. If present in significant amount, this acid could be important as a Ni chelator provided the concentrations of citric and malic acids are low. Sillén and Martell (1964) reported a log K_1 value of 9.9 for one Ni-tartrate complex.

The oxo-carboxylic acid found in the green solid isolated from P. 'palawanensis' could be a precursor in the synthesis of a non-protein amino acid. The term non-protein amino acid applies to naturally occurring amino acids, amino acid amides and imino acids which are not usually found as protein constitutents. Most non-protein amino acids are regarded as secondary metabolites. However, a few may be involved in primary metabolic pathways of plants. In higher plants, the majority of non-protein amino acids exist in the free state or as condensation compounds of low molar mass such as γ-glutamyl and acetyl derivatives (Bell, 1980). In 1963, Strassman and Ceci proposed a generalised mechanism for the biosynthesis of the 2-oxo-carboxylic acid (α-keto acid) precursor of leucine and lysine in yeast cells.The mechanism (Scheme IV.6) involved the condensation of acetyl CoA and a 2-oxo-carboxylic acid to yield an α- substituted malic acid. This intermediate may then undergo reactions of the Krebs cycle type to produce a homologue of the original 2-oxo-carboxylic acid. Subsequent amination yields an amino acid. Iso-citric acid may therefore be produced by condensation of oxaloacetic acid with acetyl CoA, while the condensation of glyoxylic acid with acetyl CoA could produce malic acid.

The non-protein amino acids L-cis-2-amino-3-formyl-pentenoic acid, 2-amino-4-oxo-pentanoic acid and 2-amino-4-oxo-3-methylpentanoic acid have been reported in Bankera fulginoeoalba (mushroom), Bacillus cereus (Bacterium) and Clostridium stricklandii (bacterium) respectively (Doyle and Levenberg,1968; Bell, 1973; Perlman et al., 1977). In 1980, Morrison suggested that 2-oxo-pentanoic acid and 3-methyl-2-pentanoic acid could be present in the Ni-rich extract from the Ni-hyperaccumulating plant Alyssum virgatum E.Nyárády based on partial assignment of peaks in the mass spectrum. Among the several heterocyclic non-protein amino acids to be reported are 3-(-2-furoyl)-L-alanine and 3-(3-carboxyfuran-4-yl)alanine (Ichihara et al., 1973; Couchman et al., 1973; Doyle and Levenberg, 1974; Hatanka and Niimura, 1975). The first compound was readily extracted from buckweed seeds (Fagopyrum esculentum Moench) and as a hot acid degradation product of ascorbalamic acid in Savoy cabbage (Brassica oleracea sabauda). The second compound was found in the mushroom Phyllotopsis nidulans (Pers. ex. Fr.) Sing. and another fungus, Tricholomopsis rutilans (Fr.) Sing. There is the likelihood that 2-furylacetic acid may also be involved in the biosynthesis of 2-oxo-carboxylic acids which could result in the synthesis of amino acids. Through partial identification of fragments represented by the mass spectrum of a Ni-rich extract from another Ni-hyperaccumulating plant, Alyssum heldreichi Haussknecht, Morrison (1980) suggested the presence of a highly unsaturated ester with at least two double bonds. He proposed esters of furan or pyran with possible molecular formula $C_8H_{12}O_2$, $C_7H_8O_3$ or $C_6H_4O_4$. Due to the similarity between his spectrum and that identified here as having resulted from 2-furylacetic acid, it may be concluded that 2-furylacetic acid was also present in A. heldreichi.

Plants belonging to the genera Phyllanthus and Alyssum are members of the Euphorbiaceae and Brassicaceae respectively. These are among the 11 families of dicotyledones in which glucosinolates are known to occur (Underhill, 1980). Within the Euphorbiaceae, glucosinolates are confined to a few taxa. However, in the Brassicaceae, glucosinolate occurrence is relatively widespread (Kjaer, 1974). Glucosinolates are secondary plant metabolites characterised by their ability to be hydrolysed by the enzyme myrosinase, to yield glucose and a labile aglucone. The latter decomposes to isothiocyanate (mustard oil) with loss of sulphate. The volatile mustard oils give rise to the distinctive, pungent flavour and odour which are associated with many of the edible Crucifers [common mustards] (Vaughan and Hemingway, 1959). It is of interest to note that

Fig. IV.4. Chromatograms of purified Ni-rich fractions from

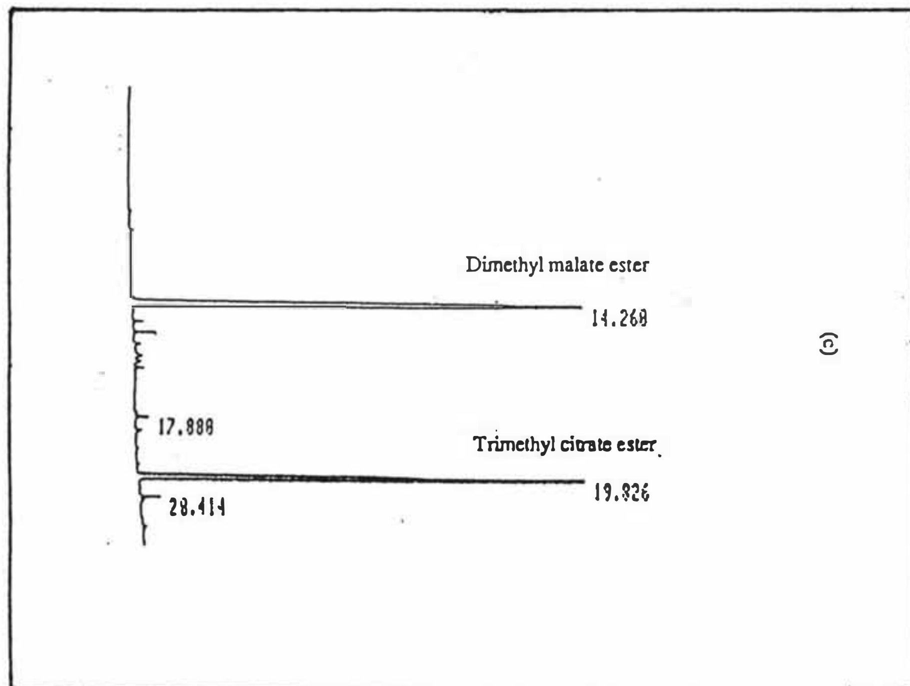
(a) Chromatogram of purified Ni-rich fraction from D.gelonioides subsp. tuberculatum [18 % Ni]

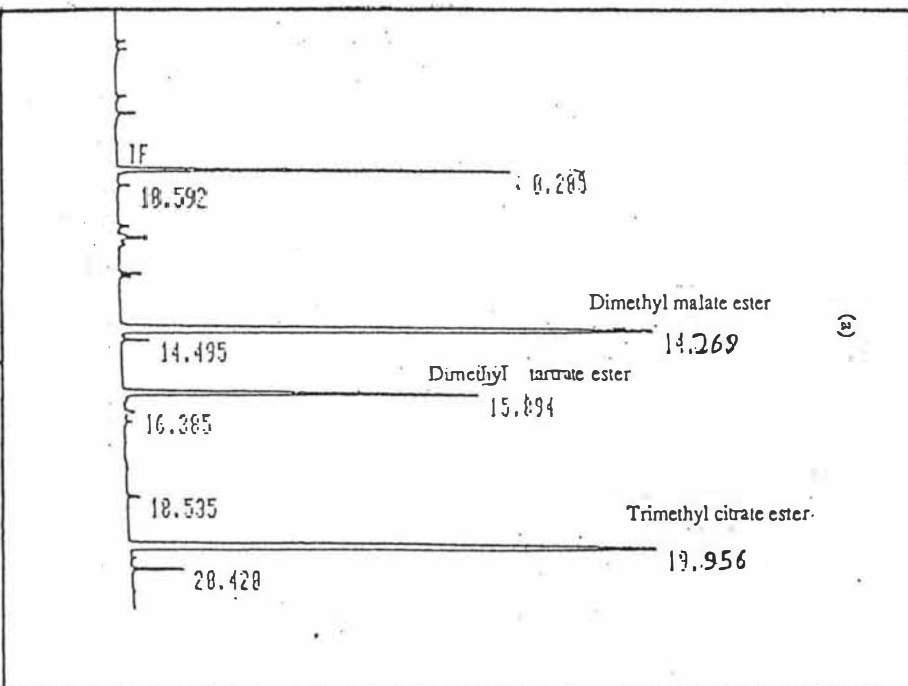
(b) Chromatogram of purified Ni-rich fraction from P. 'palawanensis' and [5 % Ni]

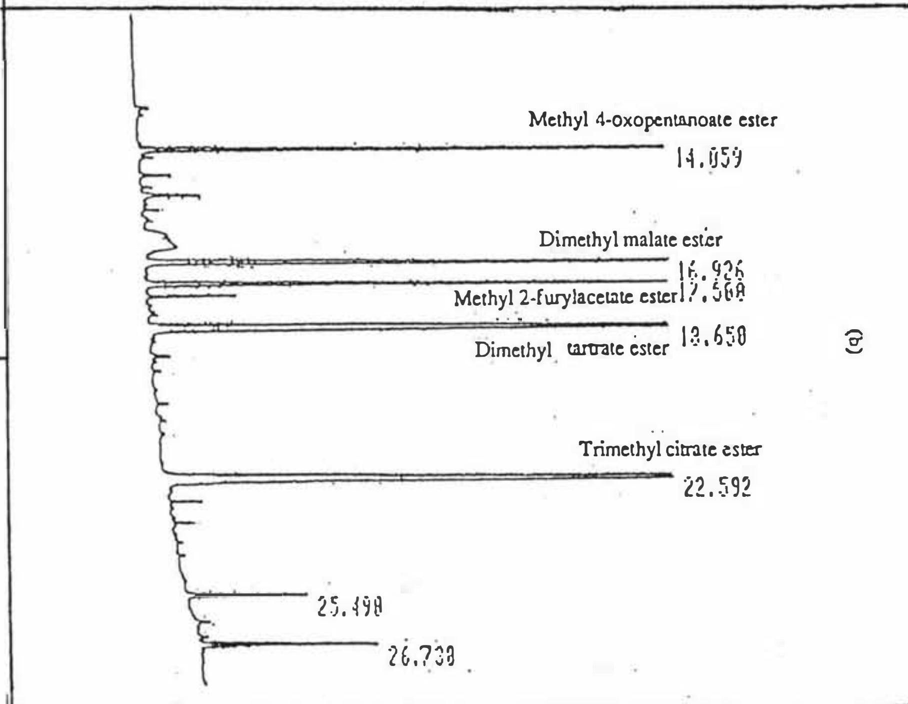
(c) Chromatogram of purified Ni-rich fraction from W. monophylla [3 % Ni]

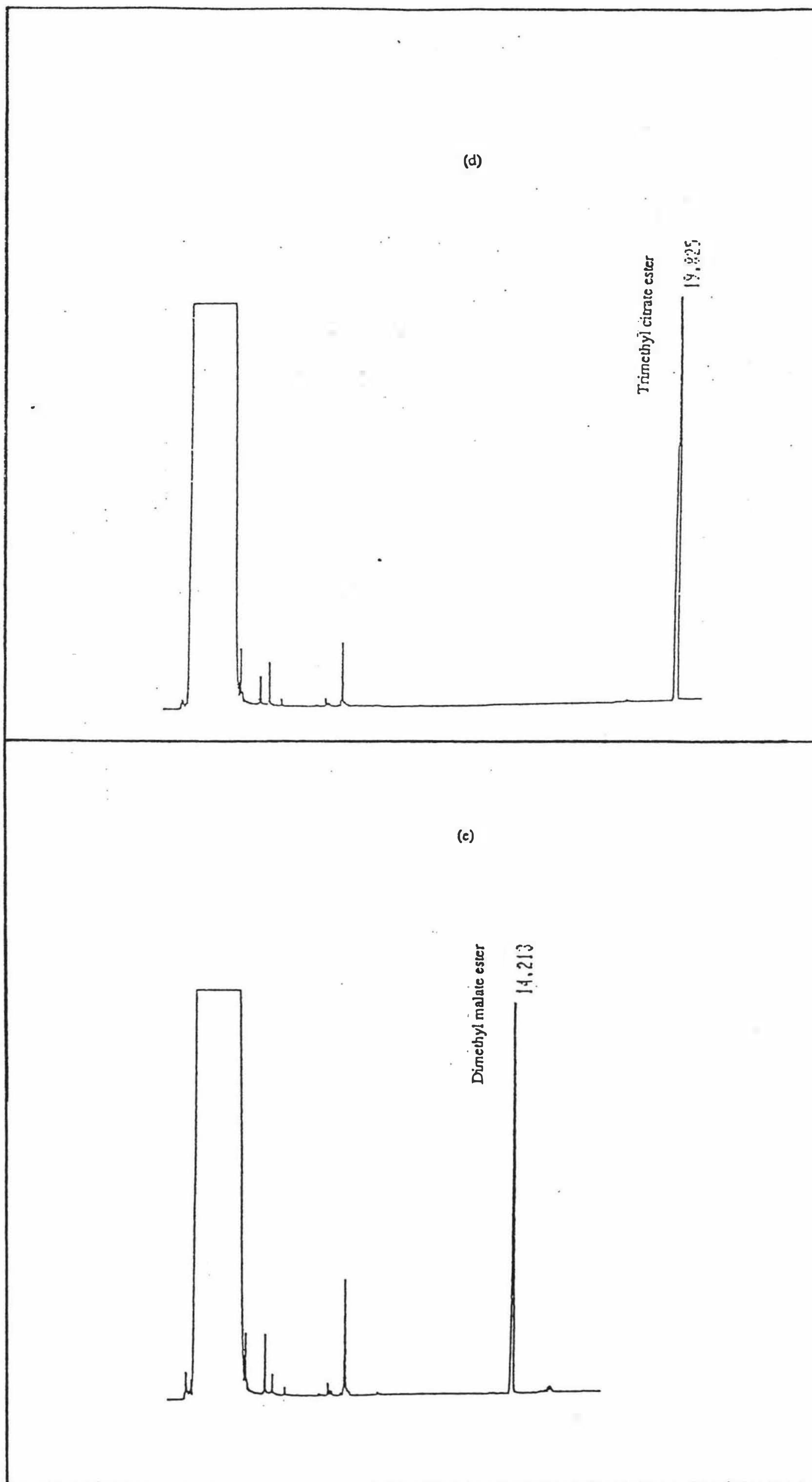
(d) Chromatogram of trimethyl citrate ester standard

(e) Chromatogram of dimethyl malate ester standard


Dimethyl malate ester
14.268
17.888
Trimethyl citrate ester
19.926
28.414
(c)


10.592
Dimethyl malate ester
14.269
14.495
Dimethyl tartrate ester
15.894
16.385
18.535
Trimethyl citrate ester
19.956
20.428
(a)


Methyl 4-oxopentanoate ester
14.059
Dimethyl malate ester
16.926
Methyl 2-furylacetate ester
17.560
Dimethyl tartrate ester
18.650
Trimethyl citrate ester
22.592
25.490
26.730
(b)


(d)
Trimethyl citrate ester
19.925
(c)
Dimethyl malate ester
14.213

the scheme of Strassman and Ceci (1963), could also be involved in sidechain modification of previously formed glucosinolates to produce new ones (Underhill, 1980). It is possible that glucosinolates may play a direct or indirect role in Ni complexation in those Alyssum species shown to contain little or no citric and/or malic acids.

The GC traces for D.gelonioides subsp. tuberculatum, P. 'palawanensis'and W. monophylla are shown in Fig. IV.4. Those for the first two species (Traces a and b) closely resemble the GC-MS total-ion chromatograms of Figs. IV.3.Ia and 3.Ib except for an unidentified peak of t_r 10.20 min. in the trace derived from D. gelonioides subsp. tuberculatum. A component did elute at 10.30 min (scan no.151) in the total-ion chromatogram, thus the two peaks may have corresponded to the same compound taking into account minor fluctations in carrier gas flow rate during chromatographic separation. Due to the low intensity of the peak in the total-ion chromatogram, no mass spectral analysis was carried out. Since the component eluted before dimethyl malate ester, it is expected to have a lower molar mass than that of dimethyl ester. The ester was not considered a decomposition product of citric, malic or tartaric acid methyl esters owing to its absence from the traces for the other two plants.

Table IV.2 shows the concentration of Ni, citric acid and malic acid in the solid material obtained from the three Ni-hyperaccumulating plants. When the percentages of citric and malic acid in the material from D.gelonioides subsp. tuberculatum were taken together with percentages of the inorganic elements in Table IV.1, it was observed that almost 96 % of the mass was accounted for. Combination of sulphur (0.83 %) as sulphate or in some other form could increase this figure. Only 25 and 22 % of the total mass of dry Ni-rich material was accounted for in P. 'palawanensis' and W. monophylla respectively. The potential for increased contribution from sulphur (2.74 %) in P. 'palawanensis' is noteworthy. The concentrations of citric and malic acid in W. monophylla were substantially higher than those reported by Freeman (1989). Improper storage of derivatives may have been the cause.

Tartaric acid, 2-furylacetic acid and 4-oxo-pentanoic acid were not quantified owing to the absence of reports providing evidence in support of their role as Ni chelators in Ni-hyperaccumulating plants. However, in the light of the proposed presence of the latter two acids

in other Ni-hyperaccumulating plants (Morrison, 1980) and the preceding discussion, a quantitative study would be worthwhile. A survey of their distribution among Ni-hyperaccumulating plants so

Table IV.2 Nickel, Citric Acid and Malic Acid Content in Purified Nickel-rich Material Obtained from D.gelonioides subsp. tuberculatum, P. 'palawanensis' and W. monophylla

PLANT	CONCENTRATION (%, w /w)			MOLE RATIO
	Nickel	Citric Acid	Malic Acid	Nickel: Citric Acid: Malic Acid
D.gelonioides subsp. tuberculatum	18	24	43	1:0.4:1
P.'palawanensis'	5.0	5:1	4.2	1:0.4:0.4
W. monophylla	3.0	2.7	8.2	1:0.2:1

far investigated could also prove useful. Both GC-MS and quantitative GC had to be carried out at the Department of Scientific and Industrial Research (DSIR), Palmerston North owing to the unavailability of these facilities in the Analytical Research Laboratory, Massey University. The tremendous work-load of the DSIR scientists militated against a rapid turnover of results. The cost of analysis was also a decisive factor. In view of this, emphasis was therefore placed on the methyl esters of acids commonly found in association with Ni in extracts from Ni-hyperaccumulating plants.

Tartaric acid in D. gelonioides subsp. tuberculatum could have accounted for some 3-4 % of the sample mass. However, in comparison to the percentages of citric and malic acid, this is quite small. The role of tartaric acid as a Ni chelator in this plant can therefore be assumed negligible. Considering the sizes of the tartaric acid, 2-furylacetic acid and 4-oxo-pentanoic acid peaks in the GC trace for P. 'palawanensis', it is possible that these acids could collectively make a significant contribution to the sample mass. Quantification of the these three acids in P. 'palawanensis' can be justified on the basis of the low concentrations of citric and malic acid, and

the correspondingly high Ni/citric acid and Ni/malic acid mole ratios (Table IV.2).

The Ni/citric acid/malic acid mole ratios were 1:0.4:1, 1:0.4:0.4 and 1:0.2:1 for D.gelonioides subsp. tuberculatum, P. 'palawanensis' and W. monophylla respectively. It is of interest to note the higher concentration of malic acid in D.gelonioides subsp. tuberculatum and W. monophylla and the comparable concentrations of citric and malic acid in P. 'palawanensis'. Similar findings were reported for P. serpentinus from New Caledonia (Kersten et al., 1980). In D.gelonioides subsp. tuberculatum and W. monophylla, all of the Ni may be complexed by malate, whereas in P. 'palawanensis', a substantial portion of the Ni may only be complexed by malate and citrate in a mixed chelate. If the number of moles of 2-furylacetic acid and 4-oxo-pentanoic acid were comparable with those of the other two acids, then a role as Ni chelator would have to be considered.

It is important to note that the findings for P. 'palawanensis' may not have reflected the true status of Ni and the associated ligands in the purified ("anionic") material owing to difficulties encountered during extraction and purification procedures (Section II.11 and 12). The presence of 2-furylacetic acid and 4-oxo-pentanoic in the material may have been the result of shifts in equilibria during sample preparation, particularly in view of our earlier observation that Ni was associated with pectate and/or proteinaceous substances. Bearing this in mind, work on P. 'palawanensis' was discontinued at this point since it was felt that more effective extraction, isolation and purification methodology should be sought in order for the Ni complexes to be properly characterised. Such developmental work was beyond the scope of this research. Due to the large percentage of mass accounted for by Ni, citric acid and malic acid in D. gelonioides subsp. tuberculatum, attempts were made to further characterise the Ni complexes present.

IV.9 Gel Filtration Chromatography Studies of Mixtures of Nickel-Citrate, Nickel-Malate and Nickel-Citrate-Malate

IV.9.I. Introduction

Citric acid is widely distributed in fungi, higher plants and animals (Miller, 1973). It has several key physiological functions, some of which are dependent on its chelating ability. A number of structural studies have been carried out on Ni(II)-citrate complexes. These have been reviewed by Glusker (1980). Strouse et al. (1977) have suggested that some metals may activate the Krebs cycle enzyme aconitase which catalyses interconversions of citrate, cis-aconitate and iso-citrate. A possible role of citrate as a zinc-binding ligand in milk has also been postulated by Lönnerdal et al., (1980).

Over the pH range 4-7, several Ni(II)-citrate complexes have been characterised by potentiometric and spectrophotometric studies (Liddle, 1979; Still and Wikberg, 1980). Their distributions are presented in Fig. IV.5. The complexes fall into two categories. One contains Ni-citrate complexes in the mole ratio 1:1, while the other contains Ni-citrate complexes in the mole ratio 1:2. At a given pH, the percentage of total Ni complexed varies depending on the mole ratio indicating different properties. Comparable studies do not appear to have been carried out on Ni-malate and Ni-citrate-malate systems. It is therefore reasonable to expect complex solutions containing Ni, citrate and malate at different mole ratios to possess different properties. One such property could be chromatographic separation behaviour. With this in mind the elution behaviour of several synthethic Ni complex solutions was investigated including one which simulated the proportions of Ni, citrate and malate in *D. gelonioides* subsp. *tuberculatum*.

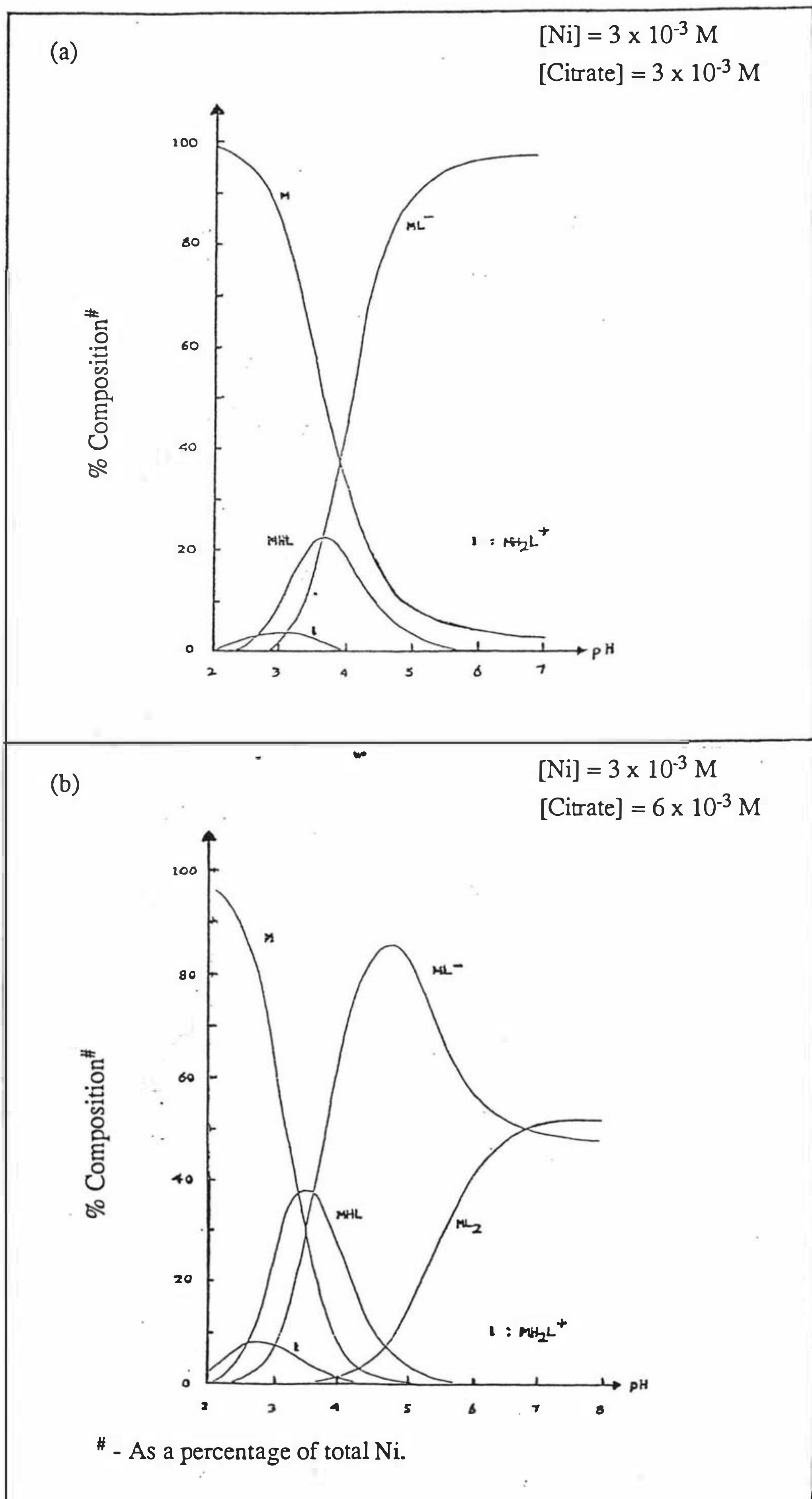


Fig. IV.5. Distribution curves for Ni(II)-citrate complexes in solution with Ni/citrate mole ratios of (a) 1:1 and (b) 1:2. (Source: Liddle, 1979).

IV.9.II. Methodology

The following samples were subjected to gel filtration chromatography using Sephadex G-10:-

i) Crude aqueous extract from D.gelonioides subsp. tuberculatum (6.14 mg Ni)

ii) Isolated Ni complexes from D. gelonioides subsp. tuberculatum obtained from two passes through Sephadex G-10 (7.40 mg Ni)

iii) Nickel citrate solution ; mole ratio 1:1 (3.05 mg Ni)

iv) Nickel malate solution ; mole ratio 1:1 (6.50 mg Ni)

v) Nickel citrate malate solution ; mole ratio 1:0.4:1 (6.44 mg Ni)

vi) Nickel citrate malate solution ; mole ratio 1:1:1 (6.50 mg Ni)

vii) Nickel citrate malate solution ; mole ratio 1:1:0.4 (6.83 mg Ni)

Nickel citrate and Ni malate solutions were prepared by the method of Baker et al. (1983). This is as follows :- a 150 cm^3 aliquot of 1M KOH (Analar) was slowly added to 50 cm^3 of 1M acid (Analar). Fifty cm^3 of 1M $NiCl_2.6H_2O$ (Analar) was then slowly added to the mixture and the pH was adjusted to 5 by further addition of 1M potassium hydroxide. Solutions containing Ni,citrate and malate in the mole ratios mentioned above were similarly prepared. All solutions were stored at 4° C. Gel filtration was carried out on Sephadex G-10 packed in a 2.5 x 67 cm glass column. The column was allowed to equilibrate by the continuous passage of 0.05 M ammonium acetate (Analar) for about 1 hr., after which the void volume was calculated at 78 cm^3 using Blue Dextran as in Section II.10.

Ammonium acetate (0.05 M,pH 6.9-7) was selected as eluant for three reasons:-

a) It demonstrates mild buffering capacity which could lead to a reduction in any dissociation that may occur during chromatographic separation.

b) The pH of the solution lies within the physiological range 5-8 ; this being the same range over which Ni-citrate complexes have been reported to be stable (Liddle, 1979).

c) Use of the solution as eluant in preliminary gel filtration studies on Ni-rich extracts from D.gelonioides subsp. tuberculatum resulted in improved resolution of the two peaks earlier obtained (Fig. II.1) when distilled deionised water was used as eluant.

No more than 2 cm^3 of sample was applied to the column on each occasion. The percentage of Ni that eluted ranged from 80 -100. Hexa-aquo Ni and solutions of Ni complexes spiked with Ni-rich extract from D. gelonioides subsp. tuberculatum were applied to the column. Elution profiles were constructed for each chromatographic separation. These are presented in Figs.IV.6.I and IV.6.II.

IV.9.III. Results and Discussion

Figure IV.6.I shows the elution patterns of Ni in the plant extracts with those of $[Ni(H_2O)_6]^{2+}$, $[Ni(citrate)]^-$, [Ni (malate)] and the mixture of Ni,citrate and malate in mole ratio 1:0.4:1. The two remaining three-component mixtures of mole ratio 1:1:1 and 1:1:0.4 exhibited identical behaviour to the 1:1 Ni-citrate complex. Small variations (± 5 %) in elution volumes of aquo Ni and Ni complexed by citrate and malate (or both) were probably due to minor changes in column loading and eluant flow rate. Whenever present, the citrate complex eluted ahead of the

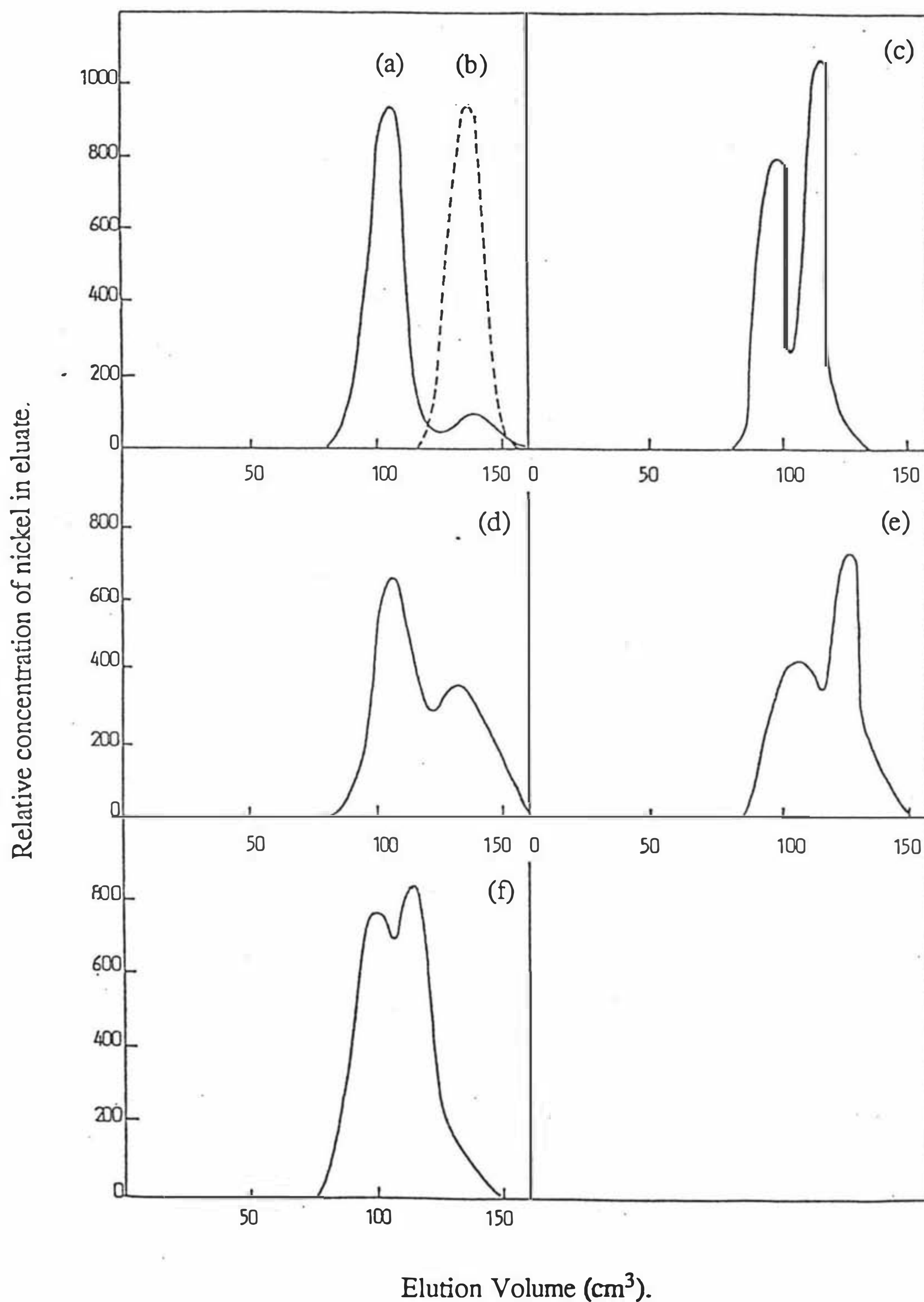


Fig. IV.6.I. Elution profiles for Ni-bearing species on Sephadex G-10 . (a) Ni-citrate (1:1) complex, (b) aquo-Ni complex, (c) Ni-malate (1:1) complex, (d) Ni-citrate-malate (1:0.4:1) mixture, (e) crude extract from D.gelonioides subsp. tuberculatum, (f) purified extract from D.gelonioides subsp. tuberculatum.

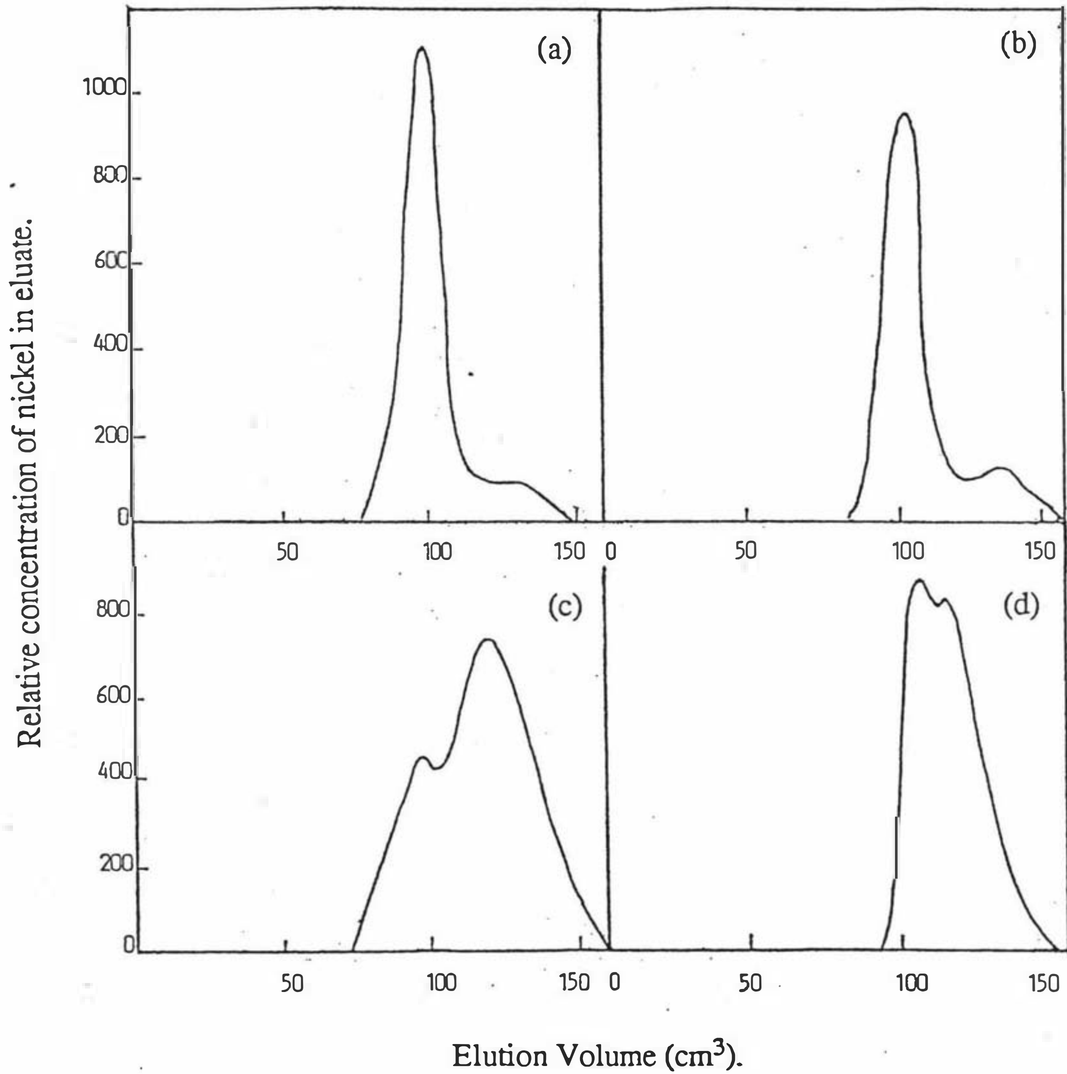


Fig. IV.6.II. Elution profiles for Ni-bearing species on Sephadex G-10.
(a) Ni-citrate-malate (1:1:1) mixture, (b) Ni-citrate-malate (1:1:0.4) mixture,
(c) Ni-citrate-malate (1:0.4:1) spiked with aquo-Ni complex,
(d) 1:1 mixture of Ni-citrate (1:1) complex and Ni-citrate (1:1) complex.

second peak ascribed to the aquo Ni complex. The aquo Ni peak was readily identified by comparing elution volumes of the second peak in chromatograms a, c, d, e and f (Figs. IV.6.I) with that of a sample of the complex which was passed through the column. Spiking the 1:0.4:1 mixture with aquo Ni was also useful (Fig. IV.6.IIc). The small aquo Ni peak that appeared when $[Ni (citrate)]^-$ eluted was probably due to a small amount of dissociation as the complex underwent dilution while passing through the column.

The complex $[Ni (citrate)]^-$ is only moderately stable ; the equilibrium

$$Ni^{2+} + Citrate^{3-} \text{<---->} [Ni (citrate)]^-$$

having a log K value of 5.47 ± 0.03 (Hedwig et al., 1980). Thus in a solution where total Ni and citrate concentrations are 0.003 M for example, at pH 6-7, about 96 % of the Ni is complexed as $[Ni (citrate)]^-$, and the remaining 4 % exists as aquo Ni. Previous studies have shown (Section III.14.II) that dissociation is reduced if higher total concentrations of Ni and citrate are maintained. The existence of a 1:2 Ni-citrate complex has been revealed from potentiometric and spectrophotometric studies (Hedwig et al., 1980). However, such a complex ($[Ni (citrate)_2]^{4-}$) with a log K value of 2.33 is only significant at pH 5-8 provided citrate is present in excess.

In D. gelonioides subsp. tuberculatum, there was insufficient citrate to complex all the Ni as the 1:1 complex. Thus the interaction between Ni and malate must be considered. For the equilibrium

$$Ni^{2+} + malate^{2-} \text{<---->} [Ni (malate)]$$

log K is 3.30 (Campi, 1963), indicating that the malate complex is 150 times less stable than the Ni-citrate complex. Lower stability renders the complex more susceptible to dissociation as the concentrations of Ni and malate are lowered. Only ~ 66 % of the Ni is complexed at pH 6-7 when the total Ni and malate concentrations are 0.003 M. This behaviour is illustrated in Fig. IV.6.Ic. When Ni-malate was applied to the Sephadex G-10 column, a substantial amount of Ni eluted as the aquo ion. The elution profile of the 1:0.4:1 Ni-citrate-malate mixture (Fig.IV.6.Id) illustrates the partial dissociation into aquo Ni predicted from the Ni-citrate and Ni-malate complexes. From

the preceding discussion of complex stabilities, the similarity in elution patterns of Ni-citrate-malate mixtures of mole ratios 1:1:1 and 1:1:0.4, and that of 1:1 Ni-citrate (Figs. IV.6.IIa and b ;IV.6.Ia) was also highly predictable. Based on similar reasoning , the purified Ni-rich material from W. monophylla containing Ni, citrate and malate in the mole ratio 1:0.2:1 would be expected to elute in a similar manner to the material from D. gelonioides subsp. tuberculatum . No separation between citrate and malate complexes was achieved on the column (Fig. IV.6.IId). Varying proportions of complexed Ni and aquo Ni were apparent in the crude extract derived from D.gelonioides subsp. tuberculatum (one pass through the column) and the purified extract (two further passes) (Fig. IV.6.Ie and f).

IV.9.IV. High Voltage Electrophoresis of Mixtures of Nickel-Citrate and Nickel-Citrate-Malate

The similarity of the behaviour of a) Ni-citrate-malate solutions of mole ratio 1:1:1 and 1:1:0.4, and Ni-citrate ; and b) Ni-rich extracts from D.gelonioides subsp. tuberculatum and the Ni-citrate-malate solution of mole ratio 1:0.4:1 during gel filtration chromatography, prompted an investigation into the electrophoretic behaviour of the solutions. The use of high voltage electrophoresis also provided an opportunity to compare the ionic nature of the mixtures prior to, and after, chromatographic separation.

High voltage electrophoresis was carried out on the following samples:

i) 1:1:0.4 Ni-citrate-malate (H)

ii) 1:1:1 Ni-citrate-malate (I)

iii) 1:0.4:1 Ni-citrate-malate (J)

iv) 1:1 Ni-citrate (K)

v) Crude aqueous Ni-rich extract from D. gelonioides subsp. tuberculatum (L)

vi) Purified Ni-rich extract from D. gelonioides subsp. tuberculatum prepared by dissolving purified green powder [Section IV.3.II] in distilled deionised water (M)

Electrophoretic separation was achieved at 1.5 kV and 30 mA for 75 min. using the phosphate buffer (pH 6.89) as in Section III.13. Hexa-aquo Ni (N) was used as the reference and Ni was visualised in the usual manner.

IV.9.V. Results and Discussion

The migratory patterns of the Ni species in the samples are shown in Fig. IV.7. Nickel-citrate (1:1), Ni-citrate-malate (1:1:0.4) and Ni-citrate-malate (1:1:1) contained both anionic and cationic nickel. The presence of anionic Ni in the three-component systems can be rationalized in terms of the stoichiometry between Ni and citrate. In this respect, these results are in agreement with those obtained from gel filtration chromatography. The experimental conditions appeared to favour dissociation of the anionic complex as evidenced by the substantial cathodic migration typical of cationic aquo nickel. Less dissociation was observed during gel filtration chromatography, probably on account of the milder experimental conditions that prevailed.

Only cationic Ni was detected in the 1:0.4:1 Ni-citrate-malate system and the sample from D. gelonioides subsp. tuberculatum. Kersten (1979) reported the complete dissociation of the 1:1 Ni-malate complex during high voltage electrophoresis. If the similarity in elution volumes of 1:1 Ni-citrate, 1:1 Ni-malate and 1:0.4:1 Ni-citrate-malate duriing gel filtration chromatography is considered along with the higher malate concentration in both the plant extract and simulated mixture, it is likely that the Ni-malate complex predominates in solution. This would explain the absence of anionic Ni in the electrophoretograms.

The results of high voltage electrophoresis also provided further evidence in support of the change in the ionic nature of Ni species during gel filtration chromatography. The electrophoretogram showed that all samples containing cationic and anionic Ni prior to chromatography contained only cationic Ni after chromatographic separation. Apparently, the

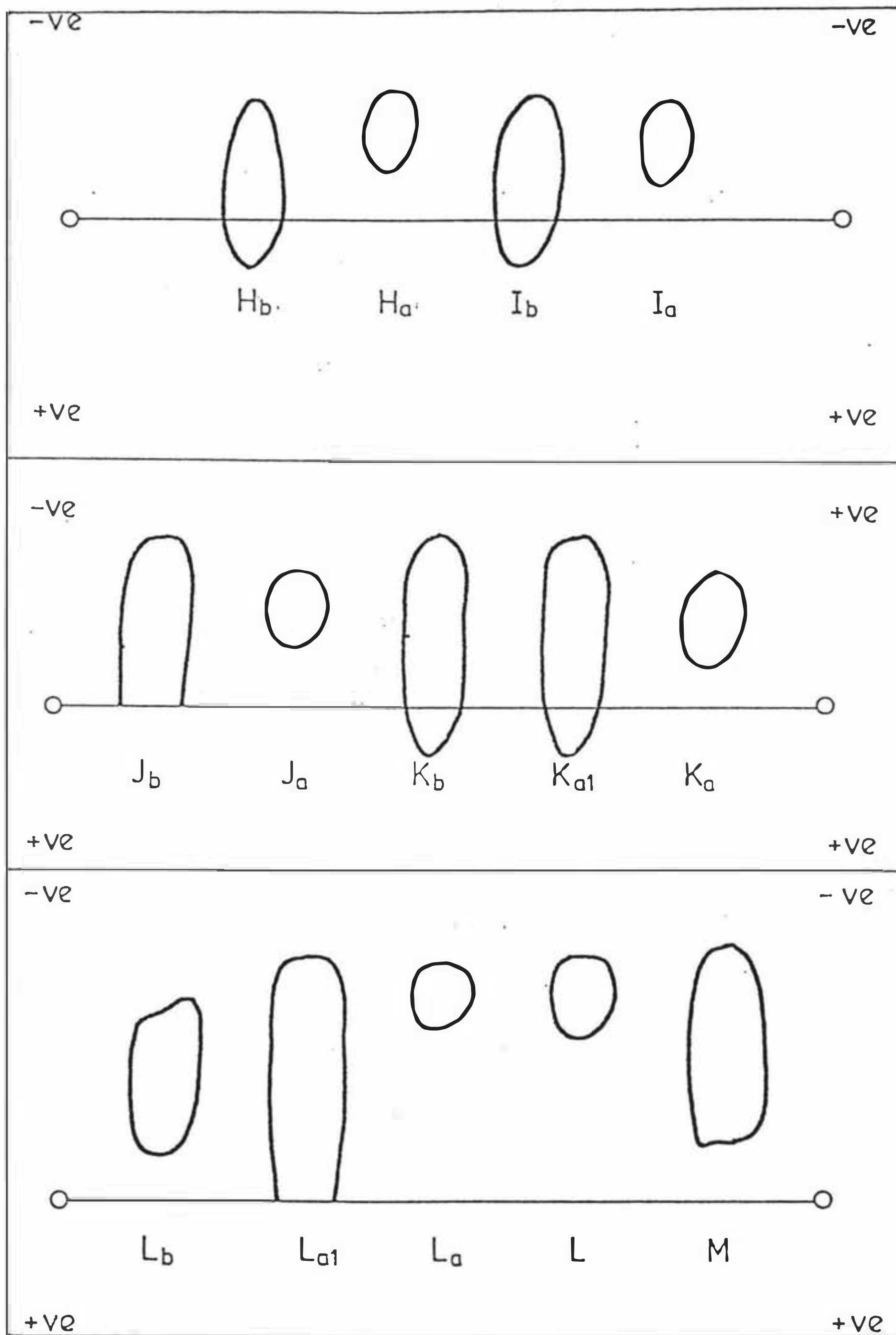


Fig. IV.7. Electrophoretogram of Ni species in (H) Ni-citrate-malate (1:1:0.4) mixture, (I) Ni-citrate-malate (1:1:1) mixture, (J) Ni-citrate-malate (1:0.4:1) mixture, (K) Ni-citrate (1:1) complex, (L) crude Ni-rich fraction from D. gelonioides subsp. tuberculatum, (M) purified Ni-rich fraction from D.gelonioides subsp. tuberculatum (b - before gel filtration chromatography, a - after gel filtration chromatography, 1 - eluted with distilled deionised water ; 0.5 M ammonium acetate was used as eluant in other cases

dilution of the complexes during passage through the Sephadex column, resulting in lowered complex concentration, was sufficient to facilitate complete dissociation during electrophoretic separation.

IV.9.VI. Conclusion

Clearly, the continuous change in the degree of dissociation of the Ni complexes as the solutions are diluted and concentrated during extraction and other separation procedures does not allow for a meaningful assignment of fractions of the plant Ni to particular species such as $[Ni\ (H_2O)_6]^{2+}$, $[Ni\ (citrate)]^-$ and [Ni (malate)]. This conclusion represents a departure from reports of previous workers in which reference is made to proportions of Ni complexed by citrate and malate (Lee, 1977; Kersten, 1979).

IV.10. Structural Elucidation of a Crystal Obtained from the Nickel-Citrate-Malate Solution of Mole Ratio 1:0.4:1

IV.10.I. Introduction

Using X-ray crystallography, Strouse et al. (1977) were the first to report the results of a crystal structural analysis of a Ni-citrate complex. Their complex, formed at pH9.2 had the formula $\{[N\ (CH_3)_4]_5\ [Ni_4\ (C_6H_4O_7)_3\ (OH)\ (H_2O)].\ 18\ H_2O\}_2$. Citrate was later shown to exist in the tetraionised state in this complex by Still and Wikberg (1980). At pH > 5-8, the tri-ionised state of citrate predominates. It may be recalled that Hedwig et al. (1980) were able to characterise several Ni-citrate complexes over the pH range 4-7 in which citrate existed in the tri-ionised state. However Still and Wikberg (1980) observed that when metal ions are bound to citrate, the hydroxyl proton can be removed at pH >8 thereby transforming citrate into a tetraionised state. Mastrapaolo et al. (1976) showed that for Cu(II), tetraionised citrate can be formed at pH 4, thus the pH at which the hydroxyl proton is removed appears to be metal dependent.

Examination of the structure of tri-ionised citrate shows that it can coordinate Ni in three possible ways (Fig. IV.8). One involves coordination between the hydroxyl group and the adjacent and terminal carboxyl groups (FIg. IV.8a). Another involves coordination between the hydroxyl group and the two terminal carboxyl groups (Fig. IV.8b). Coordination through the three carboxyl groups constitutes the third possibility (Fig. IV.8c). The relationship between the stability of a chelate complex to ring size and number is well documented (Rossotti, 1960; Douglas and McDaniel, 1965; Beck, 1970). Five-membered rings are usually the most stable, with stability decreasing as ring size increases. Stability also increases with the number of chelate rings. Based on these considerations, Liddle (1979) proposed that coordination through the hydroxyl group and the adjacent and terminal carboxyl groups (Fig. IV.8a) would provide the most stable structure. Despite the presence of two rings in each of the three structures, structure a is the only one containing a five-membered ring. The stability of the furan ring in 2-furylacetic acid will not readily facilitate coordination to nickel. The presence of only one carboxyl group and a stable keto group on 4-oxo-pentanoic acid also makes this acid unsuitable as a ligand. However tartaric acid, with its two (terminal) carboxyl groups and adjacent hydroxyl groups may show some potential as a Ni chelator.

In 1983, Baker et al. reported the structure of a Ni-citrate complex of formula $K_2[Ni (C_6H_5O_7) (H_2O)_2]_2.4 H_2O$. The complex crystallized from solution at pH 5 which is close to the physiological pH for plants. In the crystal, the complex exists as centrosymmetric dimers (Fig. IV.8d). Each citrate ion is coordinated to one Ni atom through its hydroxyl group and the adjacent and terminal carboxyl groups. This type of coordination is in agreement with the proposed coordination of Liddle (1979). The third carboxyl group is coordinated to the other Ni atom of the dimer. Two bound water molecules complete the octahedral coordination sphere of each Ni atom. The formation of a hydrogen bond between the hydroxyl proton and the carboxylate oxygen on the third carboxyl group indicates that citrate exists in the tri-ionised state in the complex. This corroborates the findings of Still and Wikberg (1980), and Hedwig et al. (1980) with respect to the ionic state of citrate at pH 5-6. The spaces between the Ni-citrate dimers are occupied by K^+ ions and molecules of water of crystallization. There appears tobe no record of similar studies carried out on the Ni-malate system.

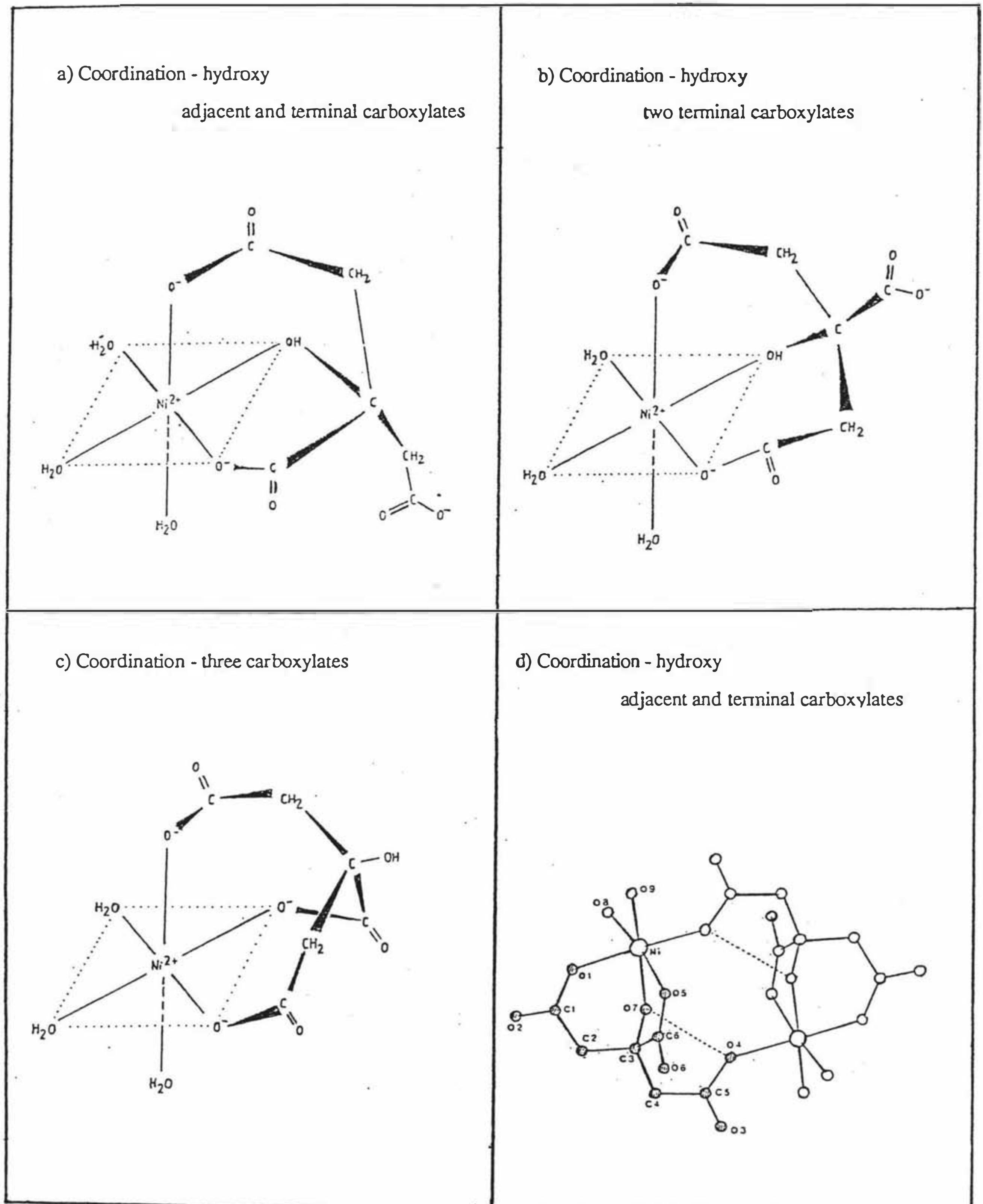


Fig. IV.8.(a)-(c) Possible structures of the anionic Ni(II)-citrato complex.(d) Structure of the anionic Ni(II)-citrato complex based on X-ray crystallography studies. Only non-hydrogen atoms are shown, and one citrate moiety is identified by cross-hatching. (Baker et al., 1983)

In view of the magnitude of structural detail obtained from X-ray crystallographic studies, the next step in the research was to attempt to procure a crystal from the synthetic solution of Ni, citrate and malate of mole ratio 1:0.4:1, and the purified Ni-rich solid from D. gelonioides subsp. tuberculatum. Microscopic examination of the green powder revealed the presence of aggregates of non-crystalline material. On account of the small amount of sample available, further refinement was not carried out.. Efforts were therefore dedicated to obtaining a crystal from the synthetic solution.

IV.10.II. Crystallisation of Nickel-Citrate-Malate Solution of Mole Ratio 1:0.4:1.

In order to obtain a crystal for structural elucidation, the two techniques of complete mixing and slow diffusion were applied using a range of Analar organic solvents. The solvents selected were ethanol, methanol, acetone, dimethyl sulphoxide, n-butanol, iso-butanol, n-propanol, iso-propanol, n-amyl alcohol and iso-amyl alcohol. All preparations were made in siliconized test-tubes. Siliconization was carried out in the following manner. The tubes were first rinsed with iso-propanol, then Serva Silicone solution (in propanol). Coating of the inner walls was then achieved by heating at 100-150° C in a drying oven for one hour.

For the complete mixing technique, 1 cm^3 of complex was mixed with solvent in a 11 x 100mm test-tube. The ratio of complex to solvent was varied from 1-2.5. Two sets of mixtures were prepared. One set was stored at room temperature while the other was refrigerated at 4° C. All tubes were stoppered with rubber bungs through which 2 mm holes had been drilled. Ethanol, methanol, acetone and dimethyl sulphoxide were used in these experiments as preliminary studies showed crystallization to be easily achieved in their presence. Crystals were observed after leaving the solutions overnight. They were removed from the mother liquor, placed on Whatman #1 filter paper, rinsed with the solvent used and allowed to air-dry overnight beneath an inverted funnel. Microscopic examination of the crystals showed them to be twinned in some instances and also highly aggregated. Such crystals were unsuitable for structure determination so the alternative technique was utilized.

In applying the slow diffusion technique, 3 cm^3 of solvent was carefully layered over 1 cm^3 of complex in a 5 x 125 mm test-tube. With the exception of ethanol, methanol, acetone and dimethyl sulphoxide improved crystallization was attempted using the solvents originally listed. Duplicate preparations were made. The test-tubes were sealed with Whatman Parafilm due to the unavailability of adequately-sized rubber bungs, and the temperature regime used earlier was applied. Crystals were observed two to three days after the experiment had begun. These were dried using the same procedure as before. When viewed under the microscope, there was a marked improvement in the quality of the crystals. However, some extraneous matter appeared to adhere to the crystals; these were considered impurities. Crystals formed using n-propanol at room temperature were the most suitable.

In an attempt to further improve the purity of the crystals, diffusion was slowed down even more by placing a layer of distilled deionised water (~ 0.5 cm^3) between the layers of complex and n-propanol. The complex solution, distilled deionised water and n-propanol were previously filtered using Millex GV 1 (3 mm) Millipore filters of pore size 0.22 mm. The rest of the experiment was carried out as before. After a period of 12 days, several single (untwinned) crystals were obtained at 4° C. One was mounted for structural determination by X-ray crystallography.

IV.10.III. X-ray Crystallography

X-ray crystallography provides information regarding the type and arrangement of atoms comprising a crystal, based on the interaction between X-rays and electrons in the atoms. Due to the short wavelength (high energy) of the radiation, this interaction usually results in scatter. When X-rays are diffracted (scattered) by an ordered environment such as a crystal lattice, both constructive and destructive interference occur owing to the fact that the distance between the scattering centres and the wavelength of the X-rays are of the same order of magnitude. In order for the X-rays to appear to be reflected from the crystal, only constructive interference must occur.

This criterion is mathematically described by Bragg's Law :-

$$n\lambda = 2d \sin \theta$$

where n = an integer ; λ = the wavelength of the X-ray ; d = interplanar distance of the crystal and θ = the angle of the incident X-rays. The angle of incidence must satisfy the condition $\sin \theta = n\lambda/2d$. At all other angles, destructive interference occurs. The intensity of the reflection varies depending on the distribution of electron density within the units making up the crystal.

Like the spectroscopic techniques described earlier, the application of X-ray diffraction in chemical analysis (X-ray crystallography) requires a source, a wavelength selection device, a sample holder, a radiation detector and output equipment. X-ray crystallography was first used to identify unknown substances during the earlier part of this century. Because the resulting X-ray diffraction pattern is dependent on the exact atomic arrangement in the sample, the technique provides unequivocal identification of the sample (Azároff, 1968). No two crystals give rise to the same diffraction pattern. The diffraction pattern is usually recorded by allowing the beam to strike and blacken a photographic film or by measuring the diffracted radiation with some form of counter (Dent-Glasser, 1977). Data analysis then furnishes information concerning the atoms present, their arrangement (unit cell) and the dimensions of the arrangement, all of which are used to characterise the crystal.

For this work, accurate cell dimensions were determined from the least squares analysis of the positions of 25 general reflections on an Enraf-Nonius CAD-4 Diffractometer using a crystal of approximate dimensions 0.5 x 0.4 x 0.2 mm and Mo-K_{α} radiation (λ = 0.7107 Å).

Table IV.3 Cell Dimensions of Crystals Derived from 1:0.4:1 Nickel-Citrate -Malate and 1:1 Nickel-Citrate Solutions

CELL PARAMETER	CRYSTAL SOURCE 1:0.4:1 Ni-Citrate-Malate Solution	1:1 Ni-Citrate # Solution
a	6.7132 Å	6.729 Å
b	9.0813 Å	9.100 Å
c	10.5955 Å	10.594 Å
α	94.863 °	94.862 °
β	100.803 °	100.762 °
γ	103.680 °	103.702 °

- Work of Baker et al. (1983)

IV.10.IV. Results and Discussion

The results in Table IV.3 show the crystal obtained from the 1:0.4:1 Ni-citrate-malate solution to possess more or less identical cell dimensions to those reported by Baker et al. (1983). Preferential binding of Ni to citrate over malate during crystallization despite the higher concentrations of the latter is therefore indicated. These findings are in agreement with those obtained from gel filtration chromatography. The lack of evidence for the existence of a mixed complex [Ni (citrate) (malate)]$^{3-}$ is predictable based on consideration of the low stability of the [Ni (malate)] complex (log K = 3.30) and the [Ni (citrate)$_2$]$^{4-}$ complex (log K = 2.33). Work of a similar nature does not appear to be documented for a Ni-malate crystal.

IV.11. Amino Acid Analysis

IV.11.I Introduction

Over the past 15 years, the consistent identification of citric acid, malic acid or malonic acid in isolated Ni-rich material from several Ni-hyperaccumulating plants has led to the conclusion that one or more of these acids must be involved in Ni chelation (Brooks and Baker, 1983). As a result, the possible role of amino acids as Ni chelators has not attracted much attention.

Nickel preferentially binds to ligands containing oxygen and nitrogen (Tiffin, 1972; Thompson and Tiffin, 1974; Nieboer and Richardson, 1980). Several amino acids are known to form complexes with divalent nickel (Table IV.4). Many of the acids involved are incorporated into plant protein. The stability constants of the complexes range from 5.12-8.67 (Sillén and Martell, 1964) compared with 3.30 and 5.47 for neutral Ni-malate and anionic Ni-citrate respectively. Provided the concentration of one of the amino acids exceeds that of citric or malic acid, Ni-amino acid complexation could be important as a means of rendering Ni non-toxic to the plant. Several Ni(III) complexes are now known with ligands that include peptides and other deprotonated amide groups (Nag and Chakravorty, 1980; Haines and McAuley 1981; Battacharya et al., 1986). The interest in this oxidation state has been greatly enhanced by the substantial documentation of biological evidence in support of its existence (Margerum and Anliker, 1988). Formerly, Ni(III) complexes were difficult to isolate and characterise owing to their highly transitory state in solution. However, the discovery of more kinetically stable complexes has facilitated some characterisation.

The investigation into the relationship between amino acid and Ni content in Ni-hyperaccumulating plants seems to be confined to only a few workers (Farago et al., 1975, 1980; Kelly et al., 1975; Lee, 1977; Bick et al., 1982). In 1975, Farago et al. suggested that Ni might be bound to an amino acid based on the results of paper chromatographic separation of ammonium oxalate extracts from the Ni-hyperaccumulating plant Hybanthus floribundus (Western Australia). Similar findings had not been reported for other Ni-hyperaccumulating plants.However, on account of Jursik's (1968) demonstration that Ni-amino acid complexes readily dissociate under

normal paper chromatographic conditions their conclusion was questionable. Additional work carried out on the same plant (Farago et al. (1980) yielded a result in agreement with that of Jursik (1968).Working on Hybanthus austrocaledonicus and Psychotria douarrei, both from New Caledonia, Kelly et al. (1975) also furnished evidence in support of Jursik's findings.Sequential use of electrophoresis and paper chromatography in a two dimensional manner to study the migratory behaviour of amino acids and Ni respectively, showed Ni to exist in a doubly-charged form and not in association with any amino acids. According to Lee (1977), no nitrogen was detected in purified Ni-rich material obtained from Sebertia acuminata (New Caledonia) and Hybanthus austrrocaledonicus when microanalysis was performed.

Bick et al. (1982) studied the relationship between aqueous acetone-soluble free amino acid composition and the total Ni content of leaves of wild and cultivated Alyssum bertolonii - a Ni-hyperaccumulating plant from Italy.They found that in leaves of wild plants, the total free amino acid - nitrogen content was directly related to the Ni content. With respect to the leaves of the cultivated plants, amino acid content appeared to be influenced by environmental factors. The amino acid studies of Farago et al. (1975), Kelly et al. (1975) and Bick et al. (1982) were all carried out on plant extracts that had not been subjected to purification by gel filtration chromatography. This was not the case with the work of Lee (1977).

The preceding research appears to represent the extent to which the relationship between amino acids and Ni in Ni-hyperaccumulating plants has been investigated. Another study is therefore justified. The number and concentrations of free amino acids present in extracts from four additional Ni-hyperaccumulating plants were determined. The aim of the study was to identify any amino acid of unusually high concentration, as this could be a Ni chelator.

Table IV.4 Stability Constants of Amino Acid Complexes of Nickel (Source: Sillén Martell, 1964).

AMINO ACID	IONIC STRENGTH (I)	LOG K_1*	LOG β_2*
Glycine	0	6.18	11.13
Alanine	0	5.83	10.48
Leucine	0.2 (20 °C)	5.45	9.71
Phenylalanine	0	5.55	10.22
Aspartic Acid	0.1	7.16	12.40
Glutamic Acid	0.1	5.60	9.76
Tyrosine	0.1	5.10	9.46
Serine	0.1	5.45	9.96
Asparagine	0.1	5.68	10.23
Glutamine	3.0	5.56	10.23
Cysteine	0.1	9.82	20.07
Methionine	0.1	5.19	9.84
Histidine	0.1	8.67	15.54
Tryptophan	0.37 (20 °C)	5.68	10.94
Proline	0.1	5.95	10.90

* - See Table III.8 for an explanation of these terms.

IV.11.II. Sample Preparation

Aqueous extracts from leaves of field specimens of D.gelonioides subsp. tuberculatum and W.monophylla, cultivated Alyssum troodii (Turkey) were freed of high molar mass, low polarity compounds by treatment with chloroform/butanol reagent. The aqueous/ethanolic extract from leaves of field specimens of P.'palawanensis' was similarly treated. Three different samples of A.troodii were used in the experiment. These were obtained from plants growing in artificial soils consisting of peat, pumice and fertiliser to which nickel was added (Analar nickel nitrate hexahydrate). Two samples were obtained from plants growing in soils containing Ni at the level of 370 and 1111 $\mu g\ g^{-1}$. Plants growing in artificial soil to which no Ni was added gave rise to the third sample. The plants which were germinated from wild seed were required for pot trial experiments undertaken to study Ni uptake characteristics (Part 2 of this thesis).

The purified extracts were then concentrated in vacuo at 33-35 °C. Nickel concentrations were determined by AAS. None of the samples was subjected to gel filtration chromatography because preliminary studies on a sample from D. gelonioides subsp. tuberculatum treated in this manner showed a low content of amino acids (Fig. IV.9b).

IV.11.III. Instrumentation

Amino acid analysis is commonly carried out by automated cation-exchange chromatography. Essentially, an automated amino acid analyser consists of an integrated system for pumping a series of appropriate buffers onto a cation-exchange column. Many amino acids are detected by the blue/purple colour formed upon reaction with ninhydrin. The acids are sequentially eluted from the column and mixed with independently pumped ninhydrin. The amino acid/ninhydrin mixture then passes through a heating coil for colour development. A dual wavelength recording spectrophotometer (or two separate spectrophotometers designed to measure absorbance at 570 and 440 nm) is commonly used for detection purposes. Most ninhydrin-treated amino acids that exhibit purple colouration show a response at 570nm. Proline, hydroxyproline and

similar amino acids which exhibit yellow colouration in the presence of ninhydrin can be detected at the shorter wavelength. Fluorescence detection is also popular. Elution profiles are obtained using suitable output equipment (Rosenthal, 1982).

In this study, amino acid analysis was performed with a Beckman 119 BL Automated Amino Acid Analyser equipped with W2 (sulphonated polystyrene) resin and a Fluorescence Ninhydrin Detection System. Elution was carried out with sodium citrate buffers of pH 3.25, 4.25 and 6.45, of ionic strength 0.20, 0.40 and 0.75 respectively. An amino acid multistandard was used to identify and quantify the amino acids present. With the exception of glucosamine (36.2 nmol / 100 μL), the concentration of amino acids in the standard was 25 nmol / 100 μL. Injection volume of the standard was 100 μL. For the samples, this varied from 25-100 μL depending on the magnitude of the signals obtained during preliminary runs.

IV.11.V. Results and Discussion

The results of the analyses are presented in Fig. IV.9. All of the amino acids in the multistandard were detected in the plant extracts except glucosamine. A small number of unidentified amino acids was also present. Proline appeared to be the most abundant amino acid in all four plants. Bick et al. (1982) also reported a high abundance of proline in A. bertolonii. Perhaps this observation is universal among Ni-hyperaccumulating plants. Large amounts of proline have been reported in roots of the copper-tolerant plant Armeria maritima, but none was reported in roots of non-tolerant plants (Farago and Mullen, 1979). High levels of proline have also been found in the tissues of sodium chloride-tolerant plants (Stewart and Lee, 1974). It is believed that elevated levels of the amino acid provide a mechanism for equalising the water potential of the vacuole and cytoplasm, where the sodium chloride is preferentially accumulated (Storey and Wyn Jones, 1975).The concentration of threonine appeared to be high in A.troodii.

It is interesting to note that leaves of cultivated A.troodii contained a higher total amino acid concentration than leaves of the field specimens of Ni-hyperaccumulating plants irrespective of the substrate Ni concentration (Table IV.5.I). This behaviour could be attributed to

the fertiliser in the artificial soils. This may be considered evidence in support of the conclusion of Bick et al. (1982), that amino caid content is influenced by environmental factors. Serpentine which support Ni-hyperaccumulating plants in the natural environment are nutrient poor. Factors such as temperature and water regime should also be considered since A. troodii plants were cultivated in a glasshouse. Farago et al. (1980) observed differences in amino acid profiles of specimens of Hybanthus floribundus grown in heavily mineralised and less mineralised areas in Queensland, Australia. No cysteine was detected in the Philippine Ni-hyperaccumulating plants.

If it is assumed that amino acids can only be of significance as Ni chelators when the amino acid : Ni mole ratio > 0.1, then the minimum molar concentration of amino acid required to satisfy this criterion can easily be calculated. Taking the average molar mass of amino acids to be about 140, the minimum concentrations are 635, 1917 and 3790 nmol cm^{-3} for W.monophylla, P. 'palawanensis' and D. gelonioides subsp. tuberculatum respectively. In W.monophylla and P.'palawanensis', the high concentration of proline, yielding proline/Ni mole ratios of 1.2 and 0.4 respectively, indicates possible involvement of this amino acid in Ni chelation. This is not the case for D.gelonioides subsp. tuberculatum which has a proline/Ni mole ratio of 0.01. In W. monophylla, possible ligands are aspartic acid (1728 nmol cm^{-3}), threonine (1236 nmol cm^{-3}), and glutamine (7980 nmol cm^{-3}) [Table IV.5.I]. Serine and glutamine have been shown to co-elute (Bick et al., 1982). A similar observation was made during these analyses (Fig. IV.9c,d, e, f and g), thus the validity of this proposal in respect of glutamine could be tenuous.

While the approach to Ni chelation by amino acids in Ni-hyperaccumulating plants based on mole ratio may be applicable to wild plants, the applicability to cultivated plants may not be valid. Examination of the total amino acid content of leaves from A. troodii plants reveals these tobe of the same order of magnitude despite differences in substrate Ni concentration, and the increase in plant Ni from 0-34.3 μmol g^{-1} as substrate Ni increased (Table IV.5.II). A similar observation was made for proline thereby discrediting it as a possible ligand. Infact, proline concentration was highest in plants obtained from soil to which no Ni was added. Slight increases in the concentrations of cysteine, alanine, valine, histidine and arginine were observed as the Ni concentrations of the leaves increased. A small decrease in leucine content was observed.

Fig. IV. 9. Amino acid elution profiles.

(a) Multistandard

Aqueous extracts from the following:

(b) D.gelonioides subsp. tuberculatum passed through Sephadex G-10 column,

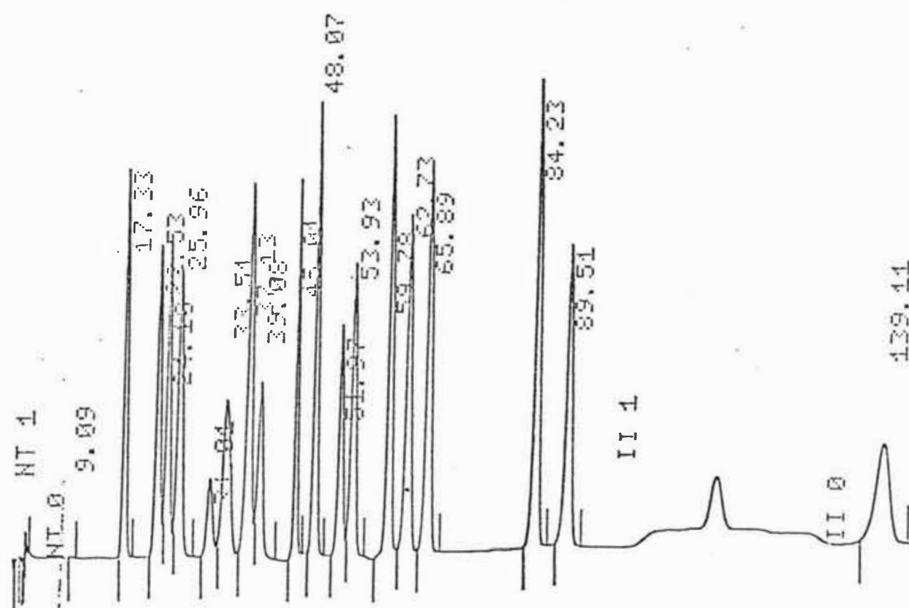
(c) D.gelonioides subsp. tuberculatum not passed through Sephadex G-10 column,

(d)-(f) A.troodii plants cultivated in soil containing 0, 370 and 1111 $\mu g\ g^{-1}$ Ni respectively

(g) P. 'palawanensis',

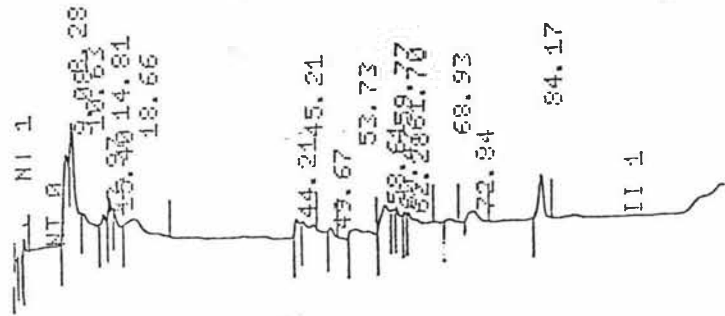
(h) W.monophylla.

(a)



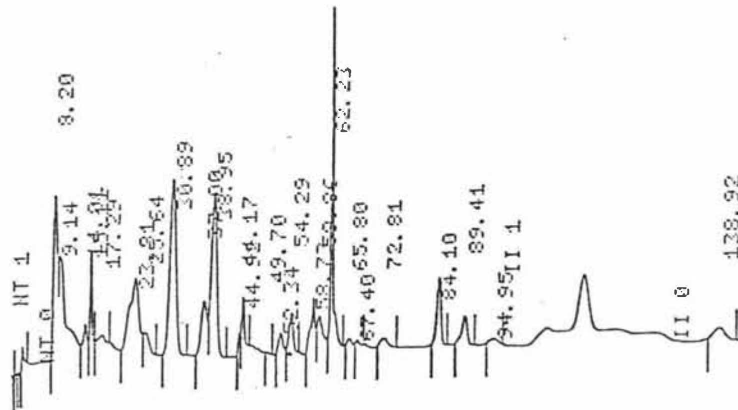
NAME	NMOLES	RT	AREA	BC	RF
ASP	25.	17.33	2806019	01	112240.76
THR	25.	22.53	3028161	02	121126.44
SER	25.	24.1	3055705	02	122228.2
GLU	25.	25.96	3428436	03	137137.44
PRO	25.	31.01	1011268	02	40450.72
CYS	25.	33.51	2841852	02	113674.08
GLY	25.	37.13	4701889	02	188075.56
ALA	25.	39.08	2419258	03	96770.32
VAL	25.	45.01	2383611	01	95344.44
MET	25.	48.07	3854644	01	154185.76
ILE	25.	51.97	2406605	02	96264.2
LEU	25.	53.93	3356511	03	134260.44
TYR	25.	59.78	4571122	02	182844.88
PHE	25.	62.73	4139250	02	165570.
GLUCN	36.2	65.89	4749842	03	131211.105
HIS	25.	84.23	5497387	01	219895.48
LYS	25.	89.51	4180736	01	167229.44
ARG	25.	139.11	3178376	01	127135.04
TOTALS	461.2		61610672		

(b)



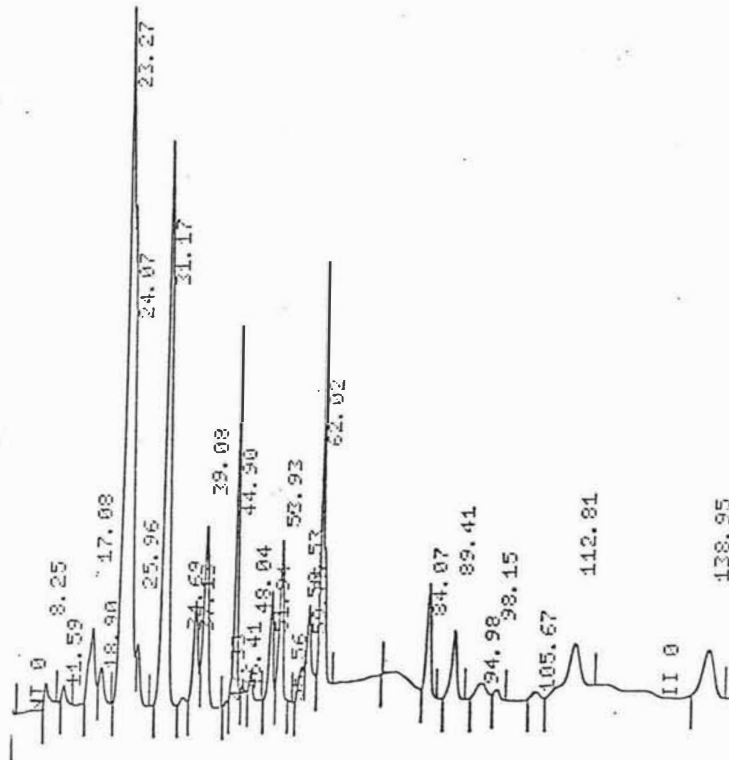
NAME	NMOLES	RT	AREA	BC	RF
1	0.	1.94	95182	03	
2	0.	8.28	892592	02	
3	0.	9.08	1509850	02	
4	0.	10.63	814647	02	
5	0.	13.97	378829	02	
6	0.	14.81	342733	02	
7	0.	15.4	389633	02	
ASP	9.057	18.66	1016603	03	112240.76
9	0.	44.21	167073	02	
VAL	2.539	45.21	242020	03	95344.44
LEU	2.202	53.73	295674	01	134260.44
12	0.	58.61	493394	02	
TYR	1.083	59.77	198021	02	182844.88
14	0.	60.47	229990	02	
15	0.	61.7	119184	02	
PHE	1.314	62.28	217511	03	165570.
17	0.	72.84	254742	01	
HIS	2.316	84.17	509415	01	219895.48
TOTALS	18.511		8167093		

(c)



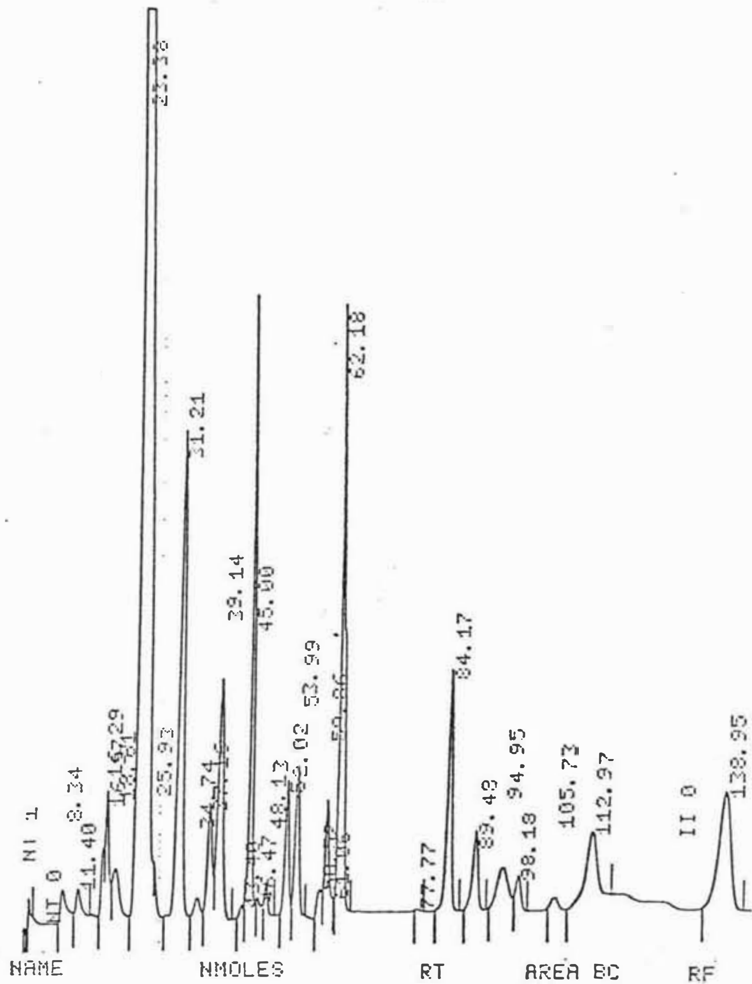
NAME	NMOLES	RT	AREA	BC	RF
1	0.	1.98	177767	03	
2	0.	8.2	2264715	02	
3	0.	9.14	2504664	02	
4	0.	14.01	242040	02	
5	0.	15.11	570518	03	
ASP	1.376	17.29	151343	01	109973.6
SER	19.779	23.81	2355864	02	119110.16
GLU	3.52	25.64	468733	03	133173.52
PRO	85.092	30.89	3430010	01	40309.28
GLY	5.946	37.	1080467	02	181709.24
ALA	37.387	38.95	3481474	03	93119.36
12	0.	44.17	133488	02	
VAL	5.983	44.92	555456	03	92850.24
ILE	3.433	52.34	327290	02	95335.84
LEU	4.635	54.29	613091	03	132272.48
16	0.	58.73	805930	02	
TYR	4.393	59.86	775170	02	176446.36
PHE	16.945	62.23	2694370	03	159905.72
GLUCN	3.0	65.8	112306	03	
20	0.	72.81	252938	01	
HIS	5.065	84.1	1040382	01	205416.32
LYS	3.582	89.41	588663	01	164314.08
23	4.0	94.95	165860	01	
ARG	3.278	138.92	423868	01	129307.96
TOTALS	200.414		25216412		

(d)



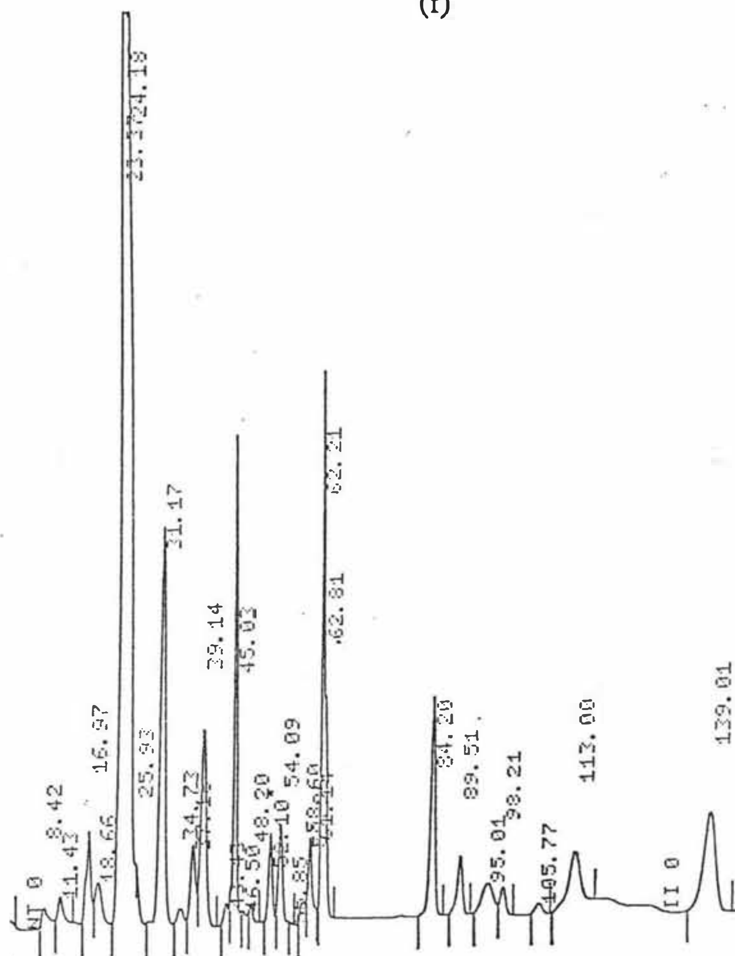
NAME	NMOLES	RT	AREA	BC	RF
1	0.	1.91	122456	03	
2	0.	8.25	368970	01	
3	0.	11.59	224784	01	
ASP	12.387	17.08	1579582	02	127520.36
5	0.	18.9	684538	03	
THR	88.017	23.27	11950424	02	135773.4
SER	45.454	24.07	6242025	02	137328.04
GLU	6.056	25.96	935654	03	154505.52
PRO	235.518	31.17	9710681	08	41231.12
CYS	1.268	34.69	153332	06	120937.2
GLY	8.68	37.13	1752244	06	201871.96
ALA	29.222	39.08	3193413	07	109278.92
VAL	34.515	44.9	3621168	02	104916.16
14	0.	46.41	301380	02	
MET	2.512	48.04	414064	03	164806.54
ILE	14.38	51.94	1530943	02	106464.16
LEU	16.718	53.93	2416846	03	144564.44
18	0.	58.53	608976	02	
TYR	8.368	59.75	1616664	02	193206.44
PHE	28.36	62.02	4894026	03	172570.36
HIS	7.778	84.07	1751321	01	225167.16
LYS	7.727	89.41	1393690	01	180354.24
23	0.	94.98	674021	02	
24	0.	98.15	248476	03	
25	0.	105.67	184942	02	
26	0.	112.81	2024473	03	
ARG	13.234	138.95	1843102	01	139267.6
TOTALS	560.194		60442195		

(e)



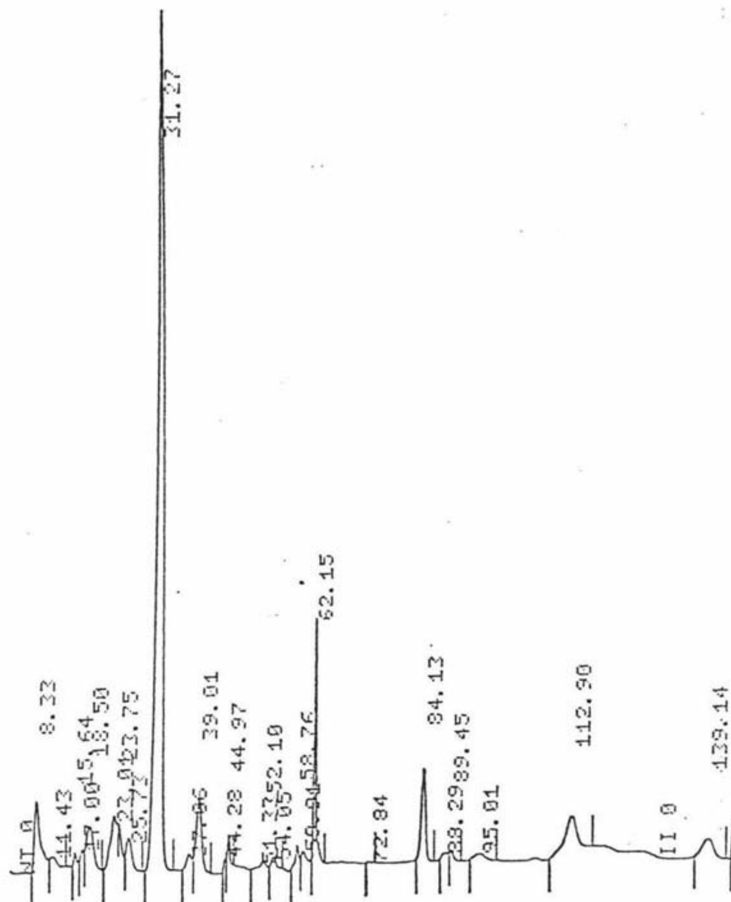
NAME	NMOLES	RT	AREA	BC	RF
1	0.	1.91	162128	03	
2	0.	8.34	690518	02	
3	0.	11.4	502043	03	
4	0.	16.29	601663	02	
ASP	12.995	16.97	1657099	02	127520.36
6	0.	18.61	1144329	02	
THR	295.153	23.38	40073934	02	135773.4
GLU	3.825	25.93	590939	02	154505.52
PRO	212.231	31.21	8750527	02	41231.12
CYS	3.861	34.74	466972	02	120937.2
GLY	9.911	37.16	2000757	02	201871.96
ALA	41.152	39.14	4496999	03	109278.92
13	0.	43.4	163319	02	
VAL	55.762	45.	5850381	02	104916.16
15	0.	46.47	316334	02	
MET	2.537	48.13	417990	03	164806.64
ILE	17.14	52.02	1824797	02	106464.16
LEU	15.066	53.99	2178031	03	144564.44
19	0.	58.52	478454	02	
TYR	9.458	59.86	1827464	02	193206.44
21	0.	61.06	83500	02	
PHE	45.303	62.18	7817914	03	172570.36
HIS	17.122	84.17	3855278	01	225167.16
LYS	8.838	89.43	1593995	01	180354.24
25	0.	94.95	1740167	02	
26	0.	98.18	800731	03	
27	0.	105.73	315050	02	
28	0.	112.97	2730242	03	
ARG	34.323	138.95	4780022	01	139267.6
TOTALS	784.677		97912127		

(f)



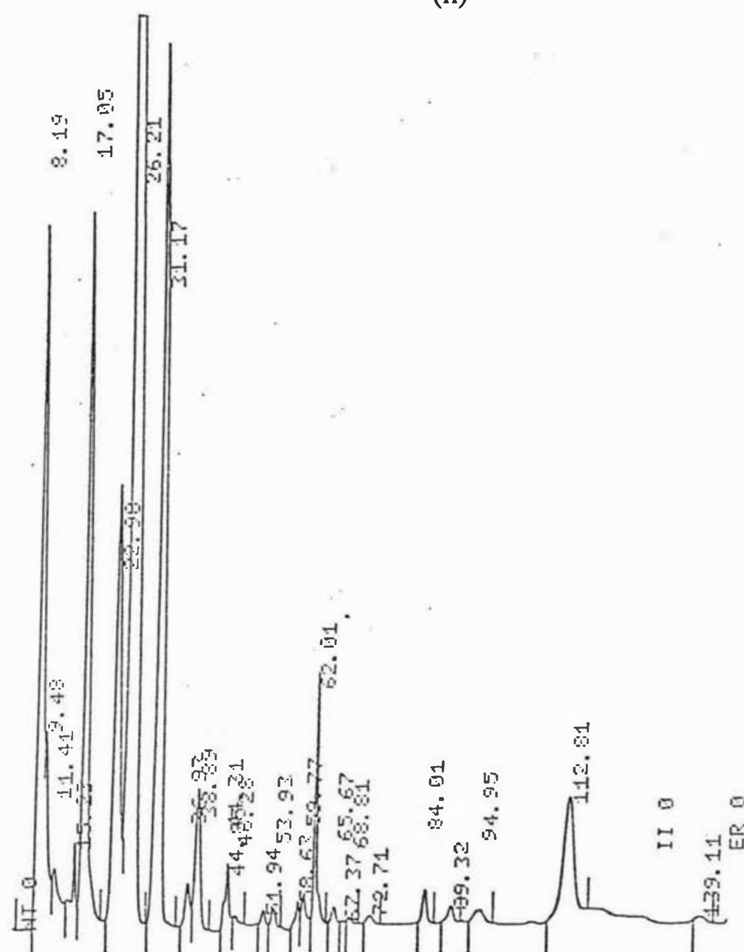
NAME	NMOLES	RT	AREA	BC	RF
1	0.	1.94	202470	03	
2	0.	8.42	463092	02	
3	0.	11.43	521081	03	
ASP	11.59	16.97	1477990	02	127520.36
5	0.	18.66	970348	02	
THR	148.843	23.37	20208933	02	135773.4
SER	93.849	24.18	12888077	02	137328.04
GLU	4.877	25.93	753447	02	154505.52
PRO	174.479	31.17	7193960	02	41231.12
CYS	3.339	34.73	403833	02	120937.2
GLY	7.077	37.16	1428678	02	201871.96
ALA	34.174	39.14	3734475	03	109278.92
13	0.	43.43	299032	02	
VAL	44.479	45.03	4666579	02	104916.16
15	0.	46.5	240146	02	
MET	1.623	48.2	267454	03	164306.64
ILE	11.744	52.1	1250314	02	106464.16
LEU	10.376	54.09	1500045	03	144564.44
19	0.	58.6.	420514	02	
TYR	6.834	59.96	1320418	02	193206.44
21	0.	62.21	4801818	02	
PHE	11.483	62.81	1981536	03	172570.36
HIS	15.488	84.2	3487538	01	225167.16
LYS	6.538	89.51	1179050	01	180354.24
25	0.	95.01	1294017	02	
26	0.	98.21	652049	03	
27	0.	105.77	255257	01	
28	0.	113.	2216661	01	
ARG	29.269	139.01	4076313	01	139267.6
TOTALS	616.062		80155125		

(g)



NAME	NMOLES	RT	AREA	BC	RF
1	0.	1.95	159408	03	
2	0.	8.33	1521676	02	
3	0.	11.43	312199	03	
4	0.	15.64	145039	02	
ASP	1.636	17.	208587	02	127520.36
6	0.	18.5	1056659	03	
THR	8.646	23.01	1173902	02	135773.4
SER	4.587	23.75	629939	02	137328.04
GLU	4.304	25.73	664991	03	154505.52
PRO	403.773	31.27	16648028	01	41231.12
GLY	1.536	37.06	310014	02	201871.96
ALA	13.902	39.01	1519151	03	109278.92
13	0.	44.28	83741	02	
VAL	3.27	44.97	343082	03	104916.16
ILE	1.322	52.1	140800	02	106464.16
LEU	1.365	54.05	197259	03	144564.44
17	0.	58.76	310656	02	
TYR	1.932	59.91	373400	02	193206.44
PHE	11.717	62.15	2021901	03	172570.36
HIS	6.434	84.13	1448751	01	225167.16
21	0.	88.29	159354	02	
LYS	0.966	89.45	174274	03	180354.24
23	0.	95.01	237484	01	
24	0.	112.9	1575588	01	
ARG	5.182	139.14	721738	01	139267.6
TOTALS	478.572		32137621		

(h)



NAME	NMOLES	RT	AREA	BC	RF
1	0.	1.89	411471	03	
2	0.	8.19	10718080	02	
3	0.	9.48	2926306	02	
4	0.	11.41	1394690	02	
5	0.	15.23	1258927	02	
ASP	86.407	17.05	11018611	03	127520.36
THR	61.822	22.98	8393848	02	135773.4
GLU	398.611	26.21	61587548	03	154505.52
PRO	389.864	31.17	16074540	01	41231.12
GLY	3.706	36.93	748119	02	201871.96
ALA	23.761	38.89	2596553	03	109278.92
12	0.	44.31	191056	02	
VAL	7.048	44.85	739477	02	104916.16
MET	1.229	46.28	202620	03	164806.64
ILE	1.83	51.94	194830	02	106464.16
LEU	1.863	53.93	269185	03	144564.44
17	0.	58.63	378726	02	
TYR	3.184	59.77	615199	02	193206.44
PHE	13.007	62.01	2244611	03	172570.36
GLUCN	0.	65.67	257919	02	
21	0.	68.81	104467	03	
22	0.	72.71	163503	01	
HIS	2.357	84.01	530702	01	225167.16
LYS	1.744	89.32	314532	01	180354.24
25	0.	94.95	470364	01	
26	0.	112.81	4227327	01	
ARG	1.312	139.11	182839	01	139267.6
TOTALS	997.745		128216050		

Table IV.5.I Amino Acid Content (nmol cm^{-3}) of Cultivated Alyssum troodii, and Field Specimens of Walsura monophylla, Phyllanthus 'palawanensis' and Dichapetalum gelonioides subsp. tuberculatum.

AMINO ACID	CONCENTRATION (nmol cm^{-3})					
	A. troodii			W. monophylla	P. 'palawanensis'	D. gelonioides subsp. tuberculatum
	Control	370§	1111§			
Aspartic Acid	496	520	464	1728	32	14
Threonine	3520	11800	5960	1236	172	---
Serine	1820	---	3752	---	92	198
Glutamine	244	152	196	7980	86	35
Proline	9440	8480	7000	7780	8080	850
Cysteine	52	156	132	---	---	---
Glycine	348	396	284	74	32	59
Alanine	1168	1648	1368	476	278	37
Valine	1380	2232	1780	140	66	59
Methionine	100	100	64	24	---	---
Isoleucine	576	696	468	36	26	34
Leucine	668	604	416	38	28	---
Tyrosine	336	380	272	62	38	44
Phenylalanine	1136	1812	460	260	234	169
Histidine	312	684	620	46	128	51
Lysine	308	352	260	34	50	36
Arginine	532	1372	1172	26	104	33
TOTAL	22436	31384	24668	19940	9446	1619
PLANT NICKEL CONC. (nmol cm^{-3})	0	1910	5043	6354	19165	37900

§ - Concentration of nickel in soil (μg g^{-1})

Table IV.5.II Amino Acid Content (µmol g^{-1}) of Cultivated Alyssum troodii, and Field Specimens of Walsura monophylla, Phyllanthus 'palawanensis' and Dichapetalum gelonioides subsp. tuberculatum

AMINO ACID	CONCENTRATION (µmol g^{-1})					
	A. troodii			W. monophylla	P. lamprophyllus	D.gelonioides subsp. tuberculautm
	Control	370§	1111§			
Aspartic Acid	2.92	2.57	3.15	6.45	0.06	0.05
Threonine	20.8	58.4	40.5	4.61	0.30	---
Serine	10.7	---	25.5	---	0.16	0.70
Glutamine	1.44	0.75	1.33	29.8	0.15	0.12
Proline	55.7	42.0	47.0	29.0	14.3	3.01
Cysteine	0.31	0.77	0.90	---	---	---
Glysine	2.06	1.96	1.93	0.28	0.06	0.21
Alanine	6.90	8.16	9.30	1.78	0.49	0.13
Valine	8.15	11.0	12.0	0.52	0.12	---
Methionine	0.59	0.50	0.43	0.09	---	---
Isoleucine	3.40	3.45	3.18	0.13	0.05	0.12
Leucine	3.94	2.99	2.83	0.14	0.05	---
Tyrosine	1.98	1.88	1.85	0.23	0.07	0.15
Phenylalanine	6.71	8.97	8.13	0.97	0.41	0.60
Histidine	1.84	3.39	4.21	0.17	0.23	0.18
Lysine	1.82	1.74	1.77	0.13	0.09	0.13
Arginine	3.14	6.79	7.97	0.10	0.18	0.12
TOTAL	132	155	168	74.4	16.7	5.73
PLANT NICKEL CONC. (µmol g^{-1})	0	9.38	34.3	23.7	33.9	134

§ - Concentration of nickel in soil (µg g^{-1}).

The comparatively low amino acid content of D. gelonioides subsp. tuberculatum could indicate that the high concentrations of malic and citric acids found in association with Ni in the purified extract were not artifactual. It may be recalled that Ni, malic acid and citric acid accounted for 85 % of the sample analysed, with Mg, K and Ca accounting for 11%. Less than 26 % of the mass of similar material was accounted for in W. monophylla and P. 'palawanensis'. T
he notably high concentration of aspartic acid, threonine and glutamine in W.monophylla therefore warrant some consideration. In the absence of significant quantities of amino acids, quantification of tartaric acid and perhaps 2-furylacetic and 4-oxo-pentanoic acids in P. 'palawanensis' would be worthwhile. An investigation into the role of pectate substances in Ni complexation in this plant is also advocated.

CHAPTER V

Determination of Fluoride

in

Dichapetalum gelonioides subsp. *tuberculatum*

V.1. Introduction

Fluoride is a natural component of rocks and soils, and is present in plant and animal tissues. The total natural fluoride content of topsoil ranges from 20-700 $\mu g\ g^{-1}$ (Robinson and Edgington, 1946; Hall and Cain, 1972; Eysinga, 1974; Manley et al., 1975).Based on the level of natural fluoride in foliage, Robinson (1978) grouped plants into three categories as follows:-

a) Non-accumulators
b) Accumulators of toxic organic fluoride
c) Accumulators of inorganic fluoride

Plants containing fluoride in the range 1-20 $\mu g\ g^{-1}$ on a dry weight basis are considered non-accumulators (Koster, 1972; McClenahen, 1976: Webber, 1977). Among the plants reported to accumulate toxic organic fluoride are three species of Dichapetalum. Dichapetalum cymosum, D. stuhlmannii and D. toxicarium, all from Africa, have been shown to contain >100 $\mu g\ g^{-1}$ fluoride (Hall, 1972). Accumulators of inorganic fluoride include Camellia japonica and C. sinensis (tea) (Zimmerman et al., 1957).

Monofluoroacetic acid was the first naturally occurring fluoro-organic compound to be isolated (Marais, 1944). It was obtained from D. cymosum which is believed to cause sporadic poisoning of cattle in South Africa. In this plant, monofluoroacetate is reported to comprise up to 50 $\mu g\ g^{-1}$ of the dry weight (Hewitt and Smith, 1975). Monofluoroacetate has also been identified in at least three other toxic plants, none of which belongs to the genus Dichapetalum (Oelrichs and McEwan, 1961; de Oliveira, 1963; McEwan, 1964). Peters et al. (1960) have isolated ω-fluoro-oleic and ω-fluoropalmitic acids from seeds of D.toxicarium.

In all these plants, the toxic principle was identified as monofluoroacetate. According to Peters et al. (1953), ingested monofluoroacetate is converted into monofluorocitrate via Krebs cycle. Monofluorocitrate blocks the cycle by competitively inhibiting the enzyme aconitase which catalyses the conversion of citrate to isocitrate. As a result, there is an accumulation of

monofluorocitrate in tissues, notably the heart and kidney. Monofluorocitrate does not appear to acumulate in the toxic plants since their Krebs cycles remain unaffected. The means by which the plants avoid damage are discussed by Louw et al.(1970), Eloff and von Sydow (1971) and Hall (1972).

The uptake of fluoride by plants is related to soluble or available fluoride, which is influenced by factors such as pH, and the levels of phosphate, calcium, clay and organic matter (Brewer, 1965; Eysinga, 1972; Cooke et al., 1976; McClenahen, 1976). According to Bertrand and de Wolf (1970), available fluoride in Belgian soils ranged from 0.1-8 $\mu g\ g^{-1}$. In English soils, only 0.2 $\mu g\ g^{-1}$ fluoride was shown to be available (Larsen and Widdowson, 1971). Fluoride appears to be taken up by the plant in both inorganic and organic form (Hall and Cain, 1972) and accumulates in the leaves, particularly in the apical tissue (Robinson, 1978).

V.2. Aim and Rationale

In view of the accumulation of fluoride by *Dichapetalum cymosum*, *D. toxicarium* and *D.stuhlmannii*, *D.gelonioides* subsp. *tuberculatum* a Ni-hyperaccumulating plant (26000 $\mu g g^{-1}$ Ni) from the Philippines was analysed for fluoride to ascertain its fluoride status. An elevated fluoride level would be demonstrative of the ability of this plant to acumulate fluoride in addition to nickel. Such behaviour could raise questions regarding a constitutional accumulatory capacity within the genus *Dichapetalum*. A high level of fluoride in *D.gelonioides* subsp. *tuberculatum* could also be indicative of fluoro-minerals in the underlying soils.

It will be recalled that as a result of result of chemical analysis of specimens of *D. gelonioides* growing in the Philippines, Malaysia and Indonesia, both strong Ni accumulators and strong Zn accumulators were identified (Section II.2). The discovery of Zn-accumulating specimens which also exhibited enhanced Cd,Co and Pb uptake (Baker and Proctor,unpublished) was of particular interest as this was suggestive of underlying sulphide minerals.These findings are of significance since fluoride is often found in association with sulphide minerals (Prinz et al., 1978 and Frye, 1981).

V.3. Methods of Determining Covalently Bonded Fluoride in Biological Materials

Up to about 30 years ago, covalently bonded fluoride in biological materials was commonly determined by UV-VIS colorimetry. This was preceded by the release of fluorine as the fluoride ion through alkaline digestion or oxygen combustion (Ramsey and Clifford, 1949; Mirosevic-Sorgo and Saunders, 1959; Belcher et al., 1959a,1959b; Peters et al., 1960). Metal ions and radicals that interfered with the estimation were removed by extraction into appropriate organic solvents. However, interfering anions, e.g PO_4^{3-} and SO_4^{2-}, were not so easily removed.The generation of fluorine as hydrofluoric acid prior to colorimetric determination was seen as a useful alternative provided the desired speed of diffusion and complete recovery of the gas could be achieved(Hall, 1960, 1963a, 1963b).

Colorimetry was superseded by direct electrometry, the use of which was reported as early as 1955 by Baker and Morrison. Using a spontaneous current and standard fluoride solutions containing 0-10 μg fluoride, they established an almost linear relationship between the two variables. An aluminium-platinum electrode couple was used and currents were measured two minutes after immersion. The development of the fluoride ion selective electrode by Frant and Ross (1966), was seen as a significant advance in electrometry. Essentially, the electrode consists of a membrane that confines an inner reference solution and reference electrode, and allows electrolytic contact to be made with the test solution. The membrane is a single europium(II)-doped lanthanum fluoride crystal. When the electrode is immersed in a fluoride solution the potential across the membranes responds to a change in fluoride concentration over the range 10^{-5}- 10^{-1} M. The relationship determining the response is described mathematically by the Nernst equation:-

$$E = E_a + 2.3\ RT/nF \log A, \text{ where}$$

E	=	the measured total potential of the system
E_a	=	the portion of the potential due to choice of reference electrode and internal solution

2.3 RT/nF = the Nernst factor (59.16/n mV at 25° C), R and F are constants, n is the charge of the ion and T is the temperature in Kelvin

A = the activity of the ion in the solution (N.B At low concentrations, the difference between activity and concentration is negligible).

The use of a fluoride ion selective electrode is advantageous owing to its specificity and selectivity. The electrode responds to free fluoride in solution but not to bound or complex forms. With the exception of the hydroxyl ion, which interferes with electrode operation there are virtually no interferences from anions. The magnitude of hydroxyl ion interference is dependent on the level of fluoride present. As a general rule, measurements are made in solutions of pH < 8.5 to minimise this interference (Taylor and Brunt, 1973). In the presence of Fe^{3+} and Al^{3+} ions, the fluorocomplexes formed reduce the concentration of free fluoride in solution. Frant and Ross (1968) observed that an acetate buffer (pH 5-5.5) containing citrate was effective in freeing complexed fluoride in water samples. The addition of this buffer to sample and standard solutions prior to taking measurements is now standard procedure. Total ionic strength adjustment buffer (TISAB), also equalises the ionic strengths of the solutions and prevents hydroxyl ion interference. Changes in ionic strength cause changes in ion activity which could lead to misinterpretation of results. The fluoride ion selective electrode may be used for direct potentiometric measurements with an external reference electrode or as an indicator electrode in potentiometric titrations. Singer and Armstrong (1968) used the fluoride ion selective electrode to successfully determine fluoride in ashed, fat-free bones.

In the determination of fluoride in D. gelonioides subsp. tuberculatum, the fluoride was liberated by alkaline hydrolysis then quantified by direct potentiometry using the fluoride ion selective electrode. According to Taylor and Brunt (1973), both alkaline and acid hydrolysis are more effective in releasing fluoride than oxygen combustion methods. The ashing procedure of Singer and Armstrong (1968) was applied for comparison.

V.4. Experimental Procedure

V.4.I. Aqueous Extraction

One gram of dried,ground leaf material obtained from D.gelonioides subsp. tuberculatum was accurately weighed into a 25 cm^3 beaker.The sample was extracted with 6 cm^3 of distilled deionised water as in Section II.8. The slurry was vacuum-filtered, and the filtrate retained. The residue was then extracted with 5 cm^3 of distilled deionised water. Following filtration, the residue was washed with 2 cm^3 of distilled deionised water. Filtrates and washings were combined and centrifuged at 1300 rpm in a Gallenkamp Junior centrifuge for ten minutes. The supernatant (12 cm^3) was retained. The supernatant was not subjected to initial purification by extraction with chloroform/butanol reagent. This step was omitted to prevent loss of any organic-fluorine compounds present. The supernatant was stored in a refrigerator at 4° C. Extraction was carried out in duplicate.

V.4.II. Alkaline Hydrolysis

The procedure for alkaline hydrolysis was based on that described by Taylor and Brunt (1973). A 2-cm^3 aliquot of 10 M Analar KOH was added to an equal volume of each aqueous extract contained in a 100-cm^3 polyethylene bottle with a screw cap. After mixing, the caps were loosely positioned on the bottles which were then placed in a shaking water-bath at 90° C. Having allowed two minuutes for equilibration, the caps were then tightly screwed down. The digests were allowed to cool after six hours of shaking. About ten minutes after cooling had begun, the caps were loosened slightly to prevent implosion. The caps were tightened after the contents had reached 22° C and the bottles were manually shaken to ensure that condensate at the top of the bottle was included in the sample.

Fluoride standard solutions having concentrations of 10^{-5},10^{-4},10^{-3},10^{-2} and 10^{-1} M were simultaneously digested. A standard solution (0.5 M) of NaF prepared from Analar reagent was appropriately diluted to yield solutions having the above concentrations.Standards and samples were subjected to the same procedure in an attempt to match the solution matirces, in order to increase the accuracy of the determination. Solutions comprising 1.5 cm^3 of extract and 0.5 cm^3 of 10^{-3} M fluoride standard were treated in an identical manner to assess the efficiency of the determination.

V.4.III. Ashing

Two 1-gram samples of material obtained from D.gelonioides subsp tuberculatum, which was used in the previous section, were accurately weighed into 5 cm^3 borosilicate test-tubes. The samples were ashed at 500° C overnight in a McGregor SR 5000 Series muffle furnace. After cooling the samples were transferred to plastic centrifuge tubes, to which was added 10 cm^3 of 0.25 M HCl prepared from redistilled (6 M) Analar reagent. The digests were vortex-mixed, then centrifuged at 1300 rpm for ten minutes. The supernatants were stored at 4° C in the refrigerator prior to fluoride determination. A blank was similarly prepared.

V.4.IV. Fluoride Determination

Total ionic strength adjustment buffer (TISAB) was prepared according to the method of Singer and Armstrong (1968). Fifty-seven cm^3 of glacial acetic acid, 58 g of NaCl and 0.3 g of tri-sodium citrate were dissolved in 500 cm^3 of distilled deionised water. The pH of the solution was then adjusted to 5.5 using 5 M NaOH and the volume diluted to 1000 cm^3 with distilled deionised. The resulting solution was then thoroughly mixed and stored at 4° C. Analytical-grade reagents were used in the preparation.

Four cm^3 of TISAB was added to each digest obtained via alkaline hydrolysis. These mixtures were prepared in plastic beakers. The pH of the mixtures which was >12, was reduced to 5.5 using concentrated HCl, and 2 and 0.1 M HCl. Concentrated HCl was used initially, followed by 2 M HCl, with final adjustments being made by the addition of 0.1 M HCl.

In the case of the ashed samples, mixtures comprising 4 cm^3 of TISAB and 4 cm^3 of acid digest were prepared in 25 cm^3 plastic beakers. The pH of the mixtures was 4.93. Dilute NaOH of concentration 1 and 0.125 M was used for pH adjustment. Standard fluoride solutions were similarly treated.

Fluoride concentrations were determined using an Orion combination-type fluoride ion selective electrode and a 501 Digital Ionalyser. Measurements were consistently taken three minutes after immersion to allow for stabilisation of the system. Throughout the entire procedure, plastic laboratory ware was used where practial to minimise possible loss of fluoride through interaction with silica in glass.

V.5. Results

The concentration of fluoride in D.gelonioides subsp. tuberculatum was in the range 19-25 $\mu g\ g^{-1}$ when the plant was subjected to alkaline hydrolysis. When the plant material was ashed, the fluoride concentration ranged from 1.79-5.39 $\mu g\ g^{-1}$.A reasonably good recovery of fluoride (80-86 %) was achieved from the aqueous extracts spiked with the fluoride standard. A linear relationship existed between potential and fluoride concentration over the range 10^{-5} to 10^{-2} M as shown in Fig.V.

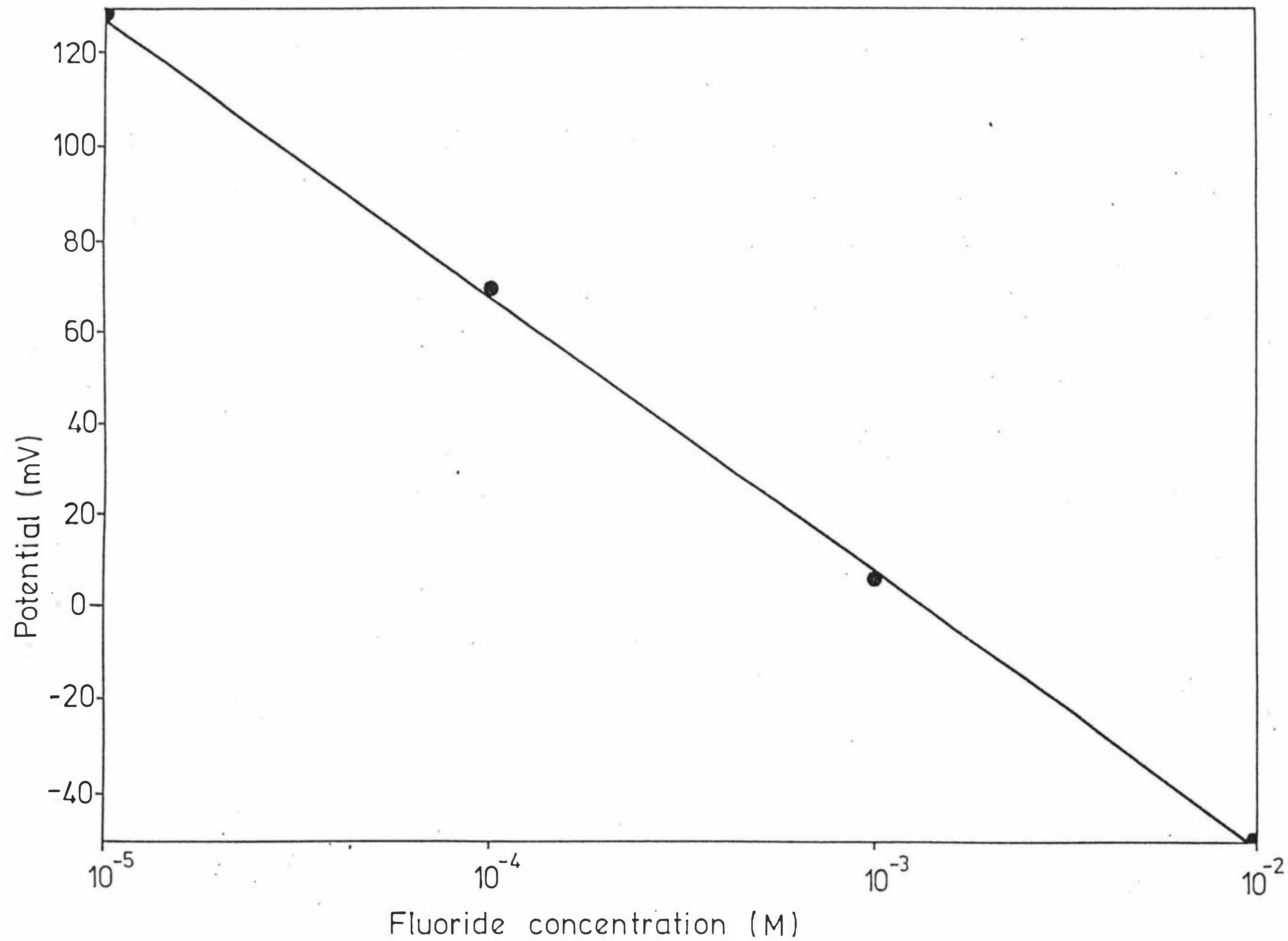


Fig. V. Fluoride calibration curve.

V.6.Discussion

Based on the results of the fluoride analysis, D.gelonioides subsp. tuberculatum does not appear to be a fluoride accumulator. A constitutional accumulator capacity for nickel and fluoride is therefore not evident. However, the fact that fluoride was detected is indicative of fluoride in the soil supporting the plant. It should be recalled that D.gelonioides subsp. tuberculatum was found growing on serpentine soils on Mt Bloomfield, Palawan, Philippines. A common feature of serpentine soils is the occurrence of nickel-rich sulphide minerals such as pentlandite [$(Fe,Ni)_9S_8$], heazlewoodite [Ni_3S_2] and millerite [NiS] (Duke, 1980). Since fluoride can be found in veins as a gangue mineral with sulphur-rich metallic ores such as galena [PbS] and sphalerite [ZnS] (Frye, 1981), its association with nickel-sulphide ores should not be ruled out. The notable affinity of the ultramafic rock-forming mineral amphibole which contains fluorine [$Ca_2(MgFe)_5S_{18}O_{22}(OH,F)_2$] for Ni may also warrant some consideration (Duke, 1980). Unfortunately, chemical analysis of the soils supporting the plants carried out by Baker and Proctor, (1988) did not include fluoride determination. The determination of fluoride in the soils would be worthwhile in the light of these results.Specimens of fluoride-accumulating plants belonging to the genus Dichapetalum were unavailable for nickel determination.

The reasons for the lower fluoride concentration obtained as a result of ashing may be twofold. Firstly, any low molar mass organic-fluorine compounds present could be readily volatilised at 500° C. The volatilised fluoride may be lost to the atmosphere, or may combine with silica in the sample to form refractory fluorosilicates. Such compounds are difficult to decompose by acid digestion, thus their presence results in serious discrepancies in the determination of organic fluoride in plant material (Hall, 1972). Remmert et al. (1953) solved this problem by fusing the ash with sodium hydroxide. Hardin et al. (1954) found potassium hydroxide to be superior to sodium hydroxide. Hall (1968) subsequently confirmed this observation.

The second reason for the lower fluoride concentration may be that the procedure was not sufficiently rigorous to render all the fluoride present amenable to detection, without an additional preceding treatment. Apart from Singer and Armstrong (1968), Hall and Cain (1972)

also used the ashing procedure to release fluoride. However, while the first two workers used fat-free bone which is completely inorganic, Hall and Cain used plant material suspended in agar, this being both inorganic and organic in nature. The suspension also contained magnesium succinate (fluoride-free), ammonia and lithium hydroxide. The resulting ash was then extracted with perchloric acid and diluted with water to facilitate fluoride determination. The inclusion of the above reagents in the suspension undoubtedly served to create conditions to reduce the possible loss of any low molar mass organic-fluorine compounds. Agar and magnesium succinate add organic bulk to the sample,and organic and inorganic anions are converted to soluble ammonium salts. Any fluorosilicates which might have been present originally and/or were formed during ashing are expected to degrade to some extent in the presence of lithium hydroxide. Clearly, application of this ashing procedure allows determination of total fluoride, and may only be used for the determination of organic fluoride if inorganic fluoride is negligible. Hall (1972) followed this procedure to assess the total fluoride content in some toxic fluoride-accumulating plants. The ashing procedure used in this study was therefore only suitable for the determination of simple inorganic fluoride.It has been suggested that extraction with ammoniacal propan-1-ol may be extremely effective in removing organically-bound fluorine (Hall, 1972). This is justified in terms of the insolubility of inorganic fluorine (including fluorophosphates, fluoroborates and fluorosilicates) in alcohols, and the high solubility of organic ammonium salts in such solvents.

It should be pointed out that the higher result obtained using alkaline hydrolysis may not be a true indication of the level of fluoride in the plant. This is due to the fact that only water-soluble fluorine compounds would have been present in the extract. Hall (1972) classified simple inorganic fluorides, organic fluoro-acids of low molar mass, fluoro-carbohydrates and possibly fluorinated nitrogenous compounds as water-soluble fluorine compounds. It may therefore be concluded that in the aqueous extract of D.gelonioides subsp. tuberculatum, 78-91 % of the fluoride is present in the organic form. While monofluoroacetate was not specifically determined, this fluoro-acid could be a major component of organic fluoride in the aqueous extracts as opposed to any of the ω-fluoro-acids identified in D.toxicarium based on molar mass considerations.

CHAPTER VI

Urease Activity and Nickel-accumulating Plants

VI.1. Introduction

Although Ni has not been shown to be a universally essential micronutrient for higher plants, there have been several reports of its beneficial effect on plant growth. A review of these is presented by Mishra and Kar (1974). A nickel requirement has been reported for the growth of the bacterium Alcaligenes eutrophus, the green alga Chlorella vulgaris and the cyanobacterium Oscillatoria sp. (Bartha and Ordal, 1965; Bertrand and De Wolf, 1967; Van Baalen and O'Donnell, 1978).

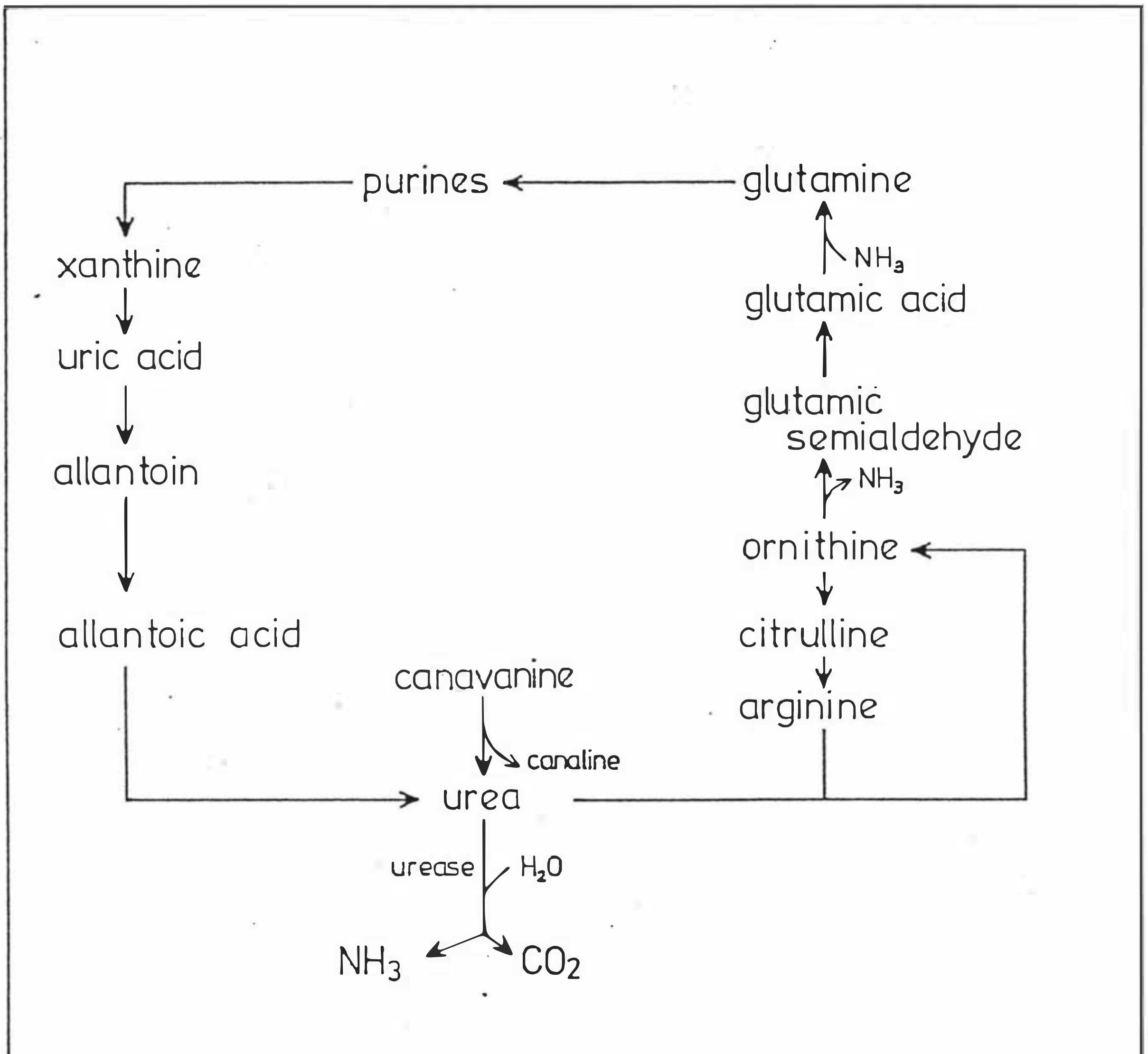
In 1975, Dixon et al. showed Ni to be an essential component of the enzyme urease, isolated from jack bean (Canavalia ensiformis). This discovery which was confirmed by Fishbein et al. (1976), resulted in the stimulation of interest in the role of Ni in plant nutrition. Urease, which catalyses the conversion of urea into ammonia and carbon dioxide, has also been found in some bacteria (Schneider and Kaltwasser, 1984; Christians and Kaltwasser, 1986; Hausinger, 1986). Other bacterial enzymes that have been reported to contain and specifically require Ni are hydrogenases, carbon monoxide dehydrogenase and methyl-coenzyme reductase (Graf and Thauer, 1981; Albracht et al., 1982; Diekert and Ritter, 1983; Ragsdale et al., 1983; Krzycki and Zeikus, 1984; Ellefson et al., 1982; Hartzell and Wolfe, 1986). To date, urease is the only known Ni-metalloenzyme found in higher plants. Its function still remains unclear. Nickel has been shown to substitute for known essential metals in several metal-activated enzymes (e.g isocitric dehydrogenase, arginase and nitrate reductase)(Kratochvil et al., 1967; Roosemont, 1969; Maranville, 1970).

Using tissue culture, in which urea was the sole N_2 source, various workers have shown Ni to be required for the growth of soybean (Glycine max), tobacco (Nicotiana tabacum), rice (Oryza sativa), barley (Hordeum vulgare), species of duck weed (Lemma paucicostata, Spirodela polyrhizza and Wolffia globosa), marine algae (Tetraselmis subcordiformis and Phaeodactylum tricornutum) and fungi (Aspergillus nidulans and Penicillium) (Polacco, 1977a, 1977b; Eskew et al., 1983,1984; Brown et al., 1987; Gordon et al., 1978; Mackay and Pateman,

1980, 1982; Rees and Bekheet, 1982). In 1981, Welch noted reports of urease in higher plants despite their cultivation in media containing NO_3^- and NH_4^+ ions, or under N_2-fixing conditions. Nickel is postulated to play a role in the following aspects of plant growth:-

a) Symbiotic and non-symbiotic N_2 fixation (Matsumoto et al., 1977; Welch, 1981)
b) Stimulation of germination (Matsumoto et al., 1977)
c) Control of N_2 economy in young seedlings and mature plants (Fujihara et al., 1977; Matsumoto et al., 1977)

Fujihara et al. (1977) and Matsumoto et al. (1977) observed increased synthesis of some major free amino acids (e.g arginine, asparagine and glutamine), allantoin and allantoic acid during the life-cycle of soybeans grown in media containing NO_3^- and NH_4^+ ions. Similar observations were made with respect to nodulated plants. Soybeans understandably commanded substantial interest owing to the agronomic value of the Leguminosae family to which they belong. However, increased allantoin production has also been observed in wheat (Krupka and Towers, 1959). Similar studies do not appear to have been carried out on Ni-hyperaccumulating plants. Beevers (1981) and Welch (1981) suggested that higher plants may require urease and therefore Ni, for the metabolism of ureides (uric acid, allantoin, allantoic acid and urea) and guanidines (arginine, canavaline and agmatine). According to Matsumoto et al. (1977), ureides constitute an important N_2 reserve in seeds from both nodulated and non-nodulated soybean plants. Guanidines may serve the same purpose. Provided urea is a product of their catabolic transformations, urease may be required for the utilisation of N_2 in anabolic processes. Proposed pathways for such transformations are presented in Scheme VI. Both ureides and guanidines may also be important means of translocating N_2 (Ankel-Fuchs and Thauer, 1988).



Scheme VI. Proposed pathways of ureide and guanidine synthesis and degradation (Source: Welch, 1981).

VI.2. Aim and Rationale

While researchers have demonstrated an association between Ni in urease and N_2 metabolism in higher plants containing normal levels of Ni (0.01 - 5 $\mu g\ g^{-1}$ on a dry weight basis), Ni-hyperaccumulating plants which contain > 1000 $\mu g\ g^{-1}$ on a dry weight basis (Brooks et al., 1979), have not stimulated comparable interest. The concentration of Ni in highly purified urease is ~1220 $\mu g\ g^{-1}$ (Dixon et al., 1975). Since urease activity has been observed among non-accumulating plants containing substantially less Ni, an investigation of urease activity in Ni-hyperaccumulating plants seemed appropriate. To this end, seeds of Alyssum tenium (Greece), and leaves of Alyssum troodii, Dichapetalum gelonioides subsp. tuberculatum and Phyllanthus 'palawanensis' were assayed for urease.

VI.3. Methodology

VI.3.I Principle

Methods for determining urease (and urea) usually take advantage of the specificity of the action of the enzyme on urea. The ammonia released may be determined colorimetrically following reaction with a suitable colour-development reagent, e.g Nessler's reagent. Alternatively, the gas may be removed from the reaction mixture by distillation or diffusion prior to estimation. Ion-exchange chromatography has proved to be effective for the removal of NH_4^+ ions.Quantitative determination may then be carried out by acid-base titration or colorimetry. During the determination, preformed ammonia (i.e ammonia originally present in the substrate) must be taken into account in order to obtain an accurate result.

In this study, the method used was based on that of Chaney and Marbach (1962). Although the method was described for urea determination, it was easily adapted to urease determination. Ammonia produced in situ was estimated colorimetrically following reaction with a phenol-sodium hypochlorite mixture to give indophenol blue. Sodium nitroprusside was added to

increase the sensitivity of the reaction for ammonia. Urease prepared from jack bean meal was used as a control, and plant material was treated as a potential source of urease.

VI.3.II. Experimental Procedure

VI.3.IIa. Sample Preparation

One gram of accurately weighed Sigma jack bean meal was digested with 5 cm^3 of distilled deionised water in a 50 cm^3 beaker at room temperature for one hour. The digest was then centrifuged at 1300 rpm for ten minutes. The resulting supernatant was transferred to a 5 cm^3 volumetric flask and diluted to mark with distilled deionised water. This process was repeated on another 1 gram sample of meal. Alyssum tenium seeds collected by Mr J.M Foskolov in August 1978, were ground with an agate mortar and pestle. One gram of this material was digested as above. The amount of ground seed available was insufficient to allow duplicate preparations to be made. Aqueous extracts of ground leaves (1g) from specimens of Alyssum troodii, obtained from soil amended with Ni at concentrations of 0, 47, 130, 370, 1111 and 3333 $\mu g\ g^{-1}$, as a result of Ni uptake studies (Section VII.3.I) were prepared according to the procedure of Section II.8. An aqueous extract of Dichapetalum gelonioides subsp. tuberculatum was similarly obtained. One gram of ground leaves from Phyllanthus 'palawanensis' was extracted by the procedure outlined in Section III.11.I. Jack bean meal and seeds of A. tenium were originally stored at 4°C. Leaf material from A.troodii was air-dried and stored at room temperature (22°C). Ground leaves from D.gelonioides subsp. tuberculatum and P.'palawanensis' were stored at room temperature. Alyssum tenium seeds and leaf material from the latter two plants were air-dried in the sun prior to storage. All the prepared extracts were stored at 4°C when not in use.

The concentration of Ni in all the extracts except those of jack bean meal and A.tenium seeds was determined directly by AAS. In the case of jack bean meal and seeds of A.tenium, 1.005 and 0.057 g respectively were ashed at 500°C overnight in a McGregor SR 5000 Series Muffle Furnace. The ash was then taken up in 1 cm^3 of 2 M HCl prepared from 6 M redistilled Analytical

grade reagent, mixed and centrifuged at 1300 rpm for ten minutes. The supernatants were then analysed for nickel. The concentration of Ni in µg g^{-1} was used to calculate the concentration of the metal in the extract in each instance. The Ni concentrations of all the extracts are given below in Table VI.

Table VI Concentration of Nickel in Aqueous Extracts Subjected to Urease Assay

EXTRACT #	NICKEL CONCENTRATION (µg cm^{-3})
Jack bean (meal)	0.50
A.tenium (seeds)	404
A.troodii $_{0}$ (leaves)	12
A.troodii $_{47}$ (")	367
A.troodii $_{130}$ (")	1029
A.troodii $_{370}$ (")	4277
A.troodii $_{1111}$ (")	10752
A.troodii $_{3333}$ (")	24028
D.gelonioides subsp.tuberculatum (leaves)	5671
P.'palawanensis' (")	321

- Subscripts indicate the original concentration of Ni (µg g^{-1}) in the soil from which the specimens were obtained.

Five cm^3 aliquots of each extract were purified by dialysis. These were dialysed against distilled deionised water overnight using Visking 27/32 No. 6 tubing of molar mass cut-off 12000 - 14000 and pore size 2.4 nm. Tightly secured lengths of tubing containing the aliquots, were placed in individual beakers to which 150 cm^3 of distilled deionised water had been added. The contents

of the beakers were magnetically stirred and the water was changed twice. The purified extracts were then centrifuged at 1300 rpm for 20 minutes. The supernatants were retained and their volumes noted.

One cm^3 of 0.016 M urea (Analar) was pipetted into each of nine test-tubes. After transferring 1 cm^3 of purified extract from all samples (except jack bean meal) into separate tubes, a drop of bromothymol blue indicator was added. The same procedure was applied to the two jack bean meal extracts using a 0.1 cm^3 aliquot. The contents of the latter two tubes turned blue instantly indicating the release of ammonia. Solutions containing the remaining extracts showed no blue colour, even after an increase in volume. Dilute HCl (0.01 and 1 M) was added dropwise to the blue solutions until a green colour was observed. They were then heated at 37°C in a water-bath until a blue colour could no longer be observed. The reaction mixtures were then quantitatively transferred to 250 cm^3 volumetric flasks, diluted to the mark with distilled deionised water and thoroughly mixed. Since no ammonia was detected in the other solutions, these were not subjected to the remainder of the procedure. A 0.0016 M urea solution was prepared for the determination of preformed ammonia.

VI.3.IIb. Urease Assay

Test-tubes were set up containing the following:-

a) 1.0 cm^3 of distilled deionised water (i.e the blank).

b) A series of ammonium sulphate (Analar) standards (1 cm^3 each) of concentration 1, 2, 3, 4, and 5 $\mu g\ cm^{-3}$ nitrogen. These were prepared by appropriately diluting a 10 $\mu g\ cm^{-3}$ stock solution.

c) 1.0 cm^3 of 0.0016 M urea (in duplicate).

d) 1.0 cm^3 of 0.016 M urea treated with purified jack bean meal extract (in duplicate).

Two and a half cm^3 of phenol-nitroprusside (1 g of Analar phenol and 0.005 g Analar sodium nitroprusside per 100 cm^3) was added to each tube. The solutions were then vortex-mixed and 2.5 cm^3 of alkaline-hypochlorite (0.5 g of Analar sodium hydroxide and 0.042 g of Analar sodium hypochlorite per 100 cm^3) was added. The tubes were sealed, mixed thoroughly by inversion and then heated in a water-bath at 37°C for 20 minutes to facilitate the completion of the reaction. This was signalled by a permanent blue colour. The solutions were then cooled to room temperature. Their absorbances were measured at 560 nm using a Shimadzu 160 UV-VIS Recording Spectrophotometer. The water blank proved to be suitable as a reference. The urease number (i.e mg N per 100 g of sample) and the number of enzyme units per gram were calculated using the method described by Hoffman (1965).

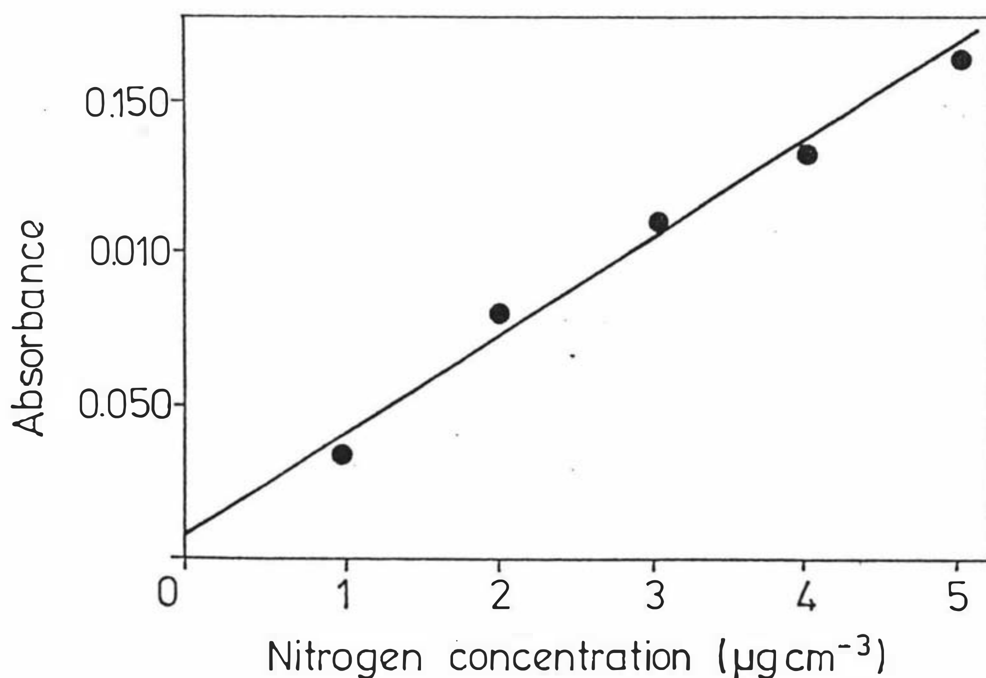


Fig. VI. Nitrogen calibration curve.

VI.4. Results and Discussion

No urease appeared to be present in extracts from A.tenium, A.troodii, D.gelonioides subsp. tuberculatum and P. 'palawanensis' as evidenced by the lack of ammonia released in the presence of urea, despite the relatively high levels of nickel. As expected, a positive result was obtained for the jack bean meal extract. The negative result may be attributed to a genuine lack of urease or possibly, the unsuitability of the sample for enzyme assay.

Ideally, enzyme assays should be carried out on fresh or well preserved material. Because such material was unavailable for this study, air-dried material was used instead. According to Hoffman (1965), methods for determining urease in plants may be carried out using tissue that was air-dried in the absence of sunlight or glycerol extracts of such tissue. Thus the drying regime used would have had only a negligible effect if any on the urease activity except perhaps for material from A.tenium, D.gelonioides subsp. tuberculatum and P. 'palawanensis', as specimens of these plants were air-dried in the presence of sunlight. The negative results were therefore probably due to a genuine lack of urease rather than the unsuitability of the samples for urease determination.

As shown in FigVI, good linearity was observed between absorbance and N concentration. The mean urease number was calculated at 2835 which corresponded to ~9 urease units per gram of meal. Since this level of enzyme activity corresponded to a concentration of 2.50 $\mu g\ g^{-1}$ Ni in jack bean meal, then, assuming a positive correlation between urease activity and Ni concentration, a positive result of a substantially higher order of magnitude would have been expected in extracts of Ni-hyperaccumulating plants containing 60 - 120140 $\mu g\ g^{-1}$ nickel. This may be considered further justification for the conclusion that urease is virtually absent in the Ni-hyperaccumulating plants investigated. The possibility of minute quantities being present which were undetected by this method is also to be considered. The evaluation of this and other methods for determining urease would be worthwhile, using soybean plants as test material, as the presence of urease in these plants appears to be well established (Polacco, 1977a; 1977b).

PART 2

METAL UPTAKE STUDIES

CHAPTER VII

Uptake of Nickel, Cobalt and Copper by Alyssum troodii and Aurinia saxatilis.

VII.1. Introduction

Plants that hyperaccumulate metals such as Ni, Co and Cu have attracted great attention. The phenomenon of plants taking up and surviving phytotoxic levels of metals irrespective of whether their essentiality is proven raises many interesting questions. According to Farago et al. (1975), there are three aspects to be considered. These are:-

a) Metal uptake via the root
b) Transport of the metal through plant tissue
c) The deposition of the metal in the aerial part of the plant

To date, several approaches have been used to investigate these aspects. In 1958, Jowett proposed elucidation of the nature of the chelating agents. Since then, several workers have reported possible metal-chelating agents in metal-hyperaccumulating plants. In this respect, the work of Pelosi et al. (1976), Lee (1977), Pancaro et al. (1978b) and Kersten et al. (1980) on Ni-hyperaccumulating plants from Europe, the Mediterranean and New Caledonia is noteworthy (see Chapter IV). The findings in the first part of this thesis are also to be considered. Similar studies have been carried out on plants from Southcentral Africa that hyperaccumulate copper and cobalt (Morrison, 1980). The chelating agents appeared to be confined to the cell cytoplasm, particularly the vacuole. Such phytochemical studies have been encouraged because it is possible to isolate milligram quantities of metal-rich fractions for further investigation instead of the microgram quantities to be expected from non-accumulating species. The work of Tiffin (1966, 1967, 1970, 1971), on exudates from non-accumulating plants containing normal levels of Ni, Fe and Cu is not to be forgotten, as it provided information regarding the binding of metals to ligands during translocation. Such an approach could result in a better understanding of the hyperaccumulation of metals by plants.

A study of the distribution of metals within the plant tissue via microscopy and radio-isotope labelling constitutes another approach. The results of several of these studies have led to the suggestion that some metals may be bound by structural components of the cell wall

(Reilly, 1973; Turner and Gregory, 1967; Vergnano Gambi, 1967; Peterson, 1969; Turner, 1970; Farago et al. (1975). A possible role of root cell wall in the binding of Zn in Agrostis tenuis has been suggested by Turner and Gregory (1967) and Peterson (1969). Farago et al. (1975) observed Ni in association with the cell wall in leaves of Hybanthus floribundus.

Several pot trial experiments have been carried out to investigate the nature of metal uptake. Brooks et al. (1979) showed that pot trials could be used to distinguish unequivocally between hyperaccumulating and non-accumulating plants belonging to the genus Alyssum. In 1980, Morrison et al. studied Ni uptake in 11 species of Alyssum, all of which belonged to Section Odontarrhena. This section is of interest because it has been shown to contain the largest number of Ni-hyperaccumulating plants (Brooks et al., 1979). Baker et al. (1983) used pot trial experiments to study the Cu and Co tolerance in three closely related taxa within the genus Silene L. These form an ecophyletic series exhibiting a range of tolerance.

Despite all of the above studies, it is still not certain whether plants (even closely related taxa) accumulate the above metals by a common mechanism. The question as to whether tolerance of one metal by a selected species endows the plant with the ability to exhibit tolerance towards another metal has been addressed by several workers.Reeves and Baker (1984) concluded that a constitutional metal tolerance may exist within the hyperaccumulating plant Thlaspi goesingense Hálácsy after observing the performance of serpentine and non-serpentine populations in soils containing elevated levels of Ni, Co and Zn. Constitutional tolerance has also been suggested in Typha latifolia L. (McNaughton et al., 1974; Taylor and Crowder, 1984), Andropogon virginicus Michx (Gibson and Risser, 1982) and the moss Scopelophila cataraetae (Mitt.) Broth. (Shaw, 1987).Fiedler (1985) suggested constitutional tolerance at the genetic level in Calochortus (Liliaceae) based on her studies of Ni, Co and Cu uptake. The ability of a plant to take up unusually high levels of more than one metal (i.e co-accumulation) was also observed in T. goesingense by Reeves and Baker (1984). They attributed this behaviour to the existence of a non-specific metal detoxification system. Further evidence in support of such a system has been provided by Hajar (1987). Working with five Pennine populations of Thlaspi alpestre, a colonist of Pb mine wastes and known Zn hyperaccumulator, he observed limited variation in tolerance to and uptake of Ag, Al, Cd, Co, Cr, Cu, Fe, Mn, Mo, Ni, Pb and Zn in individuals inhabiting a wide range of

substratum (Cd, Pb and Zn) status. Thlaspi has been reported in association with Ni rather than Pb and Zn in at least one metalliferous site in Scotland (Baker and Proctor, 1990). It is of interest to note that the British metallophyte Minuartia verna (L.) Hiern. appears to have evolved races that are singly and multiply tolerant to Cd, Cu Pb and Zn (Halliday, 1960; Hajar, 1987). There is, however, no evidence for constitutional tolerance to metals in this species. Baker and Proctor (1990) concluded that the success of some species in contaminated environments cannot therefore always be explained in terms of metal-specific tolerance evolving at the ecotype level. Unexpected interspecific differences in Pb tolerance of Agrostis spp.(Craig, 1972) and Cu tolerance of Festuca spp. Al-Hijaly, 1989; Al-Hijaly et al., 1989) have been reported for different populations. The findings of these workers are suggestive of an intraspecific heterogeneous distribution of genes for tolerance in different populations.

VII.1.I. Metal Uptake Patterns

Studies of metal uptake characteristics revealed the existence of three general forms of uptake: the rise-to-saturation form (Reilly, 1969); the exclusion-breakdown form (Nicolls et al., 1965; Wu et al., 1975; Crooks, 1979) and the completely linear form (Nicolls et al., 1975). On the basis of findings presented by these and other workers, Baker (1981) proposed that the three forms were characteristic of metal-accumulating, non-accumulating and indicator plants respectively.

In accumulating plants, there is a linear increase in the elemental content of the tissue with increasing concentrations of the element in the substrate until a plateau is reached at a relatively high metal content in the soil. For non-accumulating plants, there is restricted entry of the metal at low soil concentrations until a point is reached at which the restricting mechanism breaks down. There is then unlimited absorption over a narrow range of soil element concentration. Under such conditions, the plant eventually dies. It is important to note that the two forms of metal uptake are valid for both essential and non-essential elements. This contrasts sharply with the suggestion of Timperley et al. (1970) that uptake of non-essential elements followed the rise-to-saturation form while uptake of essential elements followed the exclusion-breakdown form. The fact that the rise-to-saturation and exclusion-breakdown forms were observed for roots and leaves respectively

in Cu-tolerant Agrostis stolonifera is also of interest (Wu et al., 1975). In the completely linear form of uptake, the metal content of the plant is directly related to the concentration of metal in the soil. This behaviour typifies plants that can be used as biogeochemical indicator species. Chemical analysis of these plants can result in the detection of mineralisation at depth (Brooks and Malaisse, 1985). Completely linear uptake has been observed in metal-tolerant species and for non-essential elements in non-tolerant plants (Nicolls et al., 1965; Timperley et al., 1970; Beckett and Davis, 1977). Similar uptake patterns have been proposed by Berry (1986) following investigations into the use of plant analysis in biogeochemical prospecting.

Barry and Clark (1978) suggested that the plant-substrate relationship is dictated by the range of substrate metal concentrations over which measurements are made. This is reinforced by the view of Baker (1981) that a plant may exhibit all of the uptake patterns over different concentration ranges of the element in the substrate. The interaction of edaphic factors such as pH and availability of the metal to the plant also warrant some consideration. Hunter and Vergnano (1952), Crooke and Inkson (1955) and Proctor (1971) noted that the addition of lime to serpentine soils resulted in improved fertility. The first pair of workers attributed reduced growth and increased necrosis of oat plants in sand culture to low levels of Ca, N and P, and elevated levels of nickel. According to them, the application of lime along with N and P fertilisers to Ni-rich soil is the most satisfactory corrective treatment for Ni toxicity. Addition of lime increases soil pH thereby reducing the availability of Ni to the plant. During water-culture experiments Gabbrielli et al. (1990) observed that the addition of Ca reversed the Ni-induced restriction of root growth in the Ni-excluding plant Silene italica L., but reduced root growth in the Ni-hyperaccumulating plant Alyssum bertolonii. This represented a confirmation of the adaptation of the species to a calcium-deficient soil in the presence of a metal like nickel.

VIII.1.II. Quantification of Metal Tolerance

Generally, short-term (~10 days) survival tests using seedlings or vegetative propagules provide a convenient means of studying the effects of metal toxicity on individuals or species that display differential tolerance. Once seedlings (or vegetative propagules) become established, yield and relative growth rate can be used as a measure of performance and tolerance (Baker, 1987). This forms the basis of several techniques that have been devised to quantitatively assess metal tolerance. They include the following :-

a) Root elongation in solution culture
b) Total biomass in soil or solution culture
c) Protoplasmic resistance and membrane damage
d) Chlorophyll fluorescence
e) Pollen tube growth
f) Germination tests

In 1957, Wilkins developed the root elongation method. He supplied tillers of the grass Festuca ovina L.with a calcium nitrate solution amended with metal salt and quantified the inhibitory effects of the metal on the plant by measuring root length over a period of time. These measurements were compared with those carried out in a control solution free of metal . Indices of tolerance so derived could then be used to rank individuals, species or populations. As a result of its simplicity and rapidity,the technique has been extensively used, albeit with some modification. It has also played a vital role in large scale screening for tolerance. Full reviews have been presented by Wilkins (1978) and Brown (1983). Disadvantages to its use have been discussed by Baker (1987). Gregory and Bradshaw (1965) and Turner (1967) observed significant correlations between the index of tolerance of Agrostis tenuis clones derived from the root elongation technique, and soil Zn status. However, Baker (1978) reported that while uptake characteristics could be correlated with soil Zn status in Silene maritima, they did not relate directly to indices of Zn tolerance. The fact that the uptake characteristics were revealed by a study of Zn uptake during long-term (12-15 weeks) water-culture experiments may be of significance. Variations of the root

elongation technique that have been successfully used include total root length (as opposed to the longest root produced), root biomass, growth rate and total biomass (Baker, 1987;Verkleij and Bast-Cramer, 1985;Ingrouille and Smirnoff, 1986).

Total biomass determination may be carried out in both solution and soil rooting media. Comments on the advantage of total biomass measurements in assessing long term effects are presented by Mathys (1977, 1980). Pot trials involving the use of artificial soils amended with Ni, and a control soil containing no added Ni, enabled the confirmation of Ni-hyperaccumulator and non-accumulator status of Alyssum pintodasilvae T. R Dudley and Alyssum serpyllifolium Desfontaines respectively (Brooks et al., 1979). Morrison (1980) used both solution and soil rooting media to study Ni and Co uptake in several Alyssum species. Similar work was also carried out on Bornmuellera tymphaea (a related species) with respect to Ni, and Haumaniastrum katangense, Haumaniastrum robertii and Aeolanthus biformifolius of Southcentral Africa with respect to cobalt and copper. Reeves and Baker (1984) studied Ni, Co and Zn uptake in specimens of Thlaspi goesingense from serpentine and non-serpentine populations using soil rooting media. The work of Hajar (1987) on Thlaspi alpestre, a colonist of Pb mine wastes is also of interest. The soil and solution culture experiments performed by these workers allowed them to assess the response of the plants to metal stress via a range of growth characteristics such as seed germination, root growth, biomass yield, and metal uptake and distribution.

The other techniques are distinct from root elongation and soil culture methods. The metal concentration required to discern between tolerant and non-tolerant genotypes may also be determined by monitoring the ability of epidermal cells to recover from plasmolysis following metal treatment. Here, metal stress is assessed at the cellular level. This is the basis of the protoplasmic resistance method used by Repp (1963), Gries (1966) and Ernst (1972), in both field and laboratory studies. Damage to the cell membrane is assessed after tolerant and non-tolerant plants have been exposed to heavy metals. Wainwright and Woolhouse (1975) measured values of K efflux from roots in solution culture following metal treatment, to ascertain the extent of membrane damage. Fluorescence measurements of chlorophyll in leaves of plants subjected to metal treatment have also been used for quick tolerance assay (Homer et al., 1980). Although the potential of the latter method has not been fully explored, some agreement between the conclusions drawn from

these measurements and those derived from the root elongation method was observed by Homer et al. (1980) in clones of Phalaris arundinacea. Searcey and Mulcahy (1985) assessed metal tolerance by studying the growth of pollen tubes in metal-enriched cultures. They observed good agreement between results of these experiments and those from root elongation experiments while investigating Zn and Cu tolerance in Silene dioica (L.) Clairv., Silene alba (Mill.) Krause and Mimulus guttatus DC. A similar method has been used to demonstrate differences in the Cu tolerance of pollen obtained from two stands of the same cultivar of Coffea arabica L. (Lepp and Dickinson, 1986)

Germination tests have proved useful in characterising tolerant and non-tolerant genotypes. Working with Becium homblei in solution culture, Horscroft (1961) observed that germination only occurred at Cu concentrations over the range 50 - 600 $\mu g\ g^{-1}$. However, the same species was later shown to germinate readily in distilled water (Howard-Williams, 1976). According to Allen and Sheppard (1971), seed germination tests were a satisfactory means of distinguishing between tolerant and non-tolerant populations based on their findings in relation to Cu-tolerant Mimulus guttatus. Their tests were carried out in soil rooting media. During soil experiments, Walley et al. (1971) observed significant differences in germination responses of seeds of a commercial, non-tolerant strain of Agrostis tenuis and a Cu-tolerant race.They also found percentage survival to be the most useful measure of performance, since many seedlings which had successfully germinated, subsequently failed to grow and remained stunted.Differential survivorship was also considered a valuable measure of tolerance by Karataglis (1980) owing to the exclusion of those individuals which germinated but failed to root. Baker and Walker (1989) have noted the usefulness of this technique in demonstrating intraspecific population differentiation. They however stressed the need to exercise caution in interpreting results of such investigations as superior survival of any individual may be related to tolerance of other factors under natural conditions. Some of these may be unrelated to metal tolerance.In 1980, Morrison investigated the ability of Haumaniastrum robertii and Aeolanthus biformifolius (both Co hyperaccumulators) to germinate in various concentrations of Co and Cu, singly and in mixtures. His results were in accordance with the Co-tolerant status assigned the two plants.

As mentioned earlier, the effects of metal stress may be observed at the cellular level. Similar observations can be made at the subcellular level. This approach has been applied to roots, since metal-induced retardation of root growth may be manifested as changes in cytokinesis, cell elongation and cell differentiation (Powell et al., 1986). The assessment of metal tolerance at the cellular and subcellular levels have been carried out on Allium cepa L. (Clarkson, 1965), Agrostis tenuis Sibth. (Wainwright and Woolhouse, 1975) and Festuca rubra L. (Powell et al., 1986). Wu and Antonovics (1978) used tissue culture to demonstrate differences in metal tolerance in non-tolerant and Zn/Cu-tolerant clones of Agrostis stolonifera L.

It is advantageous to use methods involving responses at the cellular and subcellular levels, as they are rapid, require substantially smaller amounts of material and allow quantification of tolerance without complete destruction of the individual. Other physiological responses such as inhibition of respiration, photosynthesis and N_2-fixation may also be used to study the performance of tolerant and non-tolerant plants (Baker and Walker, 1989).

VII.2. Aim and Rationale

In the work reported here, the mode and extent of Ni, Co and Cu uptake by Alyssum troodii Boissier (a Ni-hyperaccumulating plant from Cyprus) was compared with that of the non-accumulating plant Aurinia saxatilis (L.) Desv., a native of Turkey formerly classified under Alyssum (Dudley, 1966). The soil culture method was applied, and both species were also subjected to germination tests. Seed analysis facilitated an investigation into the relationship between the metal in the seed and that apparently accumulated by the plant during cultivation in metal-enriched soil. The use of artificial soils amended with metal and fertiliser over serpentine soils was justified on the basis of the lower number and greater control of variables in the former. Concern over the vigour of plants grown in serpentine soil under glasshouse conditions, also influenced the choice of substrate. Doubts regarding the suitability of serpentine soil were subsequently reinforced when several species of Alyssum, including A. troodii, cultivated under glasshouse conditions were observed by the author to have less vigour and biomass than those cultivated in artificial soils. Further, use of artificial soils provided the opportunity to ascertain

whether hyperaccumulating plants could exhibit even greater metal uptake than occurs in the natural environment.

The soil culture technique was selected after consideration had been given to the following:-

a) The reported relationship between long-term uptake characteristics and soil metal status in Silene maritima (Baker, 1978), and the promising results obtained by Kersten (1979) and Morrison (1980)

b) The ease with which the technique could be employed; the constant replacement and aeration of solution culture is not necessary in soil culture experiments

c) The availability of glasshouse facilities at the New Zealand Nursery Research Centre, Massey University, equipped with computer-controlled capillary irrigation and ventilation systems.

VII.3. Experimental Methods

VII.3.I. Media Preparation

Peat/pumice mixtures containing 10 % (w/w) of Ni, Co and Cu were prepared from the corresponding reagent-grade nitrate salts. Serial dilutions were then carried out with 1 : 1 peat/pumice mixture containing Osmocote slow-release fertiliser (N,P,K), lime, dolomite and the micronutrients Cu, B, Mo, Mn, Zn and Fe (P.G Mix) to yield media with 47,78, 130, 216, 370, 1000, 3333 and 10000 $\mu g\ g^{-1}$ of Ni, Co and Cu on a dry weight basis. Quantitative descriptions of

the composition of Osmocote fertiliser, P.G. Mix and the enriched 1:1 peat/pumice diluent are given in Appendix I. Composite soils containing Ni and Co together at equal concentrations of 24, 39, 65, 108, 300 and 500 $\mu g\ g^{-1}$ were also prepared. Constant nitrate levels were maintained by the addition of appropriate amounts of 10 % calcium nitrate (agricultural grade) in pumice powder.

VII.3.II. Plant Cultivation

Seeds of A.troodii were collected by Mr M Ch. Iacovides of Nicosia, Cyprus in August, 1978 and those of Au. saxatilis were obtained from Yates, New Zealand Limited.The seeds were stored at 4° C. Germination was carried out in the 1:1 peat/pumice diluent containing added fertiliser. After 2-3 weeks, seedlings were transplanted into plastic pots containing experimental soils (ca. 200 g). Each pot contained one seedling. There were ten replicates for each separate metal concentration. Controls consisted of the peat/pumice mixture containing fertiliser, micronutrients, lime, dolomite, micronutrients and calcium nitrate, but none of the three metals. All pot trials were performed in a glasshouse maintained at an average temperature of 22° C. Plants were watered twice a day by capillary action and harvested at the end of three months.

VII.3.III Preparation and Analysis of Plant Material

Plant samples were rinsed with distilled deionised water and air-dried. The dry weight of each plant was recorded. Alyssum troodii samples were sorted into leaves, stems and roots, while samples of Au. saxatilis were sorted into leaves and roots, as the stems are extremely short and difficult to separate.

The material (ca. 0.2 g) was weighed into borosilicate test-tubes and ashed at 500° C overnight in a McGregor SR 5000 Series Muffle Furnace. A small volume (2-5 cm^3) of 2 M HCl prepared from redistilled constant-boiling 6 M acid was added to the tubes. The mixtures were shaken and centrifuged for 10 min. in a Gallenkamp Junior Centrifuge at 3000 rpm.Supernatants were appropriately diluted and then analysed for Ni, Fe, Cu, Mn, Co, Zn, Ca and Mg by atomic absorption spectrometry (AAS). Sodium and potassium in the supernatants were determined by

flame emission spectrometry (FES). An IL 457 Atomic Absorption Spectrometer was used for both sets of analyses. However, some analyses were also carried out on a GBC 905 instrument as this replaced the IL 457 instrument towards the end of the research period. Operating conditions are presented in Appendix II. At least two duplicate determinations were made for every set of ten plants harvested from the different soils. In soils where the mortality rate was high, plant material was pooled and the number of determinations was dictated by the mass of material available. Sample blanks were subjected to the same determinations.

To determine the metal content of seeds of A. troodii and Au. saxatilis, 124 seeds of the former and 62 of the latter were weighed and ashed at 500° C overnight as before. Following the addition of 5.5 cm^3 of 2 M HCl, sample solutions were thoroughly mixed then centrifuged. The supernatants were analysed for Ni, Co and Cu as previously.

VII.3.IV Preparation and Analysis of Soils

Air-dried ground (< 60 mesh) soil samples (1 g) were weighed into boiling tubes and digested with 10 cm^3 of 2 M HCl in a water bath for 20-25 minutes. Digests were filtered through Whatman # 40 paper and the volume adjusted to 10 cm^3 with distilled deionised water. The metals were determined as before. In order to obtain another measure of the availability of the metals to the plant for comparison, ammonium-EDTA (Analar) extractions were carried out. Soil samples (0.8 g) in screw-cap glass vials were extracted with 5 cm^3 0.05 M NH_4-EDTA (pH 7) by shaking for one hour using a Kunk Junkel Electrical Shaker at 20° C (MAFF,1981). After filtration and adjustment of the volume to 5 cm^3 with extractant, the resulting extracts were analysed for Ni, Co and Cu by AAS.

The pH of the soils was determined using a combination type electrode and an Orion Research 501 Digital Ionalyser after 10 g of sample had been equilibrated overnight with 25 cm^3 of distilled deionised water. The instrument was calibrated with phosphate and acetate buffers at pH 6.89 and 4.05 respectively. Buffer solutions were prepared from reagent-grade disodium hydrogen phosphate, potassium dihydrogen phosphate, sodium acetate and glacial acetic acid using carbon

dioxide-free distilled deionised water according to the method of Bassett et al. (1978).

VII.3.V. Germination Tests

Single element aqueous solutions of Ni, Co and Cu at concentrations of 50, 100, 500, 1000, 5000, 10000, 15000, 20000, 25000 and 30000 $\mu g\ cm^{-3}$ were prepared from 10 % aqueous metal nitrate solutions. Composite solutions were similarly prepared and contained Ni and Co together at concentrations of 10, 15, 25, 50, 100, 250, 500, 1000, 2500, 5000 and 7500 $\mu g\ cm^{-3}$. Forty seeds of each of the two plant species were placed on filter papers soaked in each of the above solutions, and kept in Petri dishes. The filter papers were moistened daily with distilled deionised water to prevent dehydration. Controls consisted of filter papers moistened with distilled deionised water only. Following an interval of seven days, counts of seedlings were made every three days for a total period of 25 days. Observations of seed appearance and seedling vigour were made and the position of the dishes was rearranged regularly to minimise differences in illumination and heat.

VII.4. Results

VII.4.I. Metal Uptake

Both A. troodii and Au. saxatilis plants exhibited greatest vigour in Ni-rich soils (Plates VII.1&2). Generally, reduced vigour was observed in the other substrates.The Ni contents in the various organs of A. troodii and Au. saxatilis are shown in Table VII.1 and Fig. VII.1(vertical lines on these graphs and subsequent ones represent standard deviations). The values for the metal content of the rooting medium refer to the final concentration at the time of harvesting. These are slightly lower than the initial nominal concentrations owing to leaching effects and plant uptake. The curves for leaves and stems of A. troodii are almost completely coincident. The Ni content of these tissues greatly exceeds that of roots despite the latter having been in intimate contact with interstitial Ni solutions in the artificial media. With respect to Au. saxatilis, Ni levels in leaves and roots were fairly similar. Overall, the metal contents of organs of the accumulating plant were 1-2 orders of magnitude higher than those of Au. saxatilis. About 40 % of the A. troodii plants

Plate VII.1.Concentration of metal in media ($\mu g\ g^{-1}$):- Ni: 0, 47, 78, 130, 216, 360, 600, 1000;

Co: 0, 47, 78, 130, 216, 360, 600, 1000; Cu: 0, 47, 78, 130, 216 (no plant survived Cu concentrations in excess of 216 $\mu g\ g^{-1}$).

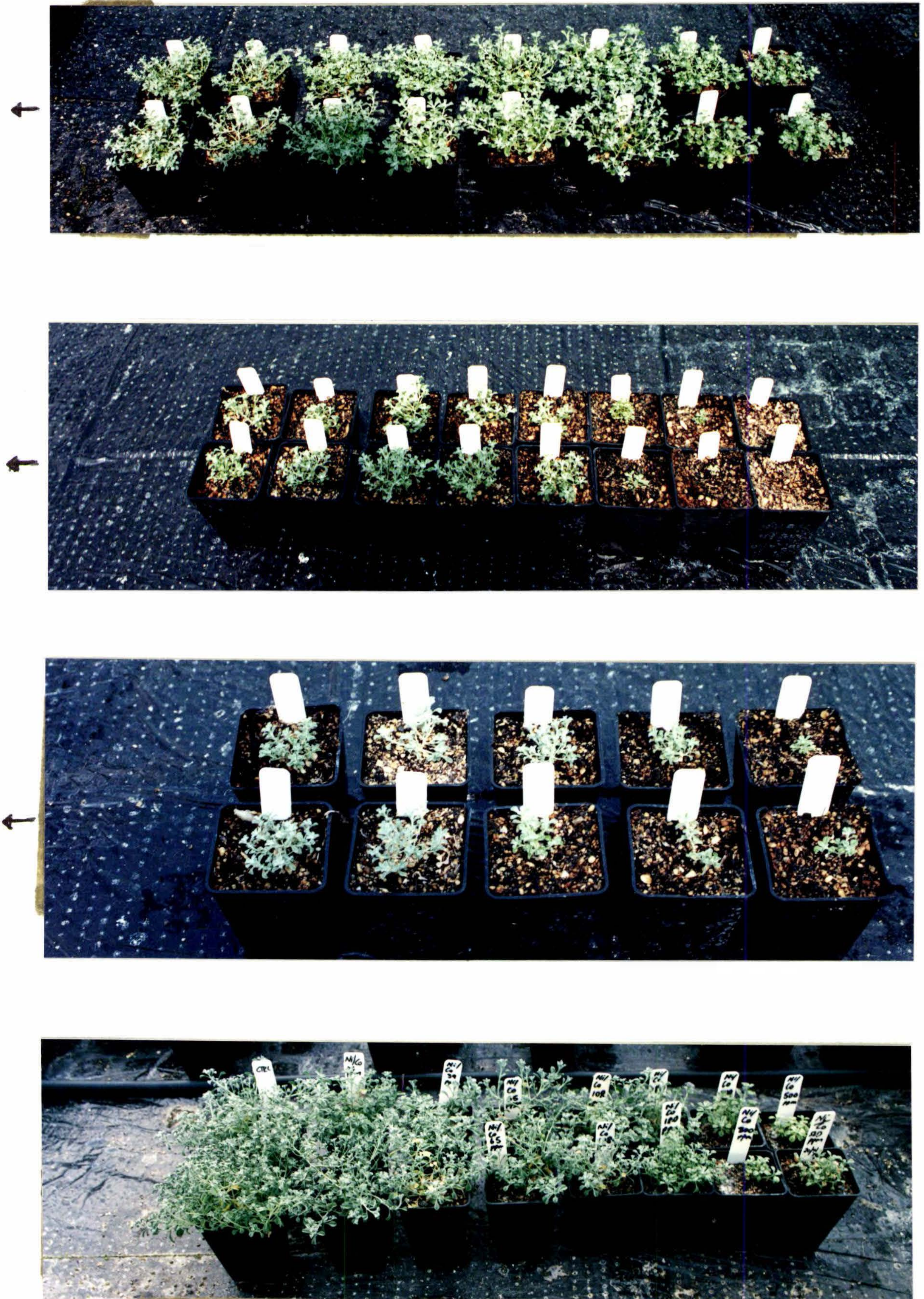


Plate VII.1 Alyssum troodii plants during cultivation. From top to bottom, plants cultivated in media containing added Ni, Co, Cu and a mixture of Ni and Co respectively.

Plate VII.2 Aurinia saxatilis plants during cultivation. From top to bottom, plants cultivated in media containing added Ni, Co, Cu and a mixture of Ni and Co respectively.

Table VII.1 Maximum and Minimum Concentrations of Nickel, Cobalt and Copper in A.troodii and Au.saxatilis.

METAL DISTRIBUTION	METAL CONCENTRATION ($\mu g\ g^{-1}$) A.troodii		Au.saxatilis	
	Maximum	Minimum	Maximum	Minimum
Ni in leaves	10084	20.7	383	1.84
Ni in stems	8540	18.6	-	-
Ni in roots	1959	16.7	65.7	3.42
	(825)[a]	(1.90)	(245)	(1.18)
Co in leaves	2325	12.2	117	0.25
Co in stems	880	6.24	-	-
Co in roots	1322	8.30	36.8	0.25
	(436)	(<0.25)	(58.0)	(1.38)
Cu in leaves	71.1	5.71	45.2	2.09
Cu in stems	82.4	5.10	-	-
Cu in roots	42.6	8.36	42.6	3.62
	(355)	(20.6)	(268)	(11.2)
Ni in leaves[b]	752	17.4	46.3	0.34
Ni in stems	528	3.81	-	-
Ni in roots	179	2.34	21.7	1.79
	(376)	(2.00)	(92.3)	(1.89)
Co in leaves[b]	2202	8.26	144	0.20
Co in stems	1109	1.84	-	-
Co in roots	752	1.46	38.1	0.40
	(340)	(2.00)	(63.1)	(1.54)

[a] - Soil metal concentraion given in brackets. No plants survived maximum concentraions.

[b] - Plants grown in soils enriched with both nickel and cobalt.

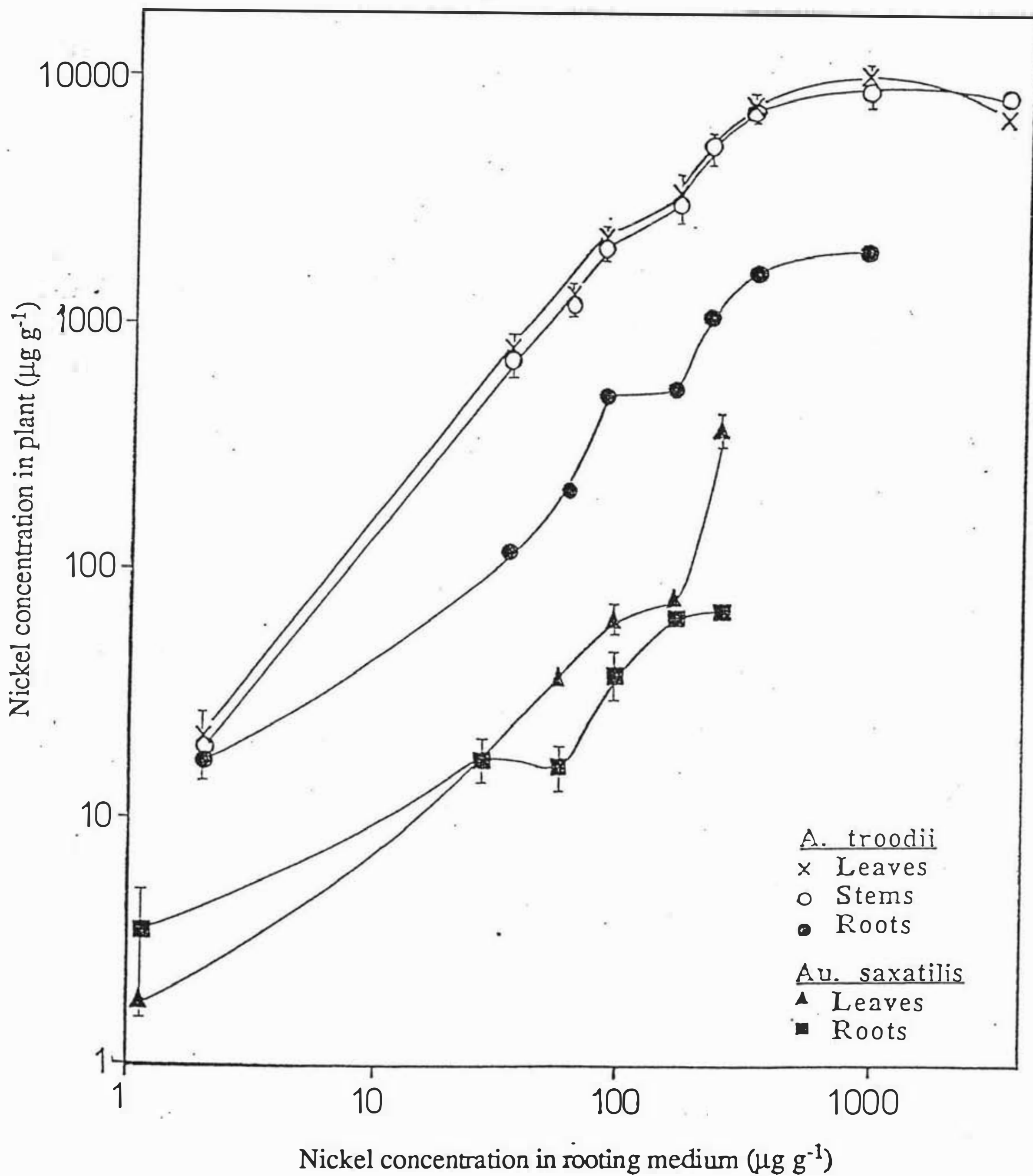


Fig. VII.1. Concentration of Ni in plants after 90-day pot trials in rooting medium containing various concentrations of Ni.

survived a Ni content of 3000 $\mu g\ g^{-1}$ in the rooting medium, but the level of this element remained about the same as in specimens grown in media containing 825 $\mu g\ g^{-1}$ (nominally 1000 $\mu g\ g^{-1}$). No specimens of Au. saxatilis survived soils containing > 245 $\mu g\ g^{-1}$.

Corresponding data for uptake of Co by both plant species are presented in Table VII.1 and Fig. VII.2. No specimen of the hyperaccumulating plant tolerated soil containing > 800 $\mu g\ g^{-1}$ cobalt. Aurinia saxatilis did not survive soil Co concentrations in excess of 58 $\mu g\ g^{-1}$. Very little difference was discerned in the tolerance to and uptake of Cu in the plants investigated (Table VII.1 and Fig. VII.3). Maximum tissue concentrations of this metal were below 100 $\mu g\ g^{-1}$ in all cases. When plants were grown in media containing equal amounts of Ni and Co (Table VII.1 and Fig. VII.4), Ni uptake was appreciably reduced. The uptake of Co was only slightly higher than that observed in soils enriched with Co only.

VII.4.II. Relative Biomass Yields and Accumulatory Capacity

Relative biomass yields for both plant species grown in experimental media containing varying amounts of the three metals are shown in Fig.VII.5. These were calculated by determining the average yield per plant in a given group and expressing this as a percentage of the highest individual yield within that group. Mean maximum yields for A. troodii were achieved in media containing 212 $\mu g\ g^{-1}$ Ni, 142 $\mu g\ g^{-1}$ Cu and 65 $\mu g\ g^{-1}$ Co. These values corresponded to average metal burdens of 22121, 53 and 1584 μg of metal per plant (Fig.VII.6). For Au. saxatilis, the corresponding maximum yields were attained at 94, 36 and 29 $\mu g\ g^{-1}$ for Ni, Cu and Co respectively. At these levels, the average weights of metal per plant were 473, 110 and 214 μg of Ni, Cu and Co respectively (Fig. VII.7).

When plants were grown in soils containing both Ni and Co, maximum yields of A. troodii occurred in substrates containing 45 and 58 $\mu g\ g^{-1}$ of Ni and Co respectively. Yields in the composite soils were lower than yields attained in soils enriched with one metal. The corresponding weights of metal per plant were 640 and 1650 μg for Ni and Co respectively. In the case of Au. saxatilis, cultivation in soils containing Ni and Co at levels of 42 and 35 $\mu g\ g^{-1}$ resulted

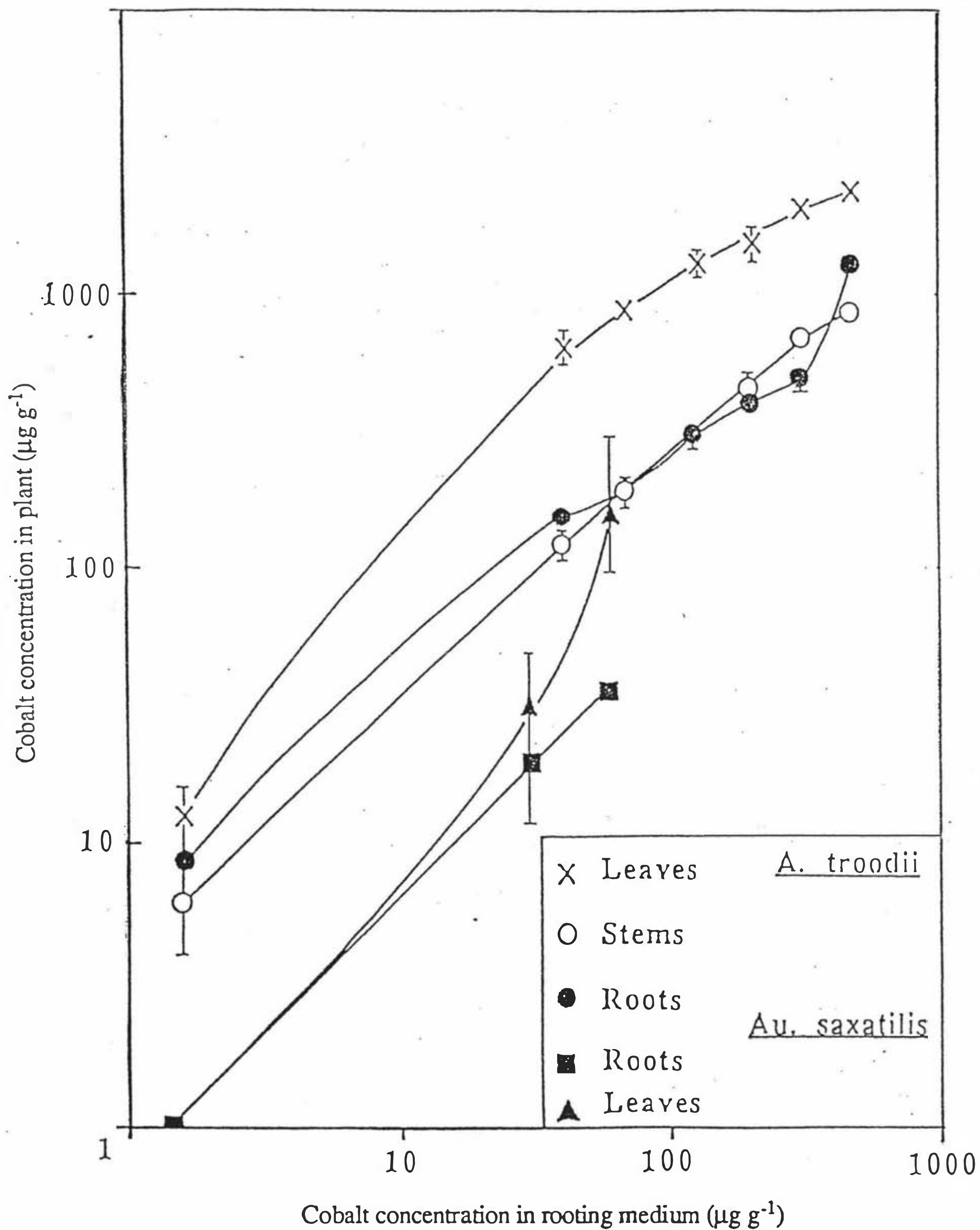


Fig. VII.2. Concentration of Co in plants after 90-day pot trials in rooting media containing various concentrations of Co.

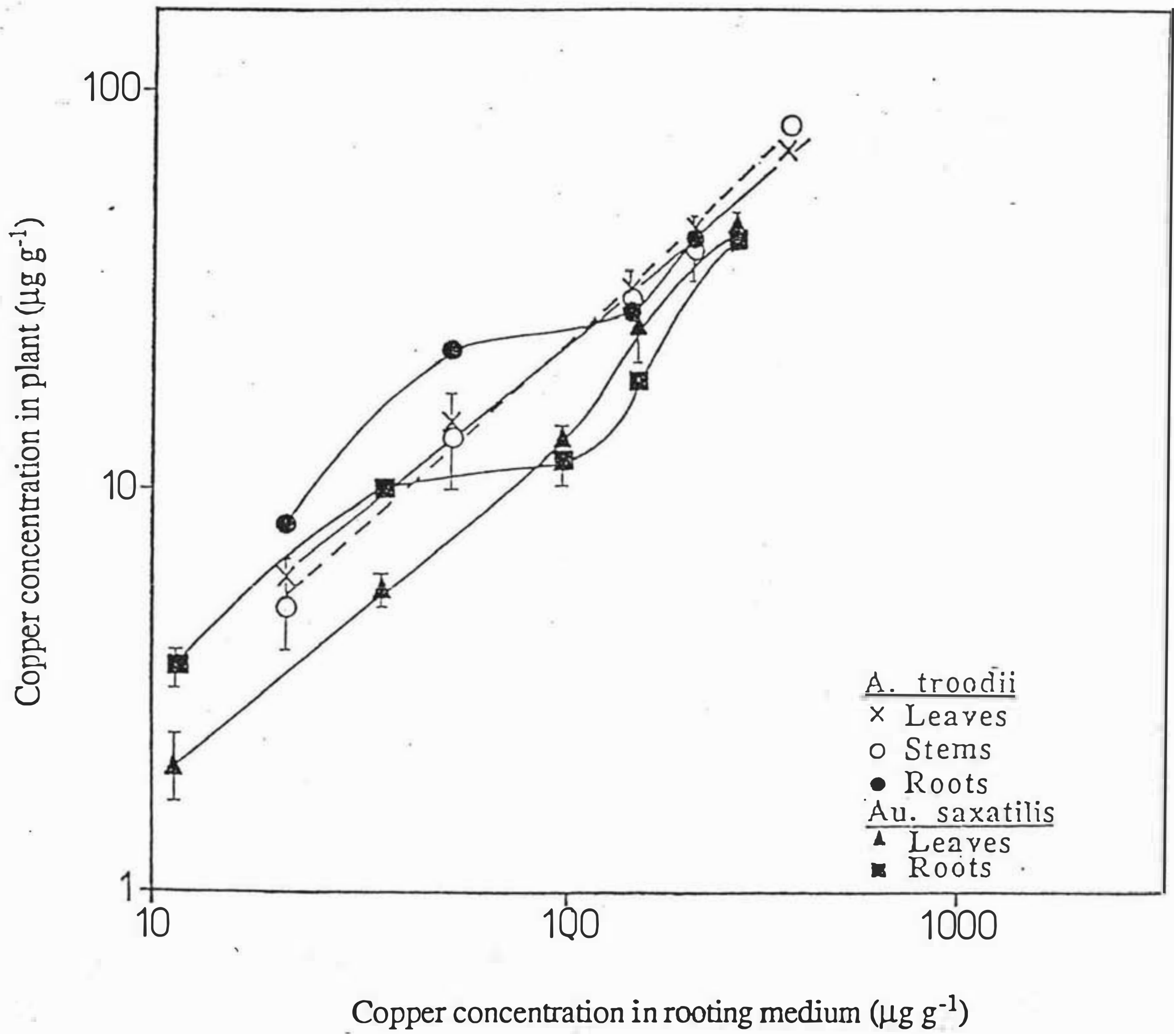


Fig. VII.3. Concentration of Cu in plants after 90-day pot trials in rooting media containing various concentrations of Cu.

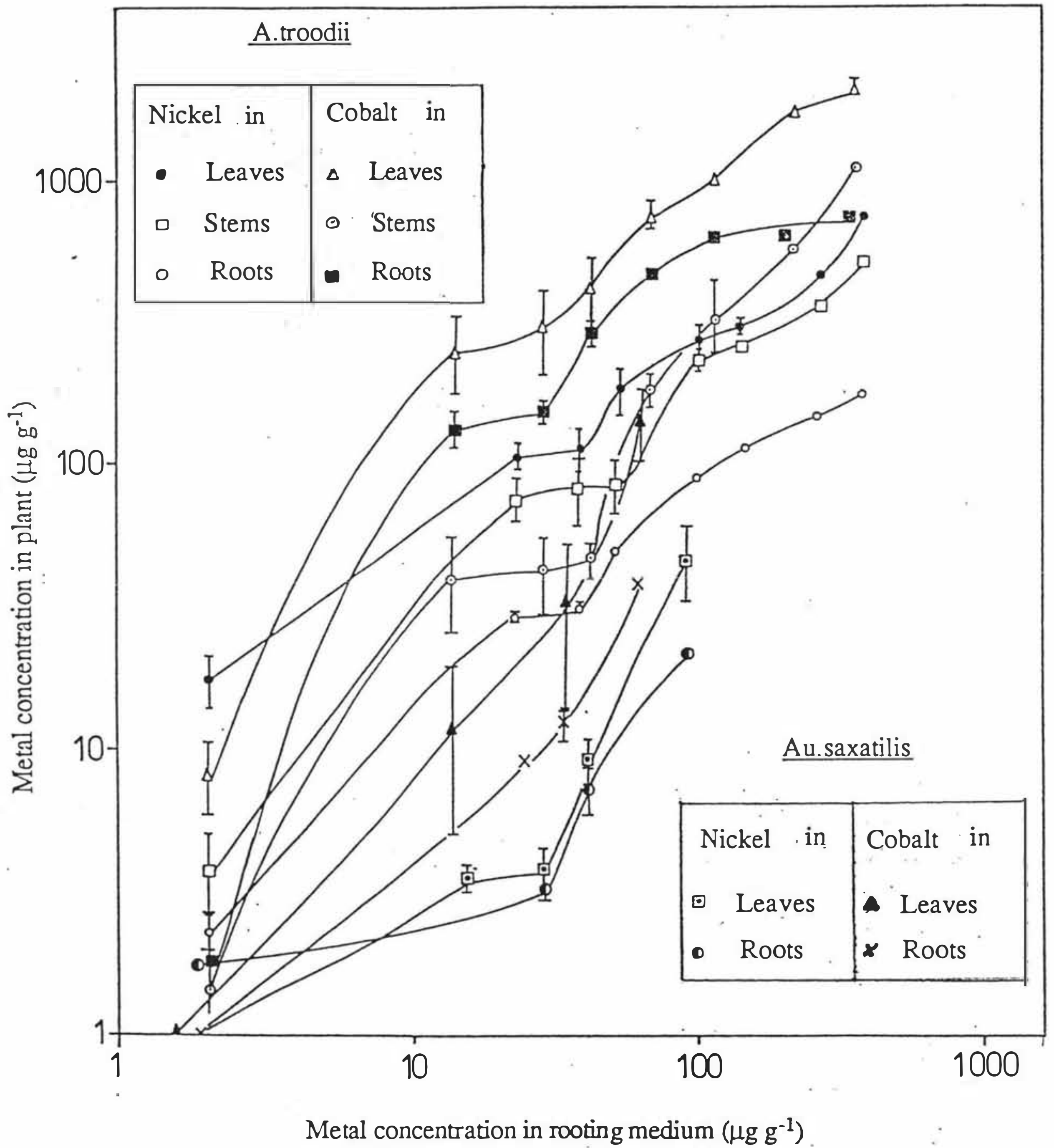


Fig. VII.4. Concentration of Ni and Co in plants after 90-day pot trials in rooting media containing various concentrations of both metals.

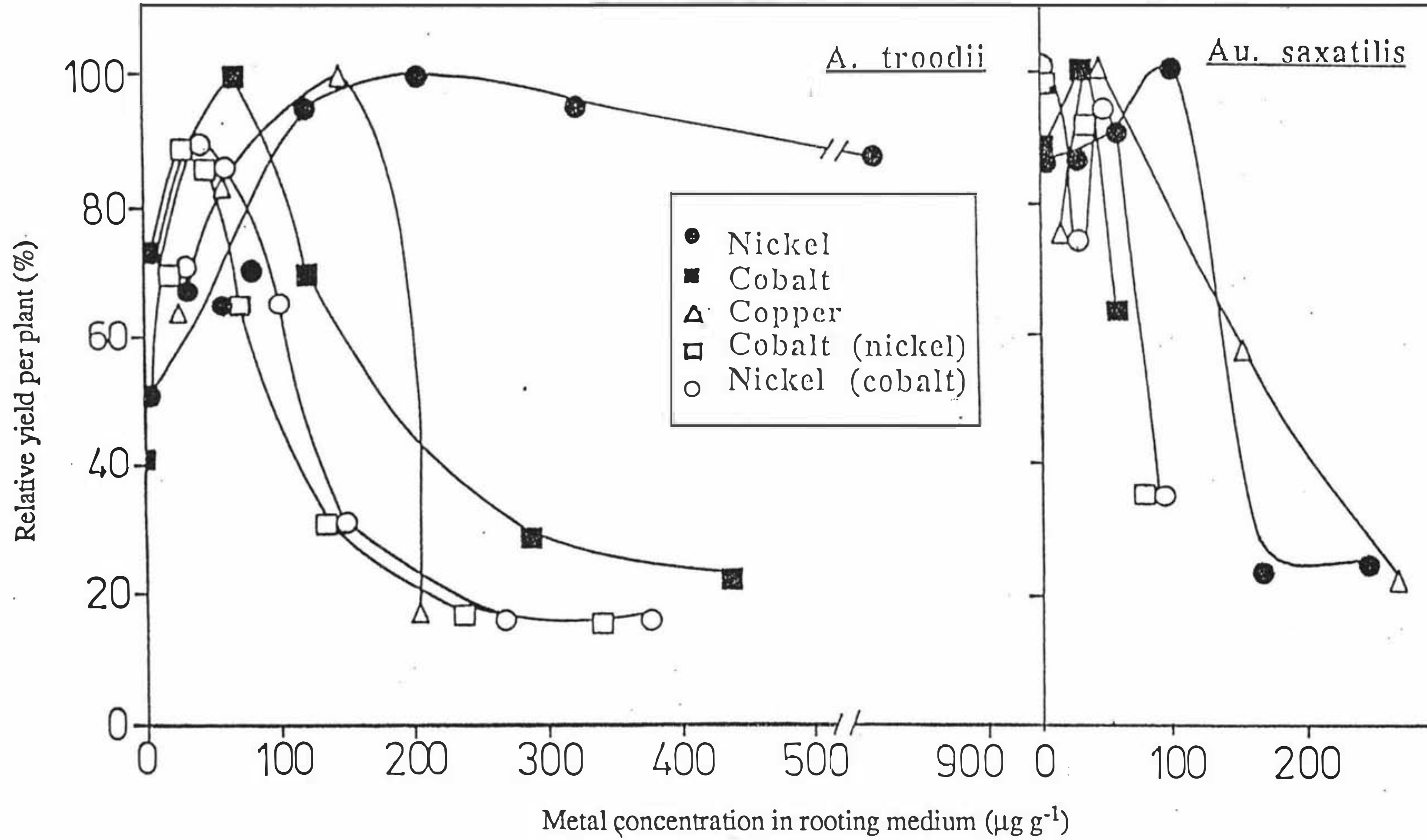


Fig. VII.5. Biomass yield curves for 90-day pot trials of plants grown in metal-enriched rooting media.

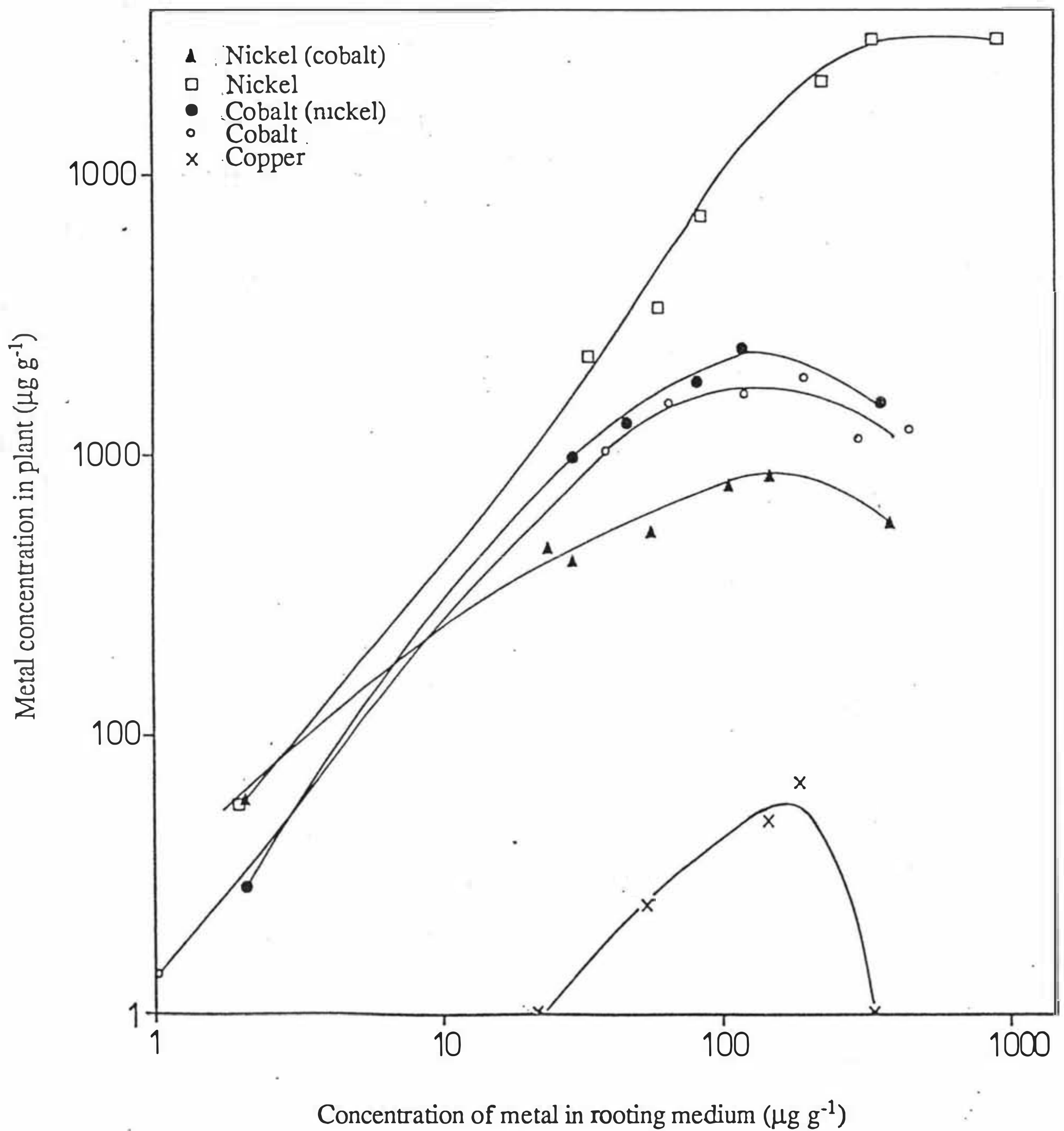


Fig. VII.6. Weight of metal in A.troodii plants in metal-enriched rooting medium.

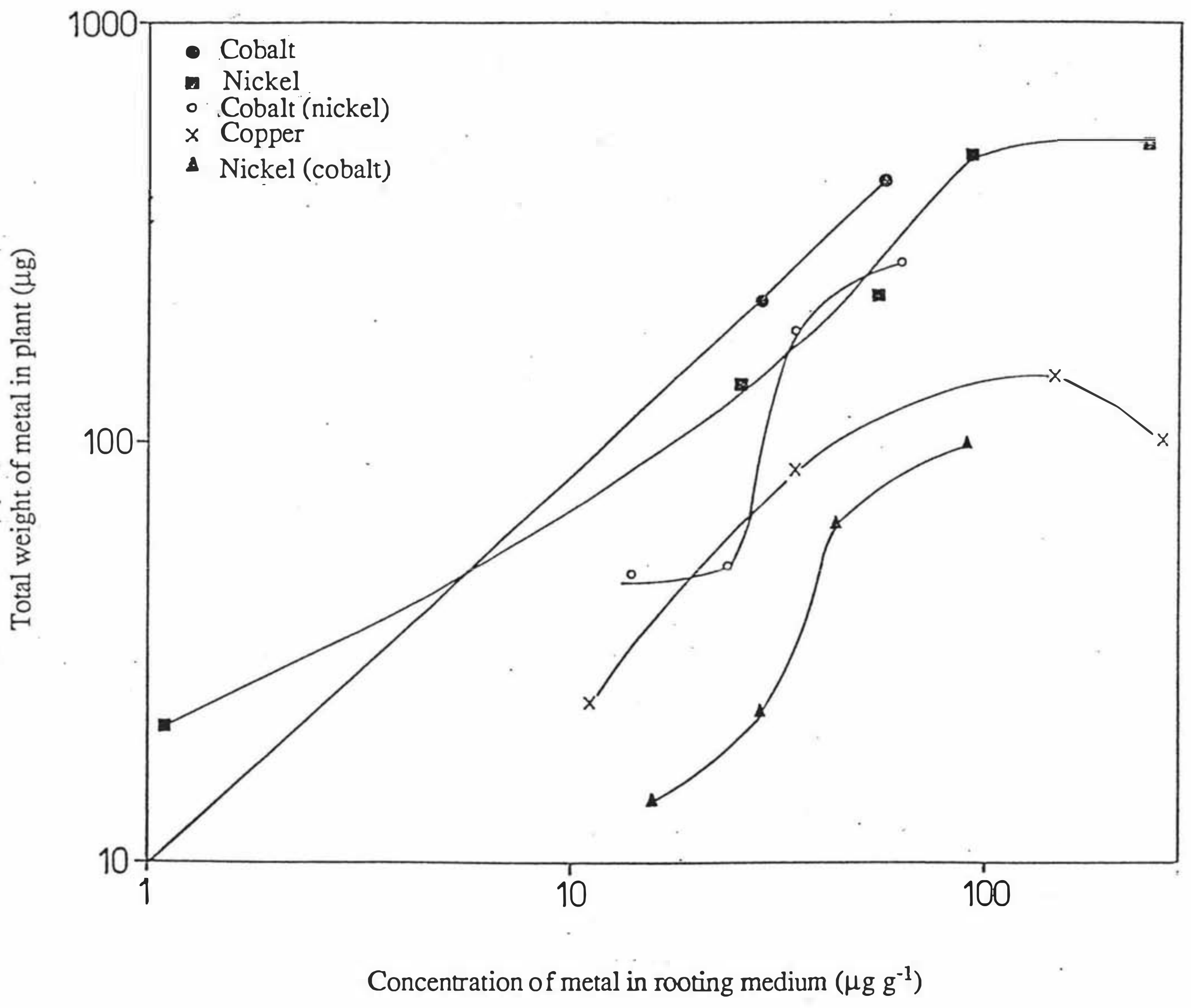


Fig. VII.7. Weight of metal in Au.saxatilis plants grown in metal-enriched media.

in a high relative yield (94 %), though initially, maximum yield appeared to be attained in the absence of the two metals (Fig. VII.5). The weights of metal per plant corresponding to the above soil metal concentrations were 55 and 165 μg . Compared to plants grown in single-element soils, plants grown in soils enriched with both Ni and Co showed restricted Ni uptake. Cobalt uptake remained virtually the same. The concentration of metals in plants cultivated in soil containing no added Ni, Co or Cu are given in Appendix IIIa.

VII.4.III. The Effect of Nickel, Cobalt and Copper on Seed Germination

Following the seven day interval, the greatest change in germination percentages was observed on day 22 (Fig. VII.8 a &b). In the controls, 75 -85 % of the A.troodii seeds and 75 % of the Au. saxatilis seeds germinated. Seedling mortality was evident beyond this period.

In the case of A. troodii, and in substrates with Ni concentrations in the range 50 -500 μg cm^{-3}, there was 80- 90 % germination. Over a wider concentration range (50- 5000 μg cm^{-3}), 70-90 % of the seeds germinated in the presence of cobalt. In the Cu-rich substrates, seeds showed maximum germination (60-80 %) when the metal was present at a level of 50 μg cm^{-3}. Seedlings showed greater tolerance to cobalt than to nickel and copper. At lower concentrations, Cu was more detrimental than were Ni and Co, and produced seedlings of poor vigour that had an unusual blue-green colour. Both growth and development were seriously retarded. For seeds exposed to all three elements at concentrations above 10000 μg cm^{-3}, mainly radicles were observed, and the few shoots that developed were chlorotic.

In experiments with seed of Au. saxatilis, the highest percentage germination (68-75 %) in the presence of Ni was observed in the medium prepared from the solution containing 100 μg cm^{-3} nickel. Some 70 -90 % of the seeds germinated in the presence of 1000 μg cm^{-3} cobalt. In the presence of Cu, maximum percent germination (70 %) was observed at the level of 100 μg cm^{-3}. Compared with the response of seeds to Ni and Cu over the range 50-1000 μg cm^{-3}, more seeds germinated in media containing cobalt.Generally, germination was severely affected at metal concentrations in excess of 15000 μg cm^{-3}, and in this respect Au. saxatilis resembled A.troodii.

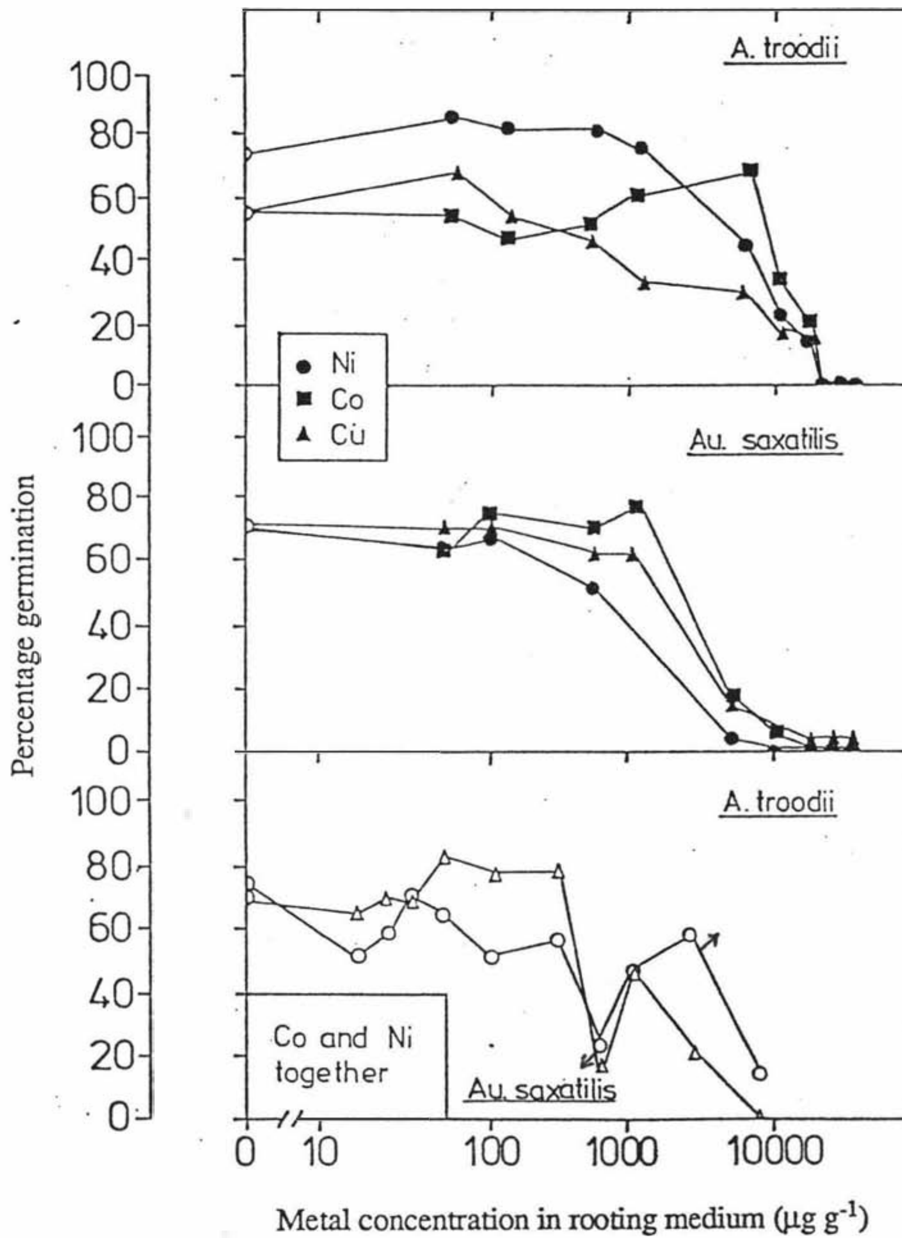


Fig. VII.8(a) Percentage germination of A. troodii and Au. saxatilis seeds 7 days after sowing as a function of metal content of the rooting medium.

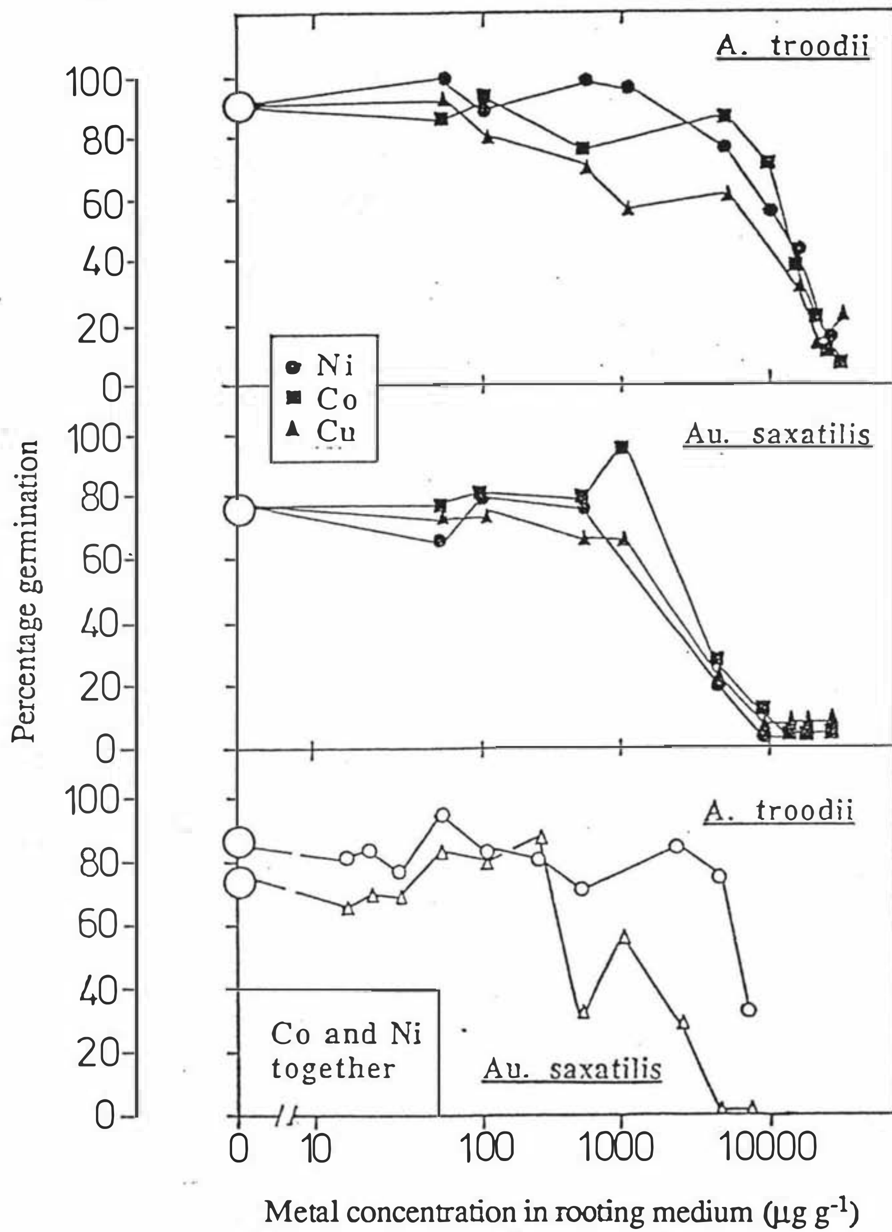


Fig. VII.8(b) Percentage of A. troodii and Au. saxatilis seeds 22 days after sowing as a function of metal content of the rooting medium.

When seeds of both plants were exposed simultaneously to Ni and Co, the highest percentage germination of A.troodii seeds (63-93 %) occurred at concentrations of 50 $\mu g\ cm^{-3}$. Some 78-85 % of Au.saxatilis seeds germinated in the concentration range of 50-250 $\mu g\ cm^{-3}$. Germination of both seed types declined beyond this level. Substantial fungal growth was observed in the case of Au. saxatilis but none was observed with the hyperaccumulating species, perhaps because of the anti-fungal nature of its metal burden. This observation was also true of specimens grown in media enriched with one metal only.

VII.4.IV. Metal Content of Seeds

The Ni content of A.troodii seeds was 1906 $\mu g\ g^{-1}$ and the concentration of Co was 1.7 $\mu g\ g^{-1}$, this being similar to the Cu concentration. Calculations revealed that the average weight of Ni per seed was 0.98 μg and that of Co (and Cu) was 10^{-4} μg. In soils containing the lowest concentration of added metal, the weights of the three metals in a single plant (2264, 1044 and 25 μg for Ni, Co and Cu) based on the average biomass, greatly exceeded these values. A similar relationship was observed in Au. saxatilis with respect to Cu (the Cu content was 2.91 $\mu g\ g^{-1}$). No Ni and Co were detected in seeds of this plant.

VII.4.V. Plant-available Metal Concentration at Time of Harvesting

The data shown in Table VII.2 indicate that for single element media, metal extractability was greatest for copper and least for nickel. In mixtures enriched with Ni and Co simultaneously Co was retained by the soil to a greater extent than was nickel. The percentage of metal extracted with NH_4-EDTA (0.05 M) was invariably lower than those extracted with HCl (2 M), except for the extraction of cobalt in the presence of nickel. In this instance, extractability in NH_4-EDTA was virtually the same as in HCl. The pH of the rooting media ranged from4.4-5.3.The concentration of metals in soil containing no added Ni, Co or Cu are presented in Appendix IIIb. Appendix IV shows the results of regression analysis.

Table VII.2 Extractable Metal Content of Substrates at Time of Harvesting

METAL		METAL CONCENTRATION ($\mu g\ g^{-1}$) or % EXTRACTION						
Nickel	Nominal Concentration	47	78	130	216	360	600	1000
	Extracted by HCl	68 %	73 %	62 %	54 %	59 %	53 %	82 %
	Extracted by NH_4-EDTA	44 %	56 %	44 %	39 %	37 %	37 %	52 %
Cobalt	Nominal Concentration	47	78	130	216	360	600	
	Extracted by HCl	80 %	80 %	91 %	87 %	79 %	73 %	
	Extracted by NH_4-EDTA	62 %	58 %	63 %	57 %	73 %	66 %	
Copper	Nominal Concentration	68	99	151	237	380*		
	Extracted by HCl	76 %	96 %	94 %	86 %	93 %		
	Extracted by NH_4-EDTA	65 %	86 %	82 %	69 %	80 %		
Nickel (with cobalt)	Nominal Concentration	24	39	65	108	180	300	500
	Extracted by HCl	97 %	97 %	82 %	94 %	81 %	89 %	75 %
	Extracted by NH_4-EDTA	68 %	75 %	68 %	73 %	66 %	84 %	70 %
Cobalt (with nickel)	Nominal Concentration	24	39	65	108	180	300	500
	Extracted by HCl	57 %	73 %	65 %	63 %	64 %	84 %	68 %
	Extracted by NH_4-EDTA	51 %	73 %	65 %	63 %	64 %	84 %	68 %

* - Higher concentrations due to copper present in soil additives.

VII.5. Discussion

The data have clearly shown that A.troodii and Au. saxatilis can be regarded as hyperaccumulator and non-accumulator of Ni respectively. While both plants accumulated Ni and Co to some degree, uptake of Cu appeared to be restricted. The observations regarding Ni and Co uptake in A.troodii are similar to those made by Morrison (1980) while studying this and other Alyssum species under somewhat similar conditions.

Eskew et al. (1983, 1984) demonstrated that Ni was essential for soybeans (Glycine max L.) and cowpeas (Vigna unguiculata L.), while Brown et al. (1987) concluded the same for barley grains (Hordeum vulgare L.). In Ni-deficient nutrient solutions containing added NO_3^- or

NH_4^+ ions, Eskew et al. (1983, 1984) observed leaflet tip necrosis caused by toxic build-up of urea. This was reduced in beans from seed containing as little as 2.5 ng Ni, and was absent when the seed Ni content was 160 ng. Brown et al. (1987) demonstrated that barley grain containing <30 ng Ni was non-viable. They attributed this response to impairment of maturation processes. Based on the findings of these workers, Ni has been shown to satisfy two of the three essentiality criteria (Epstein, 1972). Thus a rather strong case exists for Ni to be classified as an essential element for higher plants. The work of Eskew et al. (1983, 1984) and Brown et al. (1987) reduces the credibility of the proposal of Timperley et al. (1970) relating restricted metal uptake to internal control and essentiality. Copper is an essential element for higher plants, and is required at a much higher concentration level. Thus its restricted uptake and toxicity, and deficiency symptoms are more readily discerned.

An important feature of the work addressing the essentiality of Ni to higher plants is the extremely low levels of Ni dealt with. All reagents used by Eskew et al. (1983, 1984) and Brown et al. (1987) had to be purified to prevent contamination which would otherwise lead to incorrect conclusions regarding the levels of Ni required by the plant. It would appear that the background levels of Ni in natural soil and experimental media are almost always adequate for the needs of the plant, thus Ni deficiency symptoms are probably rare. It is not known whether Ni is essential for hyperaccumulating plants, particularly as they appear to grow quite well in Ni-poor soils. Reeves et al.(1981) suggested that in such soils however, they may have less resistance to fungal or insect attack and suffer competition from other more vigorous species.

Cobalt has proven to be essential for Rhizobium bacteria that associate symbiotically with legume roots (Reisenauer, 1960), free-living nitrogen-fixing bacteria (e.g Azotobacter species) and blue-green algae (Alloway, 1990). No requirement has been demonstrated in higher plants. Therefore the uptake of inordinately high levels of Ni (and Co) by hyperaccumulating plants may not reflect essentiality but may simply be an adaptation to their natural habitat. However, it has been shown that both elements stimulate vegetative growth in several ways in addition to serving as activators for enzymes (Mishra and Kar, 1974; Shkolnik, 1984).

On the basis of chemical and physiological similarities of Co and Ni, it is possible that one mechanism controls the uptake of both elements, at least in single-element rooting media. Thus one ligand may bind to Ni and/or Co at the soil/root interface, and complexation by another ligand can then effect xylem transport terminating in detoxification in the leaves. Cognisance should also be taken of changes in membrane permeability, the nature of membrane acceptors, and the role of root exudates. The lower uptake of Co by the two plant species relative to Ni in single-element substrates, coupled with the lower metal concentrations in which specimens survived, is indicative of a lower tolerance to this metal.

The higher concentrations of Co in leaves relative to stems and roots (Fig. VIII.2) has also been noted by Gustafson (1956) and Langston (1956). They reported that Co supplied through the root systems tended to accumulate in leaf margins with higher concentrations in young leaves. Hajar (1987) reported that when Thlaspi alpestre was cultivated in metal rich soil the concentrations of Ni, Cu, Mn and Zn in shoots of Thlaspi alpestre were twice as high as in the roots. Similar partitioning was observed in relation to nickel and cobalt.

When both Ni and Co were present in the rooting medium, Co levels in the plant were virtually unchanged from concentrations in single-element experiments. However, the Ni content was reduced. When the metals are present in the media at comparable levels, consideration must be given to the stability of the complexes formed during uptake. Relative to Ni, the greater influx of Co into roots and subsequent higher concentrations in leaves may be the result of a root uptake mechanism that favours complexing of Co over nickel. This could result in the suppression of Ni uptake in order to facilitate simultaneous accumulation of high levels of these two elements. Neither citrate nor malate qualify as suitable ligands as the complexes formed with Co are less stable than the corresponding Ni complexes. Sillén and Martell (1964, 1971) reported stability constants (log K) of 2.86 and 4.83 for $[Co (malate)]^+$ and $[Co (citrate)]^-$ complexes respectively. Stability constants for the corresponding Ni complexes are 3.17 (Sillén and Martell, 1971) and 5.47 (Hedwig et al., 1980) respectively. The log K value for the [Ni (malate)] complex is 3.30 (Campi, 1963).Still and Williams (1980) postulated that selectivity of Ni over Co in Ni-accumulating plants occurs at the soil/root interface. This was based on abundance (and availability) ratios of Ni/Co in serpentine soil and a study of thermodynamic stability of Ni and Co complexes. In 1983, Farago

and Mahmoud showed this postulation to be invalid in the case of Hybanthus floribundus because the Ni/Co ratio in roots and the surrounding soil were found to be the same. Support for the suppression theory presented above comes from work by Cataldo et al. (1978) who observed reduced Ni uptake and transport in soybean plants in the presence of Cu^{2+}, Zn^{2+}, Fe^{3+} and Co^{2+} ions. Further evidence for the selectivity of Co over Ni in transport systems has also been demonstrated in fungi and bacteria (Fuhrmann and Rothstein, 1968; Willecke et al., 1972).

The concentration of Co in serpentine and related soils is usually five to ten times lower than that of Ni (Brooks et al., 1977). However, this relationship is not reflected in the Ni/Co ratio in field specimens of A.troodii, where the Co content is two orders of magnitude lower than that of nickel. Despite the general rule that plants usually accumulate lower amounts of Co than Ni, there are a few exceptions, as for example accumulation of Co by the genus Nyssa (Brooks et al., 1977) where Co concentrations are usually twice those of Ni, and of course, the Cu/Co-tolerant flora of Southcentral Africa (Brooks and Malaisse, 1985), where the Co content is several orders of magnitude higher than that of nickel. The Ni content of such soils is lower than in serpentine.

The pH of the rooting medium was in the range 4.4-5.3 compared to 6.8 reported by Krause (1958) for serpentine soils. The lower pH was a result of the incorporation of peat into the soil because the low adsorptive capacity of pumice would not otherwise have allowed retention of added nickel, cobalt and copper. In this respect therefore, the experimental rooting media represent yet another departure from serpentine soils. The Ca/Mg ratios in both soils and plants were also higher.It was observed that the hyperaccumulating plant (A.troodii) survived soils with available concentrations of Ni and Co at least five times higher than those found in most serpentine soils. At the lower pH of the media used in this work, the levels of plant-available Ni and Co were higher than those of serpentines by an even larger factor. This may indicate that, in the natural habitat, plants such as A.troodii are not close to their limits of metal tolerance. Alternatively, the effects of the increased Ni and Co concentrations in the pot trials may have been ameliorated by the improved nutrient status of the media. The less marked accumulation of Co by A. troodii, is most likely attributable to substantially lower) Co concentrations found in serpentine soils. Thus some credibility must be given to the selectivity theory of Still and Williams (1980). It has been reported that at low pH, Ni and Co become more available to the plant resulting in increased metal uptake

(Alloway, 1990).

From Fig. VII.5, VII.6 and VII.7, it can be observed that the highest metal concentrations in the organs were found in plants growing in artificial media with metal contents in excess of those in which maximum biomass yields were achieved. There is therefore a concentration lag between maximum yield and maximum metal uptake. Beyond this point, the plants showed decreased vigour and biomass. Chlorosis was also evident, particularly in specimens of Au. saxatilis growing in media containing added nickel and cobalt. Leaf size in these plants was similar to that of the controls, but root size was drastically reduced.

Hunter and Vergnano (1953) found that the effectiveness of trace elements to produce chlorosis in oats followed the order Ni > Cu > Co > Cr > Zn > Mo > Mn. Dekock (1955) noted that this was the same as the order of stability of organo-metallic complexes reported by Irving and Williams (1953). Crooke and Knight (1955) demonstrated that chlorosis can be related to changes in the Ni/Fe ratio, and Bollard (1983) showed that cobalt toxicity symptoms are linked to Fe deficiency symptoms. Mengel and Kirkby (1987) observed a similar inverse association between copper and iron. Similar conclusions were reached in this work based on results of statistical correlation analysis of the data. There was a very highly significant inverse relationship ($p < 0.001$) between Co in the substrate and Fe in the leaves of A.troodii. Highly significant inverse relationships were also observed between Fe in plants and the Cu and Ni content of the rooting media.

The high extractability of Cu into 2 M HCl compared to Ni and Co is somewhat unusual in view of its reportedly high affinity for humates in soil organic matter (Stevenson and Fitch, 1981). It would appear that interaction with dilute HCl significantly reduced the Cu/organic matter association. Beveridge and Pickering (1980) reported that at pH > 6, "soluble" Cu species exist. This would account for the high degree of extractability observed with NH_4-EDTA compared to nickel and cobalt. The results indicate that in experimental soils, the order of interaction between organic matter and the three metals is Ni > Co > Cu. The sequence is similar to the Irving-Williams series for organo-metallic complex stabilities.The interaction between these metals and pumice is relatively insignificant owing to the poor adsorptive power of the latter. It is interesting to note that

the extraction of Ni improved in the presence of a comparable amount of cobalt. This is suggestive of a greater affinity of Co for soil organic matter in the presence of nickel.Support for this suggestion comes from the observed lower availability of Co in the presence of Ni. Cobalt appears to compete with Ni for the exchange sites on the surface of the organic matter. It may be recalled that plant uptake did not reflect this relationship; Co was accumulated to a greater extent than Ni when both metals were present.

The availability of various metals in natural soils to plants has captured the attention of scientists for many years. As a result, a wide range of extractants has been reported in the literature. The more common ones are 0.5 M acetic acid, 0.5 M ammonium nitrate, neutral 1 M ammonium acetate, 1 M ammonium chloride, neutral 0.05 M ethylenediaminetetraacetic acid (EDTA), diethylenetriaminepentaacetic acid (DPTA), dilute acid and water (Spence, 1957; Soane and Saunder, 1959; White, 1971; Proctor et al., 1981). Many of these are specific for certain metals. In view of the artificial nature of the experimental soils, and the fact that the experiments were carried out for 90 days under controlled conditions, the adsorptive capacity of the peat was considered the major factor influencing metal availability. Leaching and diffusion during irrigation may have made a minor contribution. Given the longer sample preparation time and consistent 20 % reduction in metal extractability with NH_4-EDTA, dilute acid extraction was considered adequate for this work.

The results for the germination tests carried out on A.troodii are similar to those of Mishra and Kar (1974), and Welch (1981). They reported that Ni at low concentrations has a stimulatory effect on germination. This effect was observed in wheat grains (Triticum aestivum L.), peas (Pisum sativum L.) and castor seeds (Ricinus communis L.). Small increases in germination percentage of A.troodii were observed over a wide range of added nickel. In Au.saxatilis, the toxic effects of Ni, Co and Cu became apparent at lower concentrations than was the case with A.troodii. Copper did not appear to stimulate germination of seeds of either species as evidenced by the appreciable decline in percentage germination over the range of Cu concentrations used. Several factors must be considered before attempts can be made to rationalise the germination behaviour observed. Among these are morphological differences between seeds (e.g thickness of the seed coat), seed viability, growth conditions (e.g the nutrient status of the medium), and in the case of the tolerant plant, the age of the seedling at which metal tolerance mechanisms become operative.

Like the Becium homblei seeds studied by Howard-Williams (1976), a substantial number of seeds of both species germinated in media containing distilled deionised water only.

The findings from the germination tests are in agreement with those of pot trial experiments, i.e the tolerance to nickel is greater than to cobalt and copper. The high levels of Ni in the plants represented genuine uptake of the heavy metal and not just redistribution of the original Ni in the seeds.

CHAPTER VIII

Concluding Discussion

The aim of this research was to carry out phytochemical studies on two Ni-hyperaccumulating plants from the Philippines, and to compare Ni, Co and Cu uptake characteristics of a Ni-hyperaccumulating plant with those of a non-accumulating plant, both being cultivated under identical controlled conditions. The results of these studies have already been discussed in the relevant sections. In this chapter, the observations will be summarised and their implications discussed. Recommendations for further research will be made in view of the findings to help create a better understanding of Ni-hyperaccumulation.

VIII. 1. Summary and General Conclusions

In the first part of this thesis, extraction of leaf material from D.gelonioides subsp. tuberculatum with distilled deionised water and dilute HCl showed a large proportion (92%) of the Ni to be associated with highly polar ligands, of low molar mass. This was also observed in stems of P.'palawanensis'. These findings are suggestive of Ni complexation mainly in the cytoplasm as opposed to the cell wall. About 50 % of the Ni in leaves in P. 'palawanensis' showed this association, while 25 % appeared to be in the form of pectates or bound to proteins.

Using gel filtration chromatography, two Ni-rich components were observed in aqueous extracts derived from each plant. Nickel was shown to exist in cationic and anionic forms based on the results of ion-exchange chromatography and high-voltage electrophoresis. Assignment of fractions of plant Ni to the two forms was eventually considered meaningless, owing to the changes observed in the relative amounts of cationic and anionic Ni during ion-exchange chromatography and high-voltage electrophoresis. Nickel-rich extracts from P. 'palawanensis' were more prone to this behaviour. This prompted a molar mass determination of the supposedly cationic and anionic forms of Ni , to ascertain whether different forms (complexes) actually existed. Such action was not deemed necessary for the cationic and anionic forms of Ni in D. gelonioides subsp. tuberculatum, as ion-exchange chromatography and high-voltage electrophoresis results were less ambiguous. Molar masses of 348 and 292 were obtained for cationic and anionic

complexes respectively suggesting two distinct complexes.

It will be recalled that obtaining an aqueous extract from P. 'palawanensis' proved rather difficult, and that the extract was not amenable to gel filtration chromatography using Sephadex G-10. Infact, it was more practical to subject the sample to ion-exchange chromatography prior to gel filtration chromatography. Since one of the aims of this research was to identify possible Ni-binding ligands in the plant extracts, a modified extraction scheme was sought to ensure that an adequate quantity of purified extract was available. It was appreciated that modification would require the use of at least one other solvent which should preferably be of high polarity and low molar mass. Moistening of the ground leaf material with 95 % ethanol followed by aqueous extraction provided material suitable for gel filtration chromatography. However, it is questionable whether the Ni complexes detected in such an extract were the same as those previously detected.

Citric, malic and tartaric acids were identified in purified Ni-rich material obtained from D. gelonioides subsp. tuberculatum and P. 'palawanensis' using GC-MS. Several workers have detected citric and malic acids in aqueous extracts from other Ni-hyperaccumulating plants. Two other acids, 4-oxopentanoic acid and 2-furylacetic acid, were found in the P. 'palawanensis' extract. The Ni/citric acid/malic acid mole ratios were 1:0.4:1 and 1:0.4:0.4 in D.gelonioides subsp. tuberculatum and P.'palawanensis' respectively. While all the Ni in D. gelonioides subsp. tuberculatum can be complexed by malate, in P. 'palawanensis' a substantial portion of the Ni can only be complexed by malate and citrate as a ternary complex. Owing to the difficulties encountered during extraction and purification procedures, work on P. 'palawanensis' was discontinued at this point in the research.

When the crude, aqueous extract from D.gelonioides subsp. tuberculatum and a solution containing Ni, citric acid and malic acid in the mole ratio 1:0.4:1, were subjected to gel filtration chromatography, the elution volumes of the two components detected were similar. The component that eluted second was identified as hexa-aquo Ni based on comparison of its elution volume with that obtained for a solution of hexa-aquo Ni. The 1:0.4:1 complex therefore eluted first. There was a notable difference in the proportion of Ni distributed between the two components in the

mixtures, and also in purified extract. A solution containing Ni, citric acid and malic acid in the mole ratio 1:1:1 behaved similarly to a 1:1 Ni-citric acid solutionon the Sephadex G-10 column. Almost all the Ni present appeared to be complexed as citrate. This was not the case for the 1:0.4:1 mixture; a substantial portion of the Ni appeared as the hexa-aquo ion. During this chromatographic study, the weights of Ni applied to the column were similar and a constant volume was used to facilitate reasonable comparisons of elution behaviour.

Despite the presence of excess malate in the 1:0.4:1 Ni-citric acid-malic acid solution, the species that crystallised was the more stable anionic 1:1 Ni-citrate complex. This was evidenced by the close similarity of unit cell parameters of the crystal to those obtained for a crystal derived from a solution of the 1:1 Ni-citrate complex. Unfortunately, the Ni-rich powder from D.gelonioides subsp. tuberculatum was unsuitable for X-ray crystallography, and a sufficient quantity was unavailable for further purification. Similar unit cell parameters would be expected for a crystal from a solution containing Ni-citric acid-malic acid in mole ratio 1:0.4:0.4 as in the case of P. 'palawanensis'.

Subjection of the synthetic solutions of the Ni complexes referred to earlier, and plant extracts, to high-voltage electrophoresis before and after gel filtration chromatography, provided further evidence of the lability of the Ni species being considered. It may be concluded that as the Ni complexes are diluted and concentrated during extraction and separation procedures, they undergo a continuous change in the degree of dissociation. Confirmation of this comes from the results of an investigation of the effect of Ni concentration and column height on the adsorption of Ni in a 1:1 Ni-citrate solution. The tendency of free Ni to predominate was observed to increase as the Ni concentration decreased and the height of the cation-exchange column increased. It seems likely that similar observations could be made with respect to anion-exchange chromatography and gel filtration chromatography. An explanation for the change in ionic form of Ni, alluded to earlier has therefore been presented.

With respect to the possible role of amino acids in Ni complexation, aspartic acid, threonine, glutamine and proline may be considered candidates in W.monophylla. Proline may warrant some consideration in P. 'palawanensis'. The low levels of amino acids in D. gelonioides

subsp. tuberculatum ruled out the possibility of them being involved in Ni complexation. While proline levels in cultivated A.troodii plants were high compared to other amino acids, little difference was discerned with increasing plant Ni concentration. Proline may therefore not be considered a possible Ni ligand in A.troodii.

The level of fluoride in leaves of D. gelonioides subsp. tuberculatum was in the range 19-25 µg g^{-1}. Organic fluoride was calculated to comprise 78-91 % of this, the remainder being inorganic fluoride. This Ni-hyperaccumulating plant is therefore not a fluoride accumulator. There is therefore no evidence of a constitutional accumulatory capacity for nickel and fluoride within the genus Dichapetalum. Small amounts of fluoride can be found in the sulphide minerals galena and sphalerite. The detection of fluoride in D. gelonioides subsp. tuberculatum is probably due to the presence of minute quantities in the Ni-sulphide minerals, millerite, pentlandite and heazlewoodite known to associate with serpentine soils.

No urease activity was detected in extracts from A.tenium, A. troodii, D. gelonioides subsp. tuberculatum and P. 'palawanensis'. There appears to be no correlation between elevated Ni levels and urease activity in these Ni-hyperaccumulating plants.

In the second part of this thesis, pot trial experiments showed that Au. saxatilis did not possess the ability to accumulate Ni to the same extent as A. troodii. The amount of Co taken up by A. troodii, like Ni,was an order of magnitude higher than that taken up by Au. saxatilis over the range of substrate concentrations used. In comparison to Ni, the amount of Co taken up by both plants was an order of magnitude lower. Very little difference was observed in the tolerance to, and uptake of, Cu in the plants investigated. The levels of this metal in A.troodii were about one-tenth those of Co, while in Au.saxatilis, the levels of Cu and Co were comparable. The cobalt-accumulating ability demonstrated by A.troodii confirms the findings of Morrison (1980). He also observed Co-hyperaccumulation in A.corsicum, A. heldreichii, A. murale, A. pintodasilvae and A. tenium under conditions similar tto those used in this research.

The potential of Ni-hyperaccumulating plants to hyperaccumulate Co was observed when Co was present in a more available form than is normal in serpentine soils. Lower pH and improved nutrient status of the experimental soil were seen as contributory factors. Alyssum troodii survived soils with available concentrations of Ni and Co at least five times higher than those found in most serpentine soils. This may also be rationalised in terms of the characteristics of the experimental soil. The same pattern may well be found among the other Ni-hyperaccumualting taxa comprising >150 species of various genera.

When both plants were cultivated in soil containing equal amounts of Ni and Co, Ni uptake was reduced. The Co uptake from these soils was only slightly higher than that observed in soils containing added Co only. The greater influx of Co in roots and leaves in the presence of Ni is surprising in view of the observed lower availability of Co to the plant. Since both plants exhibited this tendency, then, a mechanism favouring Co uptake over Ni uptake at the root must be operative. The presence of a chelating agent in the root membrane having a greater affinity for Co is therefore implied. Citrate and malate are probably not involved in Co transport across the root membrane as the resulting complexes are less stable than the corresponding Ni complexes.

The highest metal concentrations found in A.troodii and Au.saxatilis plants growing in artificial media were in excess of those in which maximum biomass yields were attained. A detoxification mechanism, that becomes operative at plant metal concentrations in excess of those for which maximum biomass yields were recorded, could explain the survival of individuals at higher concentrations, albeit with slightly lower yield. Substantially reduced yields may then be attributed to a breakdown of the detoxification mechanism.

Both plants demonstrated the rise-to-saturation form of metal uptake for Ni and Co. However, saturation point did not appear to be reached in the case of Cu. With respect to A.troodii, the results of the pot trial experiments were reinforced by those of the germination tests, i.e the tolerance to Ni is greater than to Co and Cu. Cobalt appeared to exert a greater stimulatory effect on germination of Au.saxatilis seeds compared to Ni. The high levels of Ni in the plants represented genuine uptake from the substrate, not redistribution of Ni originally present in the seeds.

VIII.2. Recommendations for Further Research

In the light of the findings of this research, it is recommended that the following avenues of research be undertaken in future.

a) Alternative methodology should be sought for unequivocal identification of cationic, anionic and neutral Ni species in plant extracts. The improved scheme could then be applied not only to *P.'palawanensis'* but also to *Hybanthus floribundus*, (Australia), in which Ni is also reported in association with pectates (Farago *et al.*, 1975). Failing this, caution must be exercised in interpreting the results obtained from the techniques which have been commonly used.

b) Since the complexation of Ni by citric and malic acids cannot account for extreme uptake of Ni, although both acids can be used internally for Ni storage and transport, a study of root exudates from Ni-hyperaccumulating plants should be encouraged. This approach could unveil the role of other ligands e.g amino acids and proteins, in Ni uptake at the plant/soil interface. According to Still and Williams (1980), it is the uptake method from soil to root that must be selective. In a discussion based on equilibrium constants, these workers pointed out that a protein with at least two N donor atoms may be involved in the uptake mechanism from soil (or xylem) to root membrane (or cell membrane). They argued against the need for high selectivity in the xylem (or cytoplasm) because of the necessity to transport high levels of other metals for plant growth.

c) The literature does not reveal the detection of 4-oxopentanoic acid, 2-furylacetic acid or similar acids in Ni-rich extracts from Ni-hyperaccumulating plants. The work of Morrison (1980), to which reference has been made, has not been published. Given the putative role of such compounds in glucosinolate metabolism, it would be advisable to carry out comparative studies of glucosinolates in Ni-hyperaccumulating plants and non-accumulating plants preferably within the same genus.

The detection of tartaric acid in Ni-rich extracts of the two Philippine hyperaccumulators should not be ignored. Although the acid is reported to be of minor metabolic importance, it should be quantified, in view of the relatively high stability (log K = 9.9) of the [Ni-(tartrate)] complex (Van Uitert and Fernelius, 1954).

d) Due to the association of fluoride with sphalerite (Zn,FeS), subspecies of D. gelonioides which hyperaccumulate Zn should be analysed for fluoride to investigate the possibility of fluoride accumulation. Other questions relating to a constitutional accumulatory capacity within the genus may be answered by performing pot trial experiments. Worthwhile experiments would be i) the cultivation of African fluoride-accumulating members of Dichapetalum in Ni-rich soil and ii) the cultivation of Zn- and Ni-accumulating members in fluoride enriched soil.

e) Although there appeared to be no urease activity in the small number of Ni-hyperaccumulating plants assayed, it would be interesting to ascertain whether the same result will be obtained for Ni-hyperaccumulating plants in other genera. Owing to its association with urease, several workers have reported Ni to be an essential micronutrient for N_2 metabolism in higher non-accumulating plants (Eskew et al.,1983,1984; Brown et al., 1987) There appears to be no evidence of this in higher Ni-hyperaccumulating plants. A survey of Ni-hyperaccumulating genera for urease activity could prove worthwhile in this respect.

f) The ability of Ni-hyperaccumulating plants to hyperaccumulate Co raises questions regarding multiple and co-accumulation of metals in plants viewed as hyperaccumulators of one metal. Further studies should be encouraged on species that are considered non-accumulators of Co and Cu. Hyperaccumulators of Co (Brooks and Malaisse, 1985) may well demonstrate the capacity to take up inordinately high levels of Ni under certain conditions.

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Appendix I

a)Fertiliser Composition

ELEMENT	PERCENTAGE (w/w)	
	OSMOCOTE (3-4 month)	P.G. MIX
N	14	14
P	6.1	7.0
K	11.6	14.9
Cu	Nil	0.12
B	Nil	0.03
Mo	Nil	0.20
Mn	Nil	0.16
Zn	Nil	0.04
Fe	Nil	0.09

b)Composition of 1:1 Peat/Pumice Diluent Prior to the Addition of Metal

COMPONENT	MASS (g) PER KILOGRAM OF PEAT/PUMICE
Lime	1.88
Dolomite	1.88
P.G. Mix	0.35
Oscomote (3-4 month)	1.13

Appendix II

AAS Operating Conditions

ELEMENT	WAVELENGTH (nm)	LAMP CURRENT (mA)	SLIT WIDTH (nm)	BACKGROUND CORRECTION
Ni	232.0	4.0	0.2	ON
Ni	341.5	4.0	0.2	ON
Fe	372.0	7.0	0.2	OFF
Cu	324.7	3.0	0.5	ON
Mn	403.1	5.0	0.2	OFF
Co	240.7	5.0	0.2	ON
Zn	213.9	2.0	0.5	ON
Mg	202.5	2.5	1.0	ON
Ca	422.7	10.0	0.5	OFF
Na	589.6	-	0.2	-
K	769.6	-	0.5	-

NOTE:

Operating conditions were the same for both the IL 457 and GBC 905 instruments with two exceptions; a) the former was not equipped with a background correction system and b) calcium was determined using a N_2O-acetylene flame with a red feather on the IL instrument, but with an oxidising air-acetylene flame on the GBC instrument. In the latter case, lanthanum was present in both standards and samples at a level of 0.1 %.

Appendix III

a) Concentration of Metals in Plants Cultivated in Soils Containing No Added Nickel, Cobalt or Copper.

METAL	CONCENTRATION ($\mu g\ g^{-1}$)				
	Alyssum troodii			*Aurinia saxatilis*	
	LEAVES	STEMS	ROOTS	LEAVES	ROOTS
Ni	16.8	16.0	20.0	1.09	2.61
Fe	78.5	40.8	72.6	30.1	69.1
Cu	5.10	5.31	13.5	2.23	3.84
Mn	286	259	41.4	138	43.6
Co	11.9	6.30	8.69	0.23	0.33
Zn	64.1	162	218	6.57	59.7
Mg	5669	3544	1996	9398	2769
Ca	38789	28936	4951	22462	3558
Na	261	431	1131	1068	1146
K	21545	30233	19873	7395	8753

b) Concentration of HCl (2 M)-Extractable Metals in Soils Containing No Added Nickel, Cobalt or Copper.

METAL	CONCENTRATION ($\mu g\ g^{-1}$)
Ni	1.80
Fe	3750
Cu	14.5
Mn	97.8
Co	1.40
Zn	38.4
Mg	973
Ca	2416
Na	422
K	342

Appendix IV

Elemental Relationships in Cultivated A.troodii.#
(Significances calculated from correlation coefficients. Negative signs indicate inverse relationships)

a) Nickel-enriched Soil

	Soil Ni	Ni	Fe	Cu	Mn	Co	Zn	Mg	Ca	Na
Fe	-S**									
Cu	-S	-S*	S							
Mn	S*	S*	-S*	-S*						
Co		S*		-S	S**					
Zn		-S**								
Mg	S**		-S							
Ca							-S**			
Na							S**		-S**	
K										S

b) Cobalt-enriched Soil

	Soil Co	Ni	Fe	Cu	Mn	Co	Zn	Mg	Ca	Na
Fe	-S**									
Cu	S**		-S**							
Mn	S									
Co	S**		-S**	S**						
Zn		-S**								
Mg		S*	S							
Ca				S*	S*					
Na				-S		S				
K									-S	S

- There was insufficient data on Au.saxatilis to enable meaningful use of correlation coefficients

Legend:

S** = P≤0.001

S* = 0.01≥ P> 0.001

S = 0.05≥ P≥ 0.01

Continued overleaf

Appendix IV continued

c) Copper-enriched Soil

	Soil Cu	Ni	Fe	Cu	Mn	Co	Zn	Mg	Ca	Na
Fe	-S**									
Cu	S**									
Mn			S**							
Co										
Zn					S**					
Mg	S**		-S	S**						
Ca	-S**			-S	S			-S*		
Na										
K			S							

d) Nickel- and Cobalt-enriched Soil

	Soil Ni	Soil Co	Ni	Fe	Cu	Mn	Co	Zn	Mg	Ca	Na
Fe											
Cu											
Mn											
Co	S**										
Zn				-S*			S				
Mg											
Ca	S*						S				
Na											
K								S*			S*

Legend:

S** $= P \leq 0.001$

S* $= 0.01 \geq P > 0.001$

S $= 0.05 \geq P > 0.01$

Publications Arising from this Thesis

Homer, F.A, Morrison, R.S, Brooks, R.R., Clemens, J. and Reeves, R.D. (1991) Comparative studies of nickel, cobalt and copper uptake by some nickel hyperaccumulators of the genus Alyssum. Plant and Soil (in press).

Homer, F.A., Reeves, R.D., Brooks, R.R. amd Baker, A.J.M. (1991) Characterisation of the nickel-rich extract from the nickel hyperaccumulator Dichapetalum gelonioides. Phytochemistry **30** (7), 2141-2145.