PHYTOCHEMICAL AND BIOGEOCHEMICAL STUDIES ON NICKEL ACCUMULATION BY SOME NEW CALEDONIAN PLANTS.

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A B S T R A C T

A herbarium survey of Homalium and Hybanthus species was successful in the detection of a number of accumulators of nickel. The term 'hyperaccumulator' was defined as those plant species that contain more than 1000 µg/g of nickel in their leaves on a dry weight basis. Their ability to indicate areas of specific geology and their use as indicators in mineral exploration was evaluated. All new hyperaccumulators of nickel that were 'discovered' were confined to the ultramafic complex in New Caledonia. The possible evolutionary significance of nickel accumulation in the order Violales is noted.

Plant-soil relationships for three New Caledonian hyperaccumulators of nickel; Hybanthus austrocaledonicus, Homalium kanaliense and Homalium guillainii were investigated, and the relationship between nickel uptake and the uptake of other elements examined statistically. The lack of interelement relationships, both stimulatory and antagonistic, of nickel with other minerals pointed to a mechanism of accumulation dependent on organic constituents, with the absence of competition from other ions indicating the specific nature of the absorption at the root.

The highly unusual accumulation of nickel in the 'latex' type exudate from the trunk of Sebertia acuminata is reported. A nickel citrato complex was isolated from the exudate and leaves of this species and identified by the use of spectrophotometry, high-voltage paper electrophoresis and gas-liquid chromatography - mass spectrometry. The complexed nickel comprises approximately 40% of the total weight of the crude latex. Citric acid was also implicated in the chelation of nickel in several other New Caledonian accumulators. Extraction procedures indicated that the nickel citrate was located primarily in the vacuoles.

Nickel citrate extracts from the plant material were compared with various synthetic nickel citrate solutions by electrophoresis and spectrophotometry. Evidence for a 2 : 1 anionic citrate - nickel complex was obtained for solutions in which the mole ratio of citrate to nickel approached and exceeded 2 : 1.

Gas liquid chromatography coupled with mass spectrometry was used in the identification of organic acids occurring in the investigated plants. High malic and citric acid concentrations were found in the hyperaccumulator species. A good correlation between citric acid and nickel content in the leaves of a number of New Caledonian plants growing over ultrabasic substrates was found.

Although nickel is translocated, and exists in the plant cell as a citrate complex, it appears unlikely that it is absorbed at the roots as such. A more specific mechanism based on the carrier concept is postulated to account for the specificity of the absorption.

Nickel accumulation in the Rhodesian hyperaccumulator Pearsonia metallifera is also described. Another organic acid rather than citric acid is involved with nickel chelation. Mass spectral evidence indicated an uncommon structure. A positive identification could not be made.

A C K N O W L E D G E M E N T S

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1.

Introduction

The location of nickel (atomic number 28) in the midst of the row of essential trace elements that extends from vanadium to zinc has suggested to some authors (Mertz, 1970; Schroeder, 1965; Schroeder and Nason, 1971; Shaw, 1960) that nickel is also likely to be an essential element. Some workers have concluded that nickel is essential for animal nutrition at least (Nielsen, 1971; Nielsen and Ollerich, 1974; Sunderman *et al.*, 1972a). Nomoto *et al.*, (1971, 1973) and Sunderman *et al.*, (1972b) have confirmed the existence of a nickel-containing metalloprotein (nickeloplasmin) in human and rabbit serum. Shaw (1960) and Mertz (1970) have noted that nickel is especially suited for a biochemical role as it readily undergoes transitions among several co-ordination structures. To date however, despite its wide distribution in plants, there is little firm evidence that nickel is essential for plants in the strict sense that, in the absence of the element, growth is inhibited.

Nickel is ubiquitous in the lithosphere and biosphere, constituting about 0.008% of the earth's crust. By far the largest part is in igneous rocks of which nickel constitutes approximately 0.01%. Among igneous rocks, ultramafic (or ultrabasic) rocks, containing iron and magnesium, and little or no silica, are the principal sources of nickel. Nickel concentrations range from 0.016% in basalts and gabbros to an average of 0.20% in peridotites. Figure 1.1 shows the average nickel content of various sedimentary and igneous rocks.

Nickel ore deposits are formed by magmatic segregation of ultramafic rocks in which nickel is concentrated. The nickel may be incorporated in sulphide or silicate minerals. Pentlandite ($(Fe, Ni)_9S_8$),

chalcopyrite ($Cu\ Fe\ S_2$), and pyrrhotite ($Fe_x\ S_{x+1}$) are sulphide minerals commonly found in nickel ore deposits. Deposits also occur in lateritic material formed by the weathering of (ultramafic) ferromagnesian silicate rocks. Some of the nickel occurs in the mineral garnierite, a hydrous nickel magnesium silicate.

The mean nickel content of vegetation is about 0.5 µg/g in the dried material or 10 µg/g in the ash, whilst soils normally contain nickel in the range 5 - 500 µg/g. However soils derived from serpentinites and other ultramafic rocks, may contain up to 8,000 µg/g, as in the case of some very humic, iron-rich peridotite residual soils found in New Caledonia. Vanselow (1966) has reviewed the nickel content of field-grown crops and several species of natural vegetation, and for most plants reported a range of 0.05 to 5.0 µg/g in the dry matter. In a comprehensive report on the geochemistry of some rocks, soils and plants in the United States, Connor and Shacklette (1975) give background nickel concentrations in a number of matrices ranging from unconsolidated geologic deposits to plant ash. In over twenty-five widespread plant species they observed a concentration range from less than 2 µg/g up to 1,300 µg/g.

The detection of very low levels of nickel in plants has been made possible in the last two decades by the advent of sensitive spectroscopic instrumentation. The most preferred technique utilizes the principle of absorption of energy by valence electrons of ground state atoms produced in a flame and, consequently, the only common interferences encountered are caused by those chemical and physical processes which inhibit the formation of ground-state atoms. Atomic absorption has now become a popular technique for the analysis of complex mixtures as it is specific, and has detection limits in the nanogram range. The recent development of flameless carbon-rod atomization has lent itself particularly to the analysis of extremely small samples.

Because nickel is so widely distributed in plant tissue, it may almost be regarded as a normal constituent, and more than forty years ago Martini (1930) considered it to be so. However, early reviews on the mineral nutrition of plants (Hewitt, 1951, 1963; Nason and McElroy, 1963; Bollard and Butler, 1966; Gauch, 1957) while dealing in some depth with the importance of many elements in plant nutrition, give little or no information on the role of nickel. Most of the considerable literature on the role of nickel in plants, deals with its toxicity and fungicidal properties. Much of this stems from the fact

that nickel is present at toxic levels in serpentine and other ultrabasic soils, and this has been examined with regard to their general infertility. Mishra and Kar (1974) have reviewed the role of nickel in plant growth and metabolism, and the National Academy of Sciences (1975) has reviewed the medical and biologic effects of nickel.

Ever since Haselhoff (1893) showed that excessive amounts of nickel were toxic towards plant life, the implications of high nickel levels in serpentine soils have been widely discussed. There is still no satisfactory explanation for the role of nickel in plant uptake from serpentine soils, and there is a need to clarify the mechanisms of tolerance of certain species towards high nickel concentrations. The enigma of nickel accumulation by certain indicator and accumulator plants is a related problem, and some facets of this phenomenon are investigated in the present work.

The need to study more fully plant adaptation to toxic soils, and mechanisms for tolerance, arises from the need to utilize areas of high heavy metal concentration, such as old mine dumps and industrial sites, and, more importantly, the tracts of barren serpentine land scattered throughout the world. The reclamation of toxic metalliferous wastes has been studied by Smith and Bradshaw (1970) and recently Hill (1973) has had considerable successes in the rejuvenation of Rhodesian mine - dumps through the use of heavy metal tolerant populations of plants.

It is important that an acceptable means of measuring tolerance is available. Ernst (1972) used the method of Repp (1963) to measure the plasmatic resistance of various populations of Indigofera setiflora and I. dyeri against increasing concentrations of zinc sulphate, copper sulphate and nickel nitrate. His results demonstrated that the metal tolerance of plants is highly specific for individual heavy metals. Ernst was able to assign a resistance limit against zinc, copper and nickel for tolerant and non-tolerant populations of Indigofera species. This limit was highest for populations found growing over anomalous areas.

A more acceptable technique for measuring tolerance, however, involves measuring root growth with various concentrations of the metal in question added to calcium nitrate nutrient solution and expressing this as a percentage of the increase in root growth of controls in pure calcium nitrate. This method has been used by various workers (Wilkins, 1957; Jowett, 1958, 1964; Proctor, 1971; Wu and

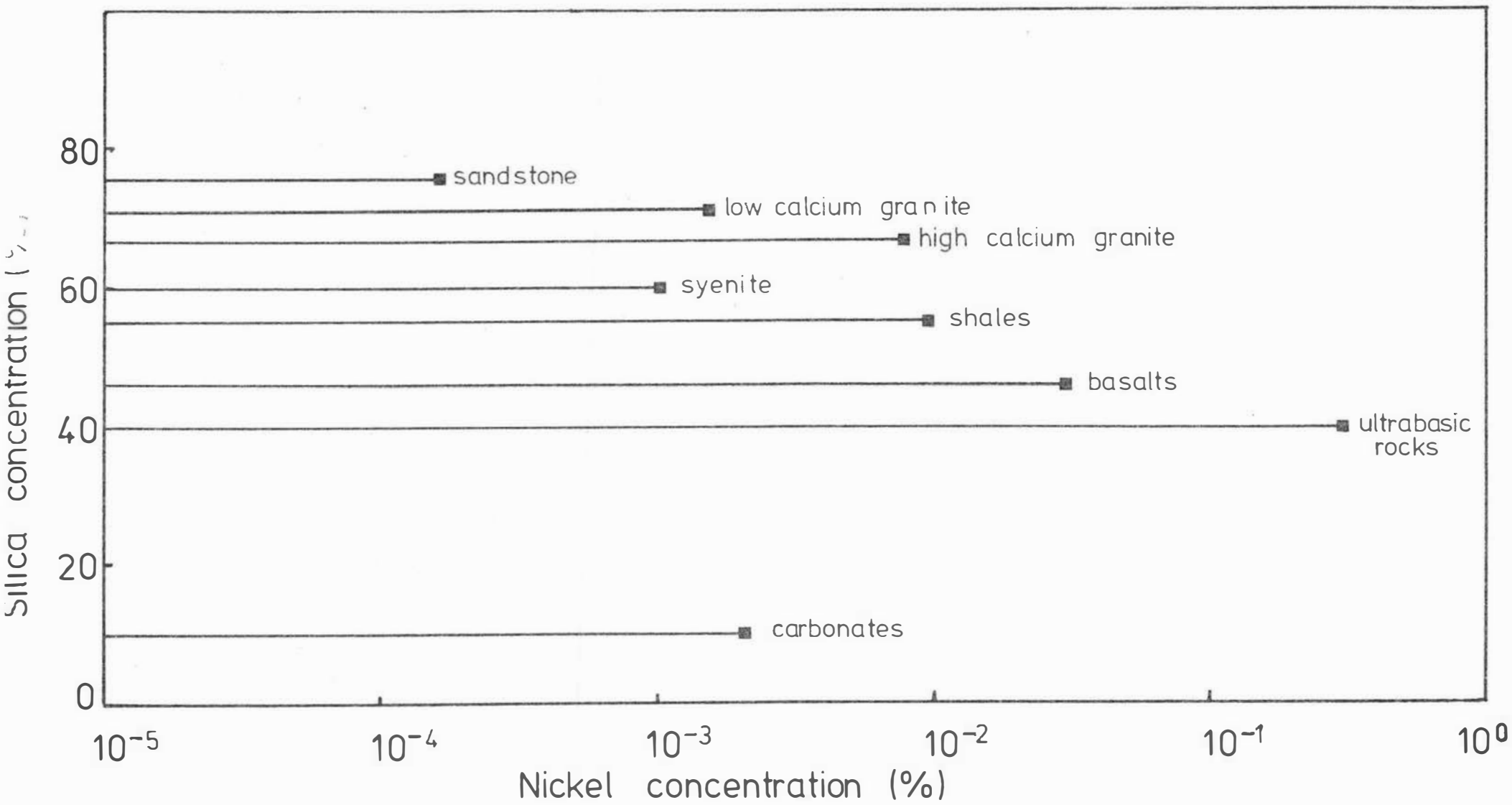


Figure 1·1 : Average nickel content in various sedimentary and igneous rocks. (Turekian, 1963)

Antonovics (1976) to demonstrate that populations growing on toxic soils were more tolerant to the metal in question than populations of the same species growing on normal substrates. Proctor (1971) showed that the roots of the serpentine Agrostis populations grew much better in the toxic solutions than the non-serpentine populations investigated. Of the tolerances investigated by Jowett (1958) and Gregory and Bradshaw (1965), nickel is the least specific. Recently Craig (1977) reported an improvement on the rooting technique for measuring heavy metal tolerance of grass roots by a method based on probit analysis. The method was used to compare the toxicities of nickel, copper, lead and zinc to roots of Zea mays L. The order of toxicity was found to be Cu > Ni > Pb > Zn. The adaptation of a serpentine population of Loudetia to nickel was demonstrated.

Ernst (1974) has claimed that heavy metal tolerant plants have no mechanism of preventing heavy metals entering the cell, except by developing a low exchange capacity in the roots. The significant feature of metal tolerance is the prevention of heavy metals from exerting disturbances in the metabolic system of the cells. In leaves and stems the cell vacuole system is the main place of heavy metal deposition. Ernst also noted that the physiological basis of tolerance is the increase in the production of malate and oxalate in zinc-tolerant plants and of phenolic compounds in copper-tolerant populations. It was suggested that these properties fulfilled the conditions of the high specificity of heavy metal tolerance, according to the different stability constants.

The literature shows that nickel is widely recognized as being harmful to a variety of plants at relatively low concentrations. The main symptoms are chlorosis or yellowing of leaves followed by necrosis. Stunting of roots and shrubs, deformation of various plant parts and unusual colourations, may also be manifested in cases of nickel poisoning. The extreme case is the death of the whole plant (Hewitt and Bolle-Jones, 1952; Vergnano, 1953; Crooke and Knight, 1955).
On serpentine soils, toxic effects of nickel may be reduced by limiting its availability to plants. This may be achieved by raising the pH by liming, as shown by Hunter and Vergnano (1952), Vergnano (1953), Chang and Sherman (1953) and Crooke (1956). Halstead, Finn and Maclean (1969) found that the amounts of extractable nickel in soils, and the concentrations of nickel in the plants, were reduced by liming of the soils and were increased by the addition of phosphate. Plants

growing on soils with a high level of exchangeable nickel have a high content in their tissues. For example, the exchangeable nickel content of the basin and hill soils of Whitecairns (Scotland) are 26-61 µg/g and 22-49 µg/g, respectively. Depending on the exchangeable nickel content, oats grown in these soil types contain 17-134 µg/g and 16-51 µg/g nickel respectively (Vergnano, 1953).

Relatively few plants are able to accumulate concentrations of nickel exceeding approximately 15 µg/g on a dry weight basis, unless they are growing over ultrabasic rocks, in which case nickel concentrations up to 100 µg/g may be found (Jaffré, 1976; Lyon et al., 1970; Severne, 1972; Lounamaa, 1956). Many species appear to be tolerant of elevated nickel levels in the soil and apparently possess certain mechanisms that exclude nickel from enzyme-active sites, the poisoning of which is thought to be the most important toxic action (Bowen, 1966). Populations of certain plant species on serpentine soils have been shown to differ genetically from those growing on non-serpentine soils. The populations represent edaphic ecotypes. The serpentine races are adapted to their substrate with its high $^{Mg}/Ca$ ratio and high concentrations of nickel, chromium and cobalt. Kruckeberg (1951) grew plants of serpentine and non-serpentine populations of Gilia capitata on serpentine soil. Those from the non-serpentine location failed to grow.

Epstein and Jefferies (1964) pointed out that the mechanisms which bring about selective ion absorption and transport in plants do not apply only to the roots. Transfer of the ions into the conducting tissues, resulting movement in the xylem and phloem, and absorption by cells in stems and leaves are all implicated. Studies with mutants and varieties can be useful in sorting out the several steps. The uptake and translocation of elements such as nickel by plants is of considerable interest, and the need to identify the carriers of this and other elements has been noted by Tiffin (1972).

Considerable controversy exists about the mechanisms of ion uptake by plants, and there is an extensive literature dealing with the various hypotheses. Much of the data available support the idea that ion transport in plants is an active metabolic process and is highly selective. Recent evidence seems to favour the carrier theory of absorption over the Donnan system or the electropotential gradient hypothesis. In contrast to the other theories, the carrier theory requires the impermeability of the membrane to free ions. Specific carriers for individual ions or closely related ions are proposed to

account for the high degree of selectivity of the ion absorption process (Epstein, 1960, 1962). Moore (1972) points out that the three hypotheses are not mutually exclusive.

Hewitt (1951) suggested a way in which non-essential elements might after all be involved in metabolism. The stimulatory effects of non-essential elements reported from time to time, may have been due to their beneficial role as enzyme activators when the normal element was deficient in the plant. Dixon et al., (1975) have adduced evidence which strongly indicates that urease isolated from jack beans (Canavalia ensiformis) is a nickel metalloenzyme, and that nickel may well be an essential trace element in jack beans.

The work summarized in the following chapters deals with various ecological and phytochemical aspects of a group of highly unusual plant species which are able to accumulate remarkably high levels of nickel. The large concentration of nickel in their tissues simplifies the problem of isolating and identifying nickel complexes from various species. If this can be achieved, it may be possible to establish some reasons as to why these plants accumulate so much nickel, and to find what differentiates them from non-accumulating neighbours growing on the same substrate, and from other species that tolerate only very low concentrations of nickel in the soil.

2.

Biogeochemical Survey

of

Nickel Accumulating Plants

2.1 INTRODUCTION.

Adaptation of plants to environments where there are marked departures from more normal conditions, such as high salinity, extremes of pH, or unusually high concentrations of various elements, is an interesting phenomenon. The ability of plants to grow under adverse conditions, such as those imposed by mineralization, has been widely studied, but there are many points of interest that have still to be fully explained. The tolerance of plants to high metal concentrations depends on their ability either to exclude the elements from their tissues, or to withstand them at accumulated levels. The mechanisms involved appear to be specific for any one element,as tolerance to one element does not necessarily hold for others.

Practically all the elements of the periodic table may be found in vegetation, and it is probable that for every one of these elements, there is at least one species that will accumulate it to an unusual extent. In many cases more of the element is found in the ash of the plant than in the soil. Sometimes even the content expressed on a dry weight is higher than that of the soil. Such species are known as 'accumulator plants.' Plants growing over ore bodies or areas of general enrichment of a certain element e.g. ultrabasic areas, may accumulate elements from areas where they occur at only normal concentrations : e.g., Amanita for selenium, brown algae for iodine and Nyssa sylvatica for cobalt (Gerloff, 1963). In both types of accumulator plants mechanisms

for tolerance of the accumulated element are presumed to be present e.g. 'extra-cellular deposition, formation of crystals within the cell, specific organo-metallic complexes, formation of unusual metabolic products.....' (Bollard and Butler, 1966).

This tolerance of plants to elevated concentrations of various elements, especially transition elements, has led to the techniques of geobotanical and biogeochemical prospecting. The first method involves visual observations, whilst the second involves chemical analysis of soils and vegetation. The fundamental factor that makes biogeochemical surveys possible is that some chemical expression will be reflected in the vegetation growing over a mineralized zone. Certain species have the ability to concentrate elements from the soil selectively whilst others endeavour to restrict their uptake. Plants growing on serpentine (formed from the alteration of ultramafic rocks) must be tolerant of high nickel and chromium. Brooks (1972) observed that indicator communities, or characteristic floras, such as those manifested on serpentine, will not necessarily indicate mineralization directly, but may delineate areas in which it occurs. Brooks (1972), Malyuga (1964) and Levinson (1974) may be consulted for a detailed study of biogeochemical methods.

The physiological basis for the tolerance of indicator plants to high concentrations of particular elements is still largely unexplained. The ash of many nickel accumulator plants can contain in excess of 10% nickel (Minguzzi and Vergnano, 1948). Moreover, the plants often develop better in ultrabasic environments than under normal conditions. In 1947, Minguzzi and Vergnano (1948) detected very high concentrations of nickel in Alyssum bertolonii Desv. from the Impruneta region near Florence. They reported 1.55% nickel oxide in the leaves, a level at least an order of magnitude greater than in other plants of the same area. The nickel content of the soil was 0.33%. They concluded that nickel may have a role in geobotanical prospecting for nickel. Malyuga (1964) wrote that species, such as A. bertolonii from Italy and A. murale from the U.S.S.R., which are well adapted to nickel-ore zones, and can grow normally in such areas, do not grow well under normal soil conditions. Such species would be ideal as indicators of nickeliferous areas. Menezes de Sequeira (1969) reported 0.52% nickel in another species of Alyssum, A. serpylifolium Desv. var. lusitanicum from Northeast Portugal, which was predominant in areas where the

biological and edaphic balance had been upset by excessive tillage, causing an increase in soil erosion.

Other biogeochemical investigations by Severne (1972), Severne and Brooks (1972) and Cole (1973) have revealed the unusually high nickel-accumulating ability of Hybanthus floribundus which, when growing over nickeliferous areas, may contain 1% nickel on a dry weight basis. This species also grows over sedimentary substrates and, as expected, the nickel content in the leaves of these plants is considerably less. Only 110 µg/g was found in a specimen growing in South Australia, but a soil concentration of 30 µg/g revealed the plant's accumulating ability. Cole (1973) concluded that while the nickel content of this species was related to the concentrations in the soil, it did not necessarily delineate nickel sulphide ore deposits. It may however be useful in indicating nickeliferous environments and hence has some value in biogeochemical prospecting.

Wild (1971) described Dicoma niccolifera which grows almost exclusively over serpentinitic substrates in a small area of Rhodesia and at one locality in Zambia. This species, which has the ability to take up as much as 0.21% nickel (dry weight) is an indicator of some anomalies, but its value as an universal indicator is somewhat weakened because of its occasional occurrence on non-nickeliferous soils.

By far the largest number of nickel accumulating plants so far discovered comes from New Caledonia. An appreciable amount of geobotanical work has been done by French workers at O.R.S.T.O.M. (Organisation de la Recherche Scientifique et Technique Outre-Mer). New Caledonia has one of the largest ultrabasic areas in the world, with a serpentine flora including a large proportion of endemic species. This flora contains several hyperaccumulators of nickel, (hyperaccumulator in this study referring to all plants containing > 1000 µg/g nickel on a dry weight basis, irrespective of the concentration in the soil). In addition to those discovered in a survey reported in this part of the thesis, other species reported include: Geissois pruinosa, Homalium guillainii and Psychotria douarrei (Jaffré and Schmid, 1974); Homalium kanaliense, Hybanthus austro-caledonicus and Hybanthus caledonicus (Brooks, Lee and Jaffré, 1974). These species are found mostly over ultrabasic substrates high in nickel, chromium, cobalt and iron.

The presence of several species of the genera Homalium and Hybanthus in the list of accumulators raises the question as to whether the nickel accumulating ability of these genera is a world-wide characteristic, or whether it is confined to New Caledonian and Australian species. Knowing the existence of other ultrabasic areas throughout the world, and the fairly widespread dispersion of these two genera throughout the tropics, sub-tropics and warm-temperate zones, it was considered that some other members may also exhibit this nickel accumulating capacity. A detailed study of the variation of nickel content in species of Homalium and Hybanthus could be made via the analysis of herbarium specimens. Herbaria throughout the world contain well over 200 million plant specimens which have been collected over the past 150 years. To date these specimens have seldom been used for other than taxonomic work. If the co-operation of herbaria could be obtained, the task of collecting many of the 240 Homalium and 150 Hybanthus species would be simplified.

Analysis of herbarium specimens would be a simple, rapid and inexpensive method of carrying out a world-wide survey of the nickel status in members of these two genera. Very little work of this nature seems to have been done in the past. Persson (1956) examined specimens of 'copper mosses' at present held in many university and government herbaria in Sweden. He noted the locality from which they were obtained and then re-examined the location by other prospecting methods. He did not however analyse the material chemically. Similarily, Cole (1971) identified cuprophytes over a copper deposit in southern Africa and then checked herbarium sheets for collection localities of other specimens of these species, again without analysis of the herbarium material. One of the earliest reports detailing the analysis of phanerogams from herbaria was given by Chenery (1948) who analysed aluminium in over 4000 herbarium specimens. His method, however, was only semi-quantitative and required a leaf sample of over 6 cm^2 in area. More recent work by Chenery and Sporne (1976) completed the investigation of representatives of all the 259 families of dicotyledons and has increased to 37 the number in which aluminium accumulators have been recorded.

Rühling and Tyler (1969) analysed various heavy metals in

samples of moss Hypnum cupressiforme collected in Skåne, Sweden from 1870 - 1943 and compared historical trends in the concentrations of these elements with levels in present day specimens. From these studies, Tyler (1970) showed how it was possible to use herbarium mosses for the study of historical changes in the deposition of heavy metals. He also noted that the risks of possible secondary contamination should be observed and controlled. More recently, Goodman and Roberts (1971) analysed bryophytes from herbaria to monitor atmospheric pollution.

The paucity of early work involving the analysis of herbarium specimens for elemental concentration possibly reflects the fact that only in recent years has it been possible to analyse samples sufficiently small to satisfy the requirements of herbarium curators. The size of sample needed for classical methods of analysis had been so large (5-10g of leaf material) that obviously valuable collections of herbarium specimens could not be disturbed. For example, when C. Minguzzi and O. Vergnano approached the Florence herbarium for material for analysis over twenty years ago, the request was rejected because even the emission spectrographic method of analysis proferred involved consumption of too large a sample (Pers. Comm. O. Vergnano Gambi to R.R. Brooks, 1977). With the use of atomic absorption spectrophotometry, and particularly the development of the even more sensitive carbon-rod atomizer attachment, it is now possible to determine concentrations of several elements using extremely small sample sizes ($< 1\ cm^2$ in area). With this the size of sample, the necessary herbarium assistance proved to be forthcoming.

For the work reported here, more than 50 herbaria throughout the world were approached for small samples of leaf material from their collections of Homalium and Hybanthus. Thirty five of these institutions supplied material (see Appendix I). These samples were analysed for the nickel content with two principal aims in mind : firstly to find additional nickel accumulators, knowing the existence of others in the genera, and secondly to assess the ability of certain species to delineate known and perhaps unknown nickeliferous areas. Although the nature of the substrate is often unknown for herbarium specimens, studies on known nickel accumulators (Severne and Brooks, 1972: Brooks, Lee and Jaffré, 1974: Jaffré and Schmid, 1974) have shown that nickel values

over 1000 µg/g dry weight are only associated with ultrabasic areas. These and other studies have also shown that values above 100 µg/g were practically always associated with ultrabasic areas. Only occasional exceptions have been found, these being principally for plants growing over laterites not overlying ultrabasic rocks, where values of up to 200 µg/g have been recorded (Severne, 1972). Those plants growing over acidic rocks were not expected to accumulate much more than 15 µg/g dry weight unless they were actual nickel accumulators. It was hoped that the analysis of a sufficient number of each species would enable a distinction to be made between nickel levels associated with ultrabasic environments and those of acidic substrates.

2.2 DISTRIBUTION AND ECOLOGY OF HYBANTHUS AND HOMALIUM

2.2.1 Hybanthus Jacquin (Violaceae)

Approximately 150 Hybanthus species (Willis, 1973) are known from the tropics and sub-tropics, with a few species extending into temperate zones. Africa, India, Malaysia, America and Australia all record native species. The main development of the species is in Central and South America, and the number of species obtained from herbaria seems to reflect this. Schulze (1936) notes that in Brazil, Paraguay and Uruguay there are about 45 species. About 20 species occur in Central America and the South-eastern United States, and 7, including endemics, in the West Indies. Bennett (1969) states that in the Old World the genus is not so common. Only one species, H. enneaspermus is widespread throughout West Africa and Australia. According to this author, there are 11 species in Africa, 5 species in tropical Asia and Indo-Malaysia, 4 species in New Caledonia and 11 species in Australia. (Present classification records only two species in New Caledonia : H. austrocaledonicus and H. caledonicus). Bennett (1969) considers that the genus has undergone no 'recent' development and that present-day distribution patterns have existed for a long time. Hybanthus is notably absent from the young oceanic islands. Species included in the subgenus Euhybanthus are restricted to Central America, the West Indies, South-eastern North America and New Caledonia, whereas species of the subgenus Ionidium are distributed

throughout the tropics, with the exception of New Caledonia. Schulze (1936) says that the subgenus Euhybanthus was probably more widespread when New Caledonia belonged to the mainland, and that the South American and New Caledonian species represent the relics, thus explaining their absence from 'recent' oceanic islands.

Jacobs and Moore (1971) have described the genus Hybanthus which is based on that given by Schulze (1936). Generally the species are small gnarly shrubs, or half shrubs and herbs, with a few trees up to 8m. In a few species the twigs are thorny and microphyllous. The leaves are often herbaceous, sometimes leathery, and vary in size from small needles to 15 by 4 cm broad-leaves.

Roots of several species of Hybanthus have been noted to store inulin-like fructans instead of starch (Kraus, 1879, cited by Jacobs and Moore, 1971). Three nickel accumulators in this genus are so far known - H. floribundus, Western Australia (Severne and Brooks, 1972), H. austrocaledonicus and H. caledonicus, New Caledonia (Jaffre' and Schmid, 1974). Bennett (1969) has divided H. floribundus into s.sp. floribundus, curvifolius, and adpressus. All these subspecies are hyperaccumulators of nickel when growing over nickeliferous substrates. Severne (1972) recorded nickel levels of 1.6%, 0.7% and 0.13%, respectively in the leaves of these plants. He also noted 0.02% nickel in H. epacroides s.sp. bilobus growing over sandstones in Western Australia.

2.2.2 Homalium Jacquin (Flacourtiaceae)

The genus Homalium is confined to tropical and sub-tropical countries and comprises about 240 species, most of which belong to lowland rainforest. Of the 24 species known in the Pacific (all of which are endemic,) about 16 belong to New Caledonia. Sleumer (1954, 1972, 1974) has done much of the work on the classification of the Flacourtiaceae to which the genus Homalium belongs. Sleumer (1974) states that the delineation of natural species in Homalium is difficult, and only a sufficient number of specimens of a species allows one to recognize clearly the extremes of shape and size of the floral parts. There are also pairs of morphologically closely related species, (ecological forms may be suggested) with separate areas of distribution. Sleumer noted that the collection of new species from intermediate localities might bring about a re-evalu-

ation of the existing treatment. Thus the closely related species H. francii and H. guillainii from New Caledonia may well be named as one when these species are studied in more detail.

It is of interest to note that the families Flacourtiaceae and Violaceae both belong to the same order (Violales) and at present no chemical characters are known which contradict the generally accepted relationship between these two families. (Hegnauer, cited by Jacobs and Moore, 1971).

To date all the known Homalium species that accumulate nickel in amounts exceeding 1000 µg/g have come from New Caledonia. The two noted in publications prior to this work are H. guillainii (Vieill.) Briq. (Jaffré and Schmid, 1974) and H. kanaliense (Vieill.) (Brooks, Lee and Jaffré, 1974). Maximum nickel values recorded in the leaves of these two species were 2.90% and 0.90% respectively. These two species differ considerably, both in nature and habitat. H. guillainii is a tall tree growing in dense rainforest, whereas H. kanaliense exists as a small xerophyte shrub found growing in scattered bush overlying laterites. The ecology of these species has been detailed by Jaffré and Latham (1974), Jaffré, Latham and Quantin (1971).

2.3 WORLD WIDE SURVEY OF THE GENERA HOMALIUM AND HYBANTHUS

2.3.1 Collection and analysis

Herbaria throughout the World were approached for small samples (~1 cm^2 or 0.03g) of leaf material from their collections of Homalium and Hybanthus. About 50 herbaria were contacted and those that responded with samples are listed in Appendix I. On arrival of the specimens, collector's reference and location data were recorded, and dried leaf samples with an average weight of approximately 0.03g were placed in 5 ml borosilicate test tubes and ignited at 500°C in a muffle furnace. Samples were sufficiently ashed within a two hour period for easy dissolution. The ash in each tube was dissolved in 10 ml of 2M hydrochloric acid which had been prepared from redistilled constant boiling hydrochloric acid. For every sample, a new, unused test-tube was used so as to minimize the risk of possible contamination. In some cases slight warming was required to effect dissolution. The solutions were analysed for nickel and cobalt by atomic absorption spectrophotometry using a small diameter aspirator tube to economize on

solution. The most sensitive nickel line (232nm) was selected for most analyses and scale expansion was usually required. Corrections for non-atomic absorption were made using a hydrogen continuum lamp with an automatic background corrector being employed for later work. For those samples where flame atomic absorption, even with scale expansion and optimum conditions, proved insensitive the carbon rod atomizer, with its increased sensitivity and ability to use samples of smaller size, could be used. All data were expressed on a dry weight basis. An approximate conversion factor of 15 may be used to convert to concentrations on an ash weight.

2.3.2 Analytical results

The co-operating herbaria provided 1926 specimens for analysis, including 121 Homalium and 103 Hybanthus species. Approximately 75% of all species had nickel levels below 15 µg/g and these are shown, along with total number analysed and their mean nickel content, in Table 2.3.1. The mean value of all Homalium species in this category was 4.45 µg/g and that for the Hybanthus species 4.34 µg/g. It is unlikely that these species are accumulators of nickel. These levels may be taken to represent those normally found in vegetation as a whole on non-nickeliferous substrates.

Figures 2.3.1 and 2.3.2. show the geographical distribution of the two genera. The Homalium species are confined almost entirely to the tropics, the exceptions being those on the east coast of South Africa and the two specimens from central China. The Hybanthus species, however, extend to the limits of the warm temperate zone (i.e. 40° north and south of the equator). Only samples with well defined collection localities were included in the figures. The nickel concentrations for all localities are represented by one of four symbols depending on the concentration range i.e. < 15 µg/g, 15-100 µg/g, 100-1000 µg/g or >1000 µg/g. The figures clearly show the predominance of New Caledonia as a source of nickel hyperaccumulators.

Figure 2.3.3 shows those species of which at least one specimen had an anomalously high nickel level (> 15 µg/g dry weight). The number of specimens analysed and the range of nickel values found are shown. Broken lines indicate extremely high values differing by a factor of two from the remainder of the range. There are some cases where unusually low values are anomalous and these are also shown by broken lines. Strong accumulators

Table 2.3.1 *Homalium* and *Hybanthus* species with nickel concentrations less than 15 µg/g (dry weight).

Homalium		
Species.	Total No.	Mean Ni (µg/g)
acuminatum Cheesem.	2	4.5
albiflorum O. Hoffm.	2	4.3
alnifolium Hutchinson & Dalziel	3	9.2
amplifolium Gilg.	1	6
aneityense Guillaumin	1	5.4
anzoateguiense Steyerm.	1	2.3
axillare Benth.	2	2.3
aylmeri Hutchinson & Dalziel	1	6.4
bailloni S. Elliot	1	3.0
barandae Vidal ex Villar	3	4.5
bhamoense Cubitt & W.W. Smith	4	6.1
brachybotrys F. Muell.	3	4.8
brachystylum Baill.	2	3.4
bracteatum Benth.	3	4.3
brevidens Gagnep.	2	3.0
brevipetiolatum	1	2.9
bullatum Gilg.	4	4.3
buxifolium Daniker	1	0.7
caryophyllaceum Benth.	8	4.5
celebicum Koord.	2	4.0
cochinchinense Druce	11	3.4
damrongianum Craib,	2	7.0
densiflorum Spruce ex Benth.	5	1.9
dewevrei Wildem. & Th. Dur.	3	7.2
dolichophyllum Gilg; Hutchinson & Dalziel.	3	7.4
elegantulum Sleum.	1	4.8
erianthum Baill.	1	4.9
eurypetalum Blake	1	7.0
fagifolium Benth.	4	2.1
fallax van Slooten	1	2.8
fasciculatum S. Elliot	1	0.9

continued...

Table 2.3.1. Homalium continued....

frutescens Warb.	1	6.8
fulviflorum Sleum.	1	13.3
gentilii Wildem.	1	0.9
grandiflorum Benth.	6	2.6
griffithianum Kurz	6	7.2
guianense (Aubl.) Oken	23	3.1
hainanense Gagnep	6	1.5
hosei Merrill	3	5.3
involucratum O. Hoffm.	1	2.6
laurentii De. Wild	1	7.6
laurifolium A.C. Smith	2	3.5
laxiflorum Baill.	2	7.0
le-testui Pellegr.	6	7.9
loheri Merrill	2	7.8
louvelianum H. Perrier	1	6.4
luzoniense F. Villar	3	2.7
macrophyllum	1	0.7
macropterum Gilg.	1	12.8
mattogrossense Malme	1	2.5
micranthum O. Hoffm.	1	6.4
microphyllum O. Hoffm.	1	4.8
molle Stapf.	2	4.3
mossambicense Paiva	1	2.1
myriandrum Merrill	2	5.3
myrianthum Gilg; Gilg ex Engl. Mildbr.	1	5.7
nitens Turrill	2	15
nudiflorum Baill.	3	6.7
obovatum Merrill	1	13.8
oppositifolium Baill.	1	1.3
paniculatum Benth.	2	2.5
parkeri Baker	1	3.5
pedicellatum Spruce ex Benth.	19	3.8
petelotti Merrill	1	7.6
pittieri Blake	2	2.9
planiflorum Baill.	1	2.7
propinquum C.B. Clarke	1	5.6

continued...

Table 2.3.1. Homalium continued....

ramosii Merrill	1	1.3
riparium Gilg.	2	2.0
rufescens Benth.	18	4.4
sarcopetalum Pierre	4	1.7
smythei Hutchinson & Dalziel	6	4.5
sorsogonense Elmer	1	0.5
stenophyllum Merrill & Chun	5	1.6
stipulaceum Welw.	13	5.3
tetramerum Baker	1	5
tomentosum Benth.	9	3.8
trichostemon Blake	5	4.1
vatkeanum O. Hoffm.	1	1.2
viguieri H. Perrier	1	0.4
zeylanicum Benth.	2	2.5

Hybanthus.

Species.	Total No.	Mean Ni (µg/g)
agateoides Melch.	2	7.8
albus Baill.	5	2.9
angustifolius Standley	2	2.6
anomalus Standley	2	0.5
atropurpureus Taub.	12	3.0
attenuatus (Humb. & Bonpl.)G.L. Schultze.	26	5.3
aurantiacus Melch.	6	5.3
bangii Rusby	2	3.3
bicolor Baill.	7	8.3
bigibbosus Hassler	17	4.5
brevis Standley	1	6
buxifolius Baill.	4	3.0
caffer Engl.	6	1.8
calceolaria (L) G.K. Schultze.	10	2.9

continued...

Table 2.3.1. *Hybanthus* continued.....

calycinus F. Muell.	7	3.7
capensis Engl.	16	6.2
caribaeus Urb.	2	3.6
circaeoides Baill	5	3.3
costaricensis Melch.	2	2.3
debilissimus F. Muell.	1	2.4
densifolius Engl.	4	4.3
domingensis Urb. & Ekman	2	8.5
elatus (Turcz.) Morton	4	2.1
filiformis F. Muell	4	4.5
fruticulosus I.M. Johnston	2	6.0
galeottii (Turcz.) Morton	2	2.8
graminifolius (Chod.) G.K. Schultze.	-	-
guanacastensis Standley	4	4.8
hasslerianus Hassler	5	3.3
heterophyllus Baill.	2	9.5
hieronymi Hassler	7	2.0
hirtus Engl.	6	5.3
ipecacuanha Baill.	28	4.6
lanatus Baill.	7	2.5
lasiocarpus	1	3.1
leucopogon Sparre	1	1.2
linearis (Torr.) Shinners	4	6.5
longifolius Melch.	2	6.9
longistylus G.K. Schultze	2	3.3
mexicanus Ging. ex DC	5	7.2
mocinsanus Morton	2	8.6
monopetalus Domin.	2	1.6
mossamedensis Mendes	1	8.6
natalensis Burtt Davy	3	3.0
nigricans Standley	1	9.2
occultus (Polak.) Standley	1	6.3
paraguariensis (Chod.) G.K. Schultze	3	3.5
poaya Baill.	1	4.2
polygalaefolius Vent.	4	2.6
portoricensis Urb.	5	4.4

continued...

Table 2.3.1. *Hybanthus* continued...

potosinus Morton	1	1.2
ramosissimus Melch	1	11.6
riparius Standley	7	6.1
runyonii Morton	1	1.2
schoenfelderi Dtr.	1	4.7
serratus Hassler	6	1.6
simplex Dtr.	1	2.9
strictus Spreng. ex Steud.	2	7.5
subpoaya G.K. Schultze.	1	13.9
suffruticosus Baill.	6	3.8
sylvicola Standley & Steyerm.	1	2.5
tarapotinus Ule.	4	1.6
tatei F. Muell.	2	3.8
tenellus Dtr.	2	3.5
thesiifolius Hutchinson & Dalziel	1	0.8
thorncroftii Burtt Davy	1	6.3
tricolor Taub.	2	2.8
velutinus G.K. Schultze	1	0.7
verbenaceus Loesen.	6	2.9
vernonii F. Muell.	2	4.0
verticillatus (Ortega) Baill.	16	4.2
villosissimus Taub.	5	2.5

(100-1000 µg/g) and hyperaccumulators (> 1000 µg/g) are clearly marked. All hyperaccumulators except for Hybanthus floribundus are from New Caledonia. The data used for the compilation of Figure 2.3.3 are given in Appendices II and III.

2.3.3 Identification of ultrabasic substrates.

Tables 2.3.2 and 2.3.3 give additional data for hyperaccumulators of nickel. All specimens were taken from different sites and, where possible, the nature of the substrate was determined from a knowledge of the sample locality.

After some years of study of serpentine flora in New Zealand (Lyon et al., 1970; Lee, 1974), Western Australia (Severne and Brooks, 1972) and New Caledonia (Jaffré and Schmid, 1974; Brooks et al., 1974), it has been observed that nickel concentrations over 1,000 µg/g in plants are always associated with ultrabasic areas. Values ranging from 100-1000 µg/g are mostly associated with ultrabasic areas, except occasionally for plants growing over laterites not overlying ultrabasic rocks. In such cases, values up to 200 µg/g have been found. Severne(1972) reports a value of 200 µg/g for a single specimen of Hybanthus epacroides s.s p. bilobus growing on leached quartz sands containing 10 µg/g nickel. Values of 15-100 µg/g are characteristic of serpentine plants and are also typical of hyperaccumulators not growing on nickelferous areas. Values greater than 15 µg/g are uncommon for plants in general growing over normal substrates.

Although this survey was carried out at a very low sampling density over a greater part of the earth between the latitudes 40° N. and 40° S. it delineated many of the World's major ultrabasic areas. (e.g. Cuba, Philippines, W. Australia, New Caledonia and Puerto Rico). Although all these areas are well known, a survey of this nature could possibly be used for the mapping of unknown ultrabasic areas in parts of the globe that are poorly mapped geologically. It is important to note that this survey has not revealed nickel-ore bodies as such but has delineated wider areas where nickel levels are higher than normal, such as in ultrabasic rocks and laterites. In many cases the collector may have been unaware of the nature of the substrate and the value of herbarium specimens for identifying possible ultrabasic areas

Table 2.3.2. Hyperaccumulators (>1000 µg/g dry weight) of nickel.

Species.	Total No.	No. above 1000 µg/g	Locality	Highest Ni. conc. (µg/g dry weight)	Nature of substrate
Homalium					
austrocaledonicum Sleum.	6	4	New Caledonia	1805	ultrabasic
deplanchei Warb.	10	2	New Caledonia	1850	ultrabasic
francii Guillaumin	7	7	New Caledonia	14500	ultrabasic
guillainii Briq.	2	2	New Caledonia	6926	ultrabasic
kanaliense Briq.	6	5	New Caledonia	9420	ultrabasic
mathieuanum Briq.	3	1	New Caledonia	1694	ultrabasic
rubrocostatum Sleum.	2	1	New Caledonia	1157	ultrabasic
Hybanthus					
austrocaledonicus Schinz et Guillaumin	4	4	New Caledonia	13750	ultrabasic
caledonicus (Turcz.) Cretz.	11	2	New Caledonia	5917	ultrabasic for values > 1000 ug/g
floribundus F. Muell.	13	2	W. Australia	6680	ultrabasic for values > 1000 ug/g

Table 2.3.3. Strong accumulators (>100 µg/g dry weight) of nickel.

Species.	Total No.	No. above 100 µg/g.	Locality	Highest Ni. conc. (µg/g dry weight)	Nature of substrate
Homalium					
angustifolium Keay	5	1	Sierra Leone	155	unknown
decurrens Briq.	5	1	New Caledonia	176	various incl. ultrabasic
gitingense Elmer	2	2	Philippines	144	unknown
le-ratiorum Guillaumin	7	4	New Caledonia	643	various incl. ultrabasic
panayum F.Villar	11	1	Philippines	507	ultrabasic
pleiandrum Blake	3	2	Puerto Rico	343	ultrabasic
rubiginosum Warb	1	1	New Caledonia	397	ultrabasic
serratum Guillaumin	6	1	New Caledonia	116	ultrabasic

CONTINUED....

Table 2.3.3. continued...

Hybanthus					
brevilabris Domin.	4	1	W. Australia	229	ultrabasic.
linearifolius Urb.	11	1	Cuba	107	ultrabasic.
malpighiifolius Standley	1	1	Mexico	638	unknown
setigerus Baill.	3	1	Brazil	130	probably ultrabasic
wrightii Urb.	2	1	Cuba	350	ultrabasic
yucatanensis Millsp.	12	1	Mexico	134	unknown

depends on the accuracy with which the collection localities have been given.

2.3.4 The Discovery of New Nickel-accumulating Plants

This herbarium survey has resulted in the discovery of five new hyperaccumulators and thirteen strong accumulators of nickel as well as the 'rediscovery' of all the previously known hyper-accumulators of nickel. They are given in Table 2.3.2 and 2.3.3 The effectiveness of the survey is shown by the fact that all accumulators were found without the necessity of fieldwork. Hybanthus floribundus was found only after the arduous collection and analysis of an entire flora (Severne, 1972) whereas this survey readily revealed its nickel accumulating capacity with the minimum of expense and labour.

The hyperaccumulators new to the literature are : Homalium austrocaledonicum, H. deplanchei, H. francii, H. mathieuanum and H. rubrocostatum; all of which are from New Caledonia. No new hyperaccumulators were found outside New Caledonia but some of the species classified here as strong accumulators may ultimately prove to be hyperaccumulators when further specimens are analysed.

2.3.5 The regional distribution of nickel accumulating plants

Figures 2.3.1 and 2.3.2 , with additional information in Tables 2.3.2 and 2.3.3 , show the regional distribution of nickel accumulators. All hyperaccumulators of the genus Homalium are confined to New Caledonia. Several of the strong accumulators are also found in New Caledonia with other notable species from the Philippines (H. panayum) and Puerto Rico (H. pleiandrum), both from ultrabasic areas. Sleumer (1974) has recognized 16 species of Homalium in New Caledonia. Of these, 7 are hyperaccumulators and 4 are strong accumulators. New Caledonia also plays host to two hyperaccumulators from the genus Hybanthus : H. austracaledonicus and H. caledonicus. The former is found only on ultrabasic substrates but as seen from Figure 2.3.3 the latter is much more widespread in its distribution. Values ranging from 2.0 µg/g to upwards of 5,000 ug/g have been detected in various subspecies of H. caledonicus, the actual nickel concentrations depending on

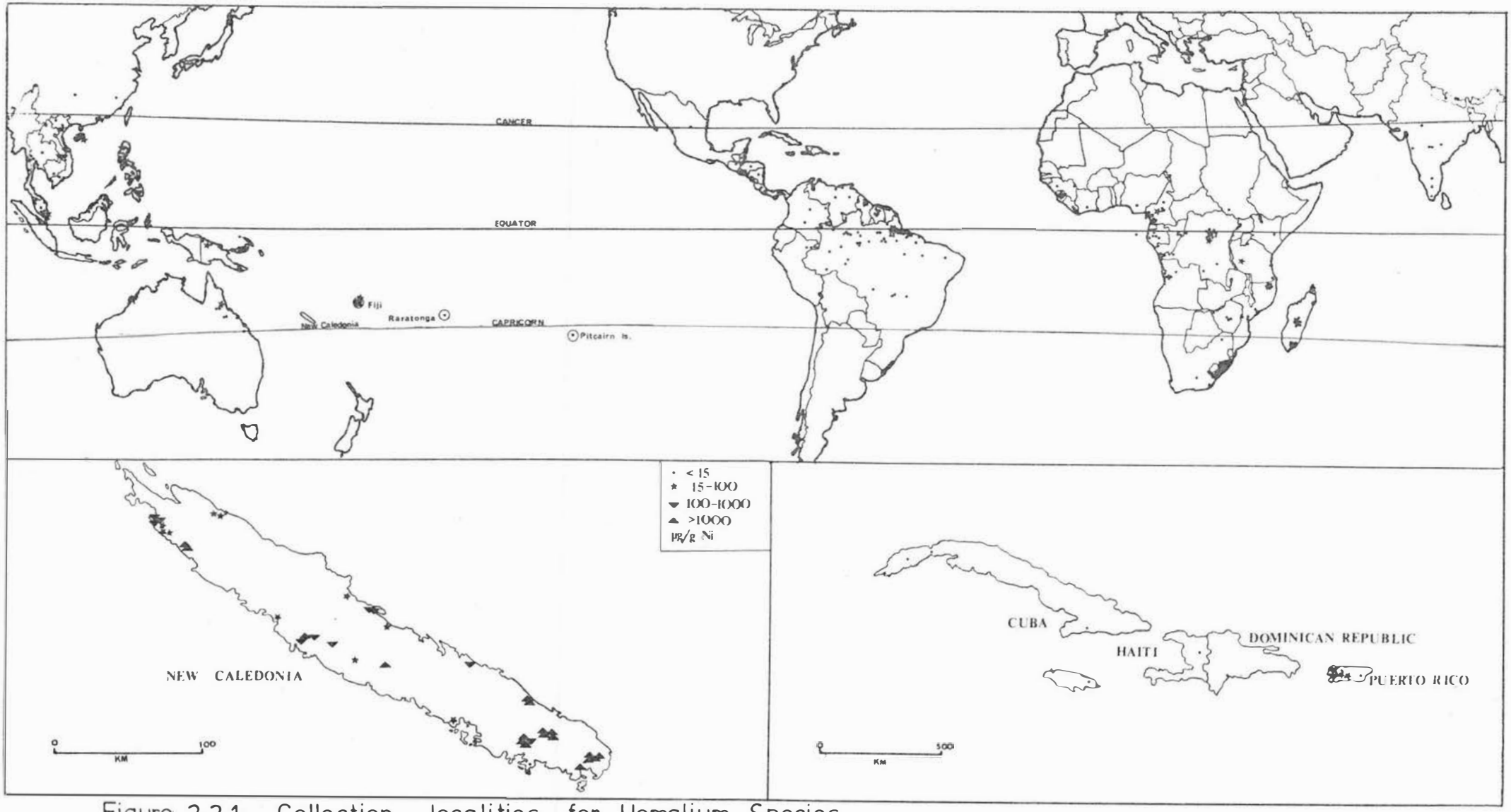


Figure 2·3·1: Collection localities for Homalium Species.

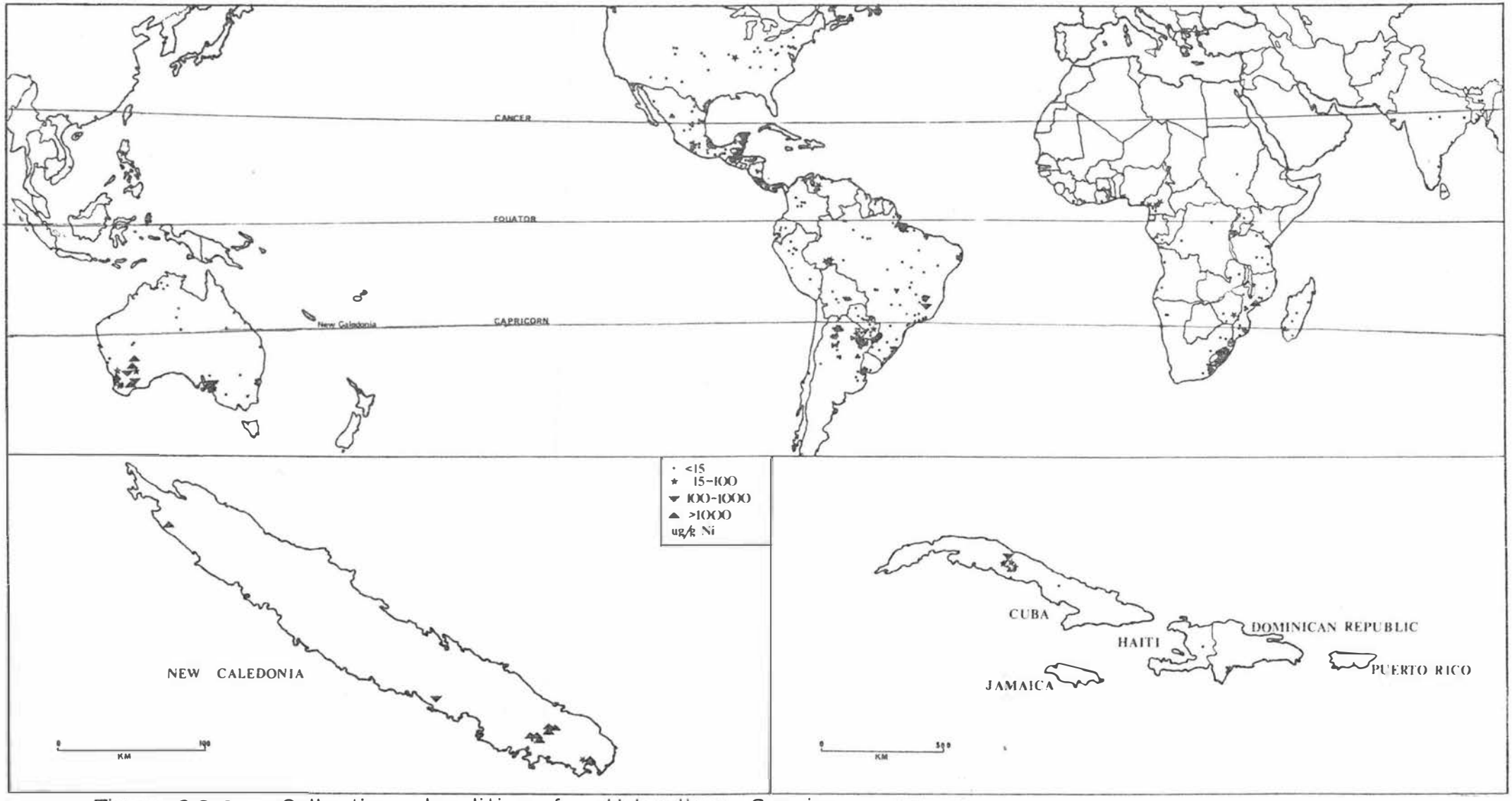


Figure 2·3·2: Collection localities for Hybanthus Species.

whether it is growing on or off ultrabasic substrates.

Outside New Caledonia the only hyperaccumulator of the genus Hybanthus is the well documented H. floribundus (which includes s.sp adpressus and s.sp floribundus). H. enneaspermus had the widest distribution of all species investigated, with specimens occurring in a broad arc from S. Africa, through India and Southeast Asia to northern Australia. Over 100 specimens of H. enneaspermus were analysed.

2.3.6 Conclusions.

The most significant results of this herbarium survey were : the discovery of new nickel accumulating plants, and their indication of areas of a specific geology. Although outside New Caledonia no new hyperaccumulators were found, many of the strong accumulators, with more intensive sampling, may warrant inclusion in the higher grouping.

In the present survey, the nickel content of vegetation was used, not to indicate nickel itself, but to delineate areas of specific geology. To this end it proved successful, particularly in the delineation of ultrabasic areas. The principles used in this survey could be applied to other genera for other elements. Mineral deposits of many elements would be too localised for herbarium surveys to be of use but areas where possible mineralization may occur could be indicated. Ultrabasic rocks in particular are favoured targets for geochemical exploration, since they are often hosts for a wide range of important ore elements such as nickel, chromium, platinum and cobalt.

Since this work, Brooks and Wither (1977) and Wither and Brooks (1977) have successfully used herbarium material to pinpoint previously unknown ultrabasic areas in South East Asia. During this work the nickel accumulating ability of Rinorea bengalensis (Wall) O.K. was discovered. This species can be classified as a hyperaccumulator when found over ultrabasic soils, but grows on a wide variety of soil types throughout S.E. Asia. R. bengalensis has two advantages over all other hyperaccumulators so far recorded: its widespread geographical distribution over S.E. Asia and northern Australia, and the fact that it is very common. It is therefore potentially ideal in defining ultrabasic areas. Brooks

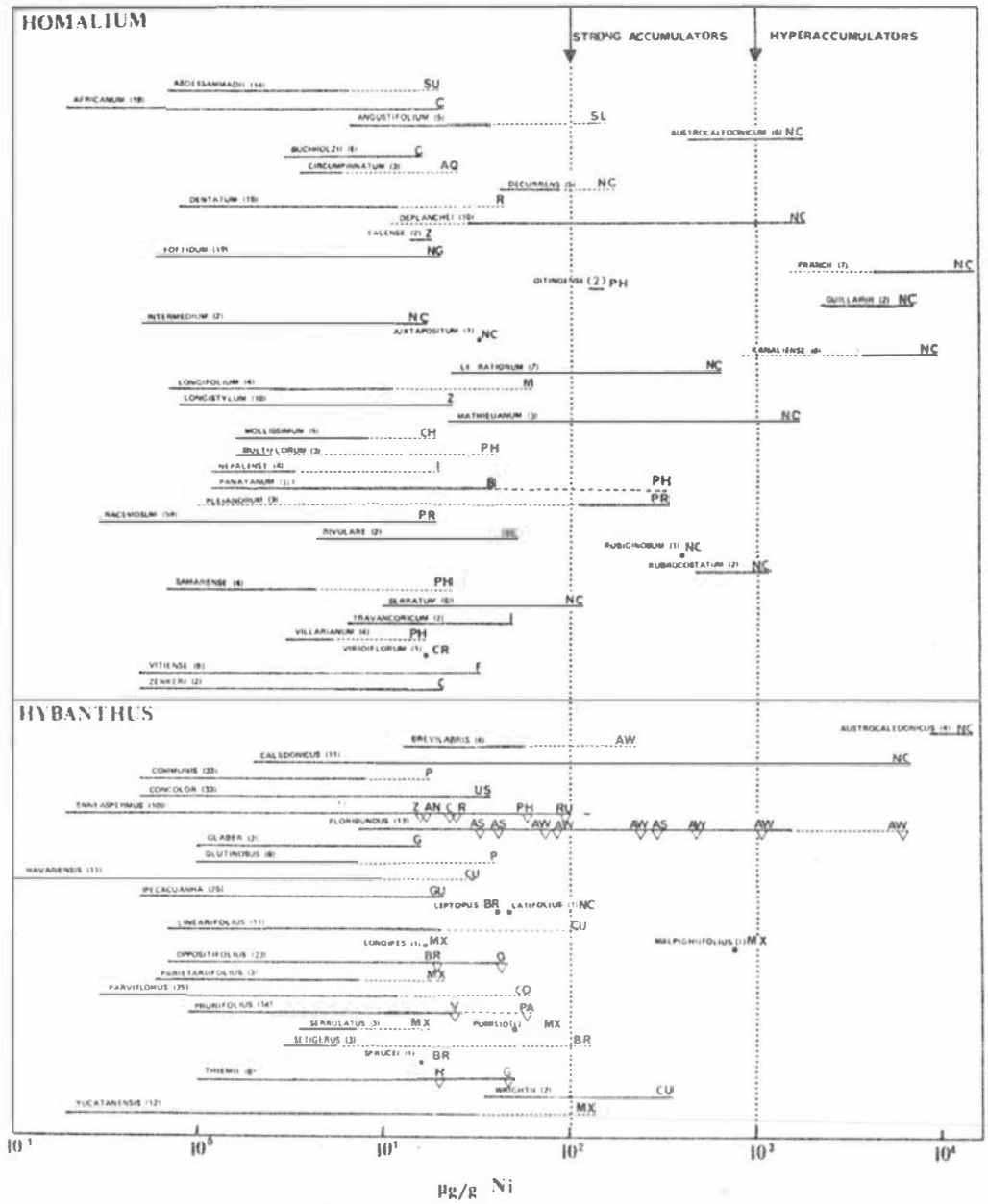


Fig. 2.3.3 Range of nickel concentrations (µg/g dry weight) in *Homalium* and *Hybanthus* species containing at least one value higher than 15 µg/g. Extreme values differing by more than a factor of two from all others are shown by broken lines. For each species, the country or countries of origin of specimens with anomalous values are indicated by the following code: AN = Australia (Northern Territory), AQ = Australia (Queensland), AS = Australia (South Australia), AW = Australia (Western Australia), B = Borneo, BR = Brazil, C = Cameroons, CH = China (Hainan), CO = Colombia, CU = Cuba, F = Fiji, G = Guatemala, GU = Guyana, H = Honduras, I = India, M = Malaya, MX = Mexico, NC = New Caledonia, NG = New Guinea, P = Paraguay, PA = Panama, PH = Philippines, PR = Puerto Rico, R = Rhodesia, RU = Ruanda Urundi, SL = Sierre Leone, SU = Sudan, US = United States, V = Venezuela, Z = Zaïre.

and Wither (1977) report a range of values from 1 - 17,500 µg/g representing three populations indicative of different rock types. Wither and Brooks (1977) also report three other hyperaccumulators of nickel : Planchonella oxyedra Dubard (1.96% nickel); Myristica laurifolia Spruce ex D.C. var. bifurcata (0.11%) and Trichospermum kjellbergii Barret (0.38%), all from Obi Island, Indonesia. Their use in predicting geology is yet to be fully assessed but will depend on their geographical distribution and accumulating ability on different substrates.

So far herbaria have been extremely co-operative in supplying small samples for work of this nature, but if heavy demands are placed upon their resources this attitude may well change. Many collections held by herbaria have been accumulated over a long period of time, and many specimens are irreplaceable. The most serious limiting factor for future surveys of this type could be an unwillingness by herbarium curators to part with valuable material.

It is interesting to note that both Rinorea and Hybanthus belong to the same family (Violaceae) whilst the genus Homalium belongs to the same order as these two (Violales). The concentration of nickel accumulators in a single order points to a genetic influence in the ability of these plants to absorb and translocate large quantities of this element.

2.4 OTHER NICKEL ACCUMULATING PLANTS FROM NEW CALEDONIA.

2.4.1. Sebertia acuminata Pierre ex Baill.

S. acuminata, a member of the Sapotaceae from New Caledonia, is the most unusual example of high nickel accumulation found to date. The species is a tree which reaches 10m in height and is found mainly in the Grand Massif du Sud. In the Tiebaghi Massif in the north of the island it is found more often in forested areas over peridotitic alluvia or colluvia relatively rich in nickel. A typical soil would contain approximately (in percentages) 0.85, nickel; 3, chromium; 0.10, cobalt, 45, iron; 8.5, aluminium; 6.0 silica; 0.02, potassium; 0.06, calcium; and 2.0, magnesium.

S. acuminata is well known in New Caledonia for the blue-green colour of its latex and is therefore known locally as Sève bleue (blue sap). It is endemic to New Caledonia, relatively rare and has never been recorded off ultrabasic areas. In the dense humid forest area in the vicinity of the Rivière Bleue, S. acuminata is the fourth hyperaccumulator of nickel to be found, after Hybanthus austrocaledonicus, Homalium guillainii and Psychotria douarrei (Jaffre' and Schmid, 1974). A more definitive description of this area is given in the next chapter.

Table 2.4.1 shows the nickel concentrations in various organs of S. acuminata. The data are based on the analysis of 3 specimens (one from the Rivière Bleue area, one from the Tontouta area and another from the Forêt Cachée). The three nickel analyses for the latex on a dry weight basis (dried for 1hr at 110°C) were 15.8%, 19.25% and 25.74% respectively.

Table 2.4.1. Mean nickel content in organs of S. acuminata

Organ	Nickel (% Dry Weight)
Latex	20.20
Leaves	1.17
Trunk bark	2.45
Twig bark	1.12
Fruits	0.30
Wood	0.17

Other elements in the latex were present at normal levels. Typical percentages were: cobalt, 0.007; iron, 0.06; chromium, 0.004;

sodium, 0.11; potassium, 0.15; magnesium; 0.052: calcium, 0.52.

The nickel concentration in the latex is nearly five times higher than for any other part of any other species and is easily the highest nickel level reported for any other living material. The nickel content of the leaves is also high, but comparable with that of other hyperaccumulating species. Chemical studies have been carried out on the latex of S. acuminata and the isolation and identification of an organic acid complex with nickel is detailed in Chapter 3.

2.4.2 Psychotria douarrei (G Beauvisage) Däniker

The hyperaccumulation of nickel by this species from New Caledonia was first recorded by Jaffre' and Schmid (1974) and their highest values (4.70% dry weight) are easily the highest recorded for any leaf material of any plant species. Freeze-dried material from the Rivière Bleue area contains 1.35% nickel and was the material used for all subsequent chemical studies of this species.

The species is found usually in dense rainforest but is not confined exclusively to ultrabasic substrates. P. douarrei is a member of the Rubiaceae.

2.4.3 Geissois pruinosa Brangn. et Gris.

Jaffre' and Schmid (1974) have reported the hyperaccumulation of nickel by this New Caledonian species. They found 0.80% in the leaves of one specimen growing over residual iron-rich soils overlying ultrabasic rocks. The soil contained 0.37% nickel. G. pruinosa is often found forming part of an overstorey 5-10m high in vegetation approaching dense forest in nature.

A water extract of freeze-dried material was found to be extremely siliceous.

2.5 General Discussion.

The survey of the Homalium and Hybanthus genera was successful in showing how herbarium specimens may be used to discover new accumulating species and to indicate areas of specific geology. Although the survey was undertaken with a low sampling density it did show quite successfully the World's major ultrabasic areas. All new hyperaccumulators were, however, confined solely to New Caledonia.

The importance of such high enrichment of nickel in certain selected species extends to a number of fields. Interesting ecological and taxonomic questions arise apart from the obvious physiological questions posed by the high nickel levels. The occurrence of hyperaccumulation may also play an important role in mineral exploration and this has already been discussed. A detailed study of the mechanisms involved in the uptake of large quantities of nickel, with particular reference to the latex of S. acuminata may be of value for the technology of low energy extraction of nickel from uneconomic ores such as laterites. Some microbiological methods of ore extraction are already economically feasible.

The evolutionary significance of several hyperaccumulators belonging to the order Violales has been mentioned. Of the 24 species of hyperaccumulators reviewed by Brooks (1977), 19 belong to the subclass Dilleniidae as defined by Cronquist (1968). It is also noted that half of the species are found in the closely-related families Violaceae and Flacourtiaceae. Chenery (1975) found that aluminium accumulators were widely scattered amoung the major subdivisions of the dicotyledons, and that groupings above the family level had little taxonomic significance. However, statistical analysis showed that aluminium accumulation was correlated with primitiveness and occurred more frequently in tropical rain forests of the world. This may simply be because highly leached tropical soils contain large quantities of available or exchangeable and ionic aluminium, and plants must be able to either tolerate or exclude aluminium from their tissues in order to survive in most tropical rain forests. A similar parallel exists with nickel accumulation. Strong or hyperaccumulation occurs only in areas where there is a large exchangeable nickel fraction and often occurs in 'relic'

species. The concentration of these plants in particular areas, especially New Caledonia, may have a hereditary basis. Ernst (1972) suggests that, "as the evolution of metal tolerance is a world wide phenomenon on heavy metal soils, the character 'zinc or copper tolerance' is a dominant hereditary character in African plants, the same way as was found for European plants."

An important distinction must be made between hyperaccumulators and plants that merely tolerate high metal areas without accumulating quantities of the element. Hyperaccumulators physiologically appear to be well adapted to extract large quantities of nickel from the substrate and actively to translocate it to higher parts of their system. This strongly differentiates them from neighbouring heavy-metal tolerant species which accumulate only minimal amounts of nickel and possibly possess defensive mechanisms which restrict uptake.

The work reported in the next chapter is concerned with the uptake of nickel and other elements by three hyperaccumulating species from New Caledonia growing on different substrates.

.3.

Plant-soil Relationships

in a

New Caledonian Serpentine flora

3.1 INTRODUCTION

New Caledonia has a rich and largely endemic flora characterised by some 3000 species growing on a tropical island of only 19000 km^2 in area. The flora is characterised by the number of species adapted to ultrabasic rocks and soils (Jaffre', Latham and Quantin, 1971), which cover about one-third of the island. These species are notable in including several hyperaccumulators of nickel (> 1000 µg/g on a dry weight basis) belonging to the genera Geissois, Psychotria, Homalium, Sebertia and Hybanthus. The characteristic infertility of these soils has been studied by Jaffre' (1969), Quantin (1969) and Latham (1972), and the peculiar effect they have on vegetation has been noted by Virot (1956), Schmid (1974) and Jaffre' (1974). The vegetation varies from dense forest in more favoured areas to scrubby, open formations on the poorly developed lateritic soils of the Plaine des Lacs in the South of the island. Areas of the latter type are known locally as the "Maquis des terrains miniers." In other parts of the World, serpentine areas have been similarly noted. In Southern California they are called "serpentine barrens," in Italy "monte pellato," in Switzerland "tot-Alpen," words that always indicate their unfavourability for normal plant habitation.

Serpentine rocks show three chemical peculiarities which have frequently been postulated as influencing their overlying vegetation. They have a high content of magnesium in relation to calcium; they are relatively rich in the transition metals, nickel, chromium and cobalt; and they are generally deficient in the major plant

nutrients. There is much experimental work to support the importance of each of these factors. Some workers have stressed the unfavourable Ca/Mg ratio (Loew and May, 1901; Walker, 1954; Kruckeberg, 1954; Proctor, 1971) whilst others point out the dominating role of the metals, particularly nickel (Robinson *et al*. 1935; Birrell and Wright, 1945; Mitchell, 1945; Hunter and Vergnano, 1952, 1953; Rune, 1953; Crooke *et al*., 1954; Spence, 1957). Krause (1958), Paribok and Alexeyeva - Popova (1966) and Sarosiek (1964) concluded that the survival of plants on serpentine soils was dependent on their ability to adapt to all of the factors operating in the serpentine ecosytem, and not just on one or two of these factors. These factors may operate in varying degrees and combinations depending on the local conditions. In a study (Lee *et al*, 1975) of the soil factors controlling the distribution of five species on a serpentine area in Nelson, New Zealand, discriminant analysis showed the strong influence of magnesium. Endemic species tended to be found in localities of highest magnesium concentration. The role of nickel appeared to be secondary to that of magnesium in discriminating between sites occupied by endemic and non-endemic species.

Proctor (1971), using *Agrostis* spp as test plants, showed that nickel and chromium are extremely harmful, and in the absence of other cations, root growth is retarded even by very low levels of these elements. However, it has frequently been observed that soils containing high levels of nickel and chromium are not toxic to some plant species. Proctor concluded that, in the serpentine soils that he studied, the role of heavy metals remained enigmatic. Better evidence for heavy metal toxicity in serpentine soils has been obtained by Soane and Saunder (1959) and Hunter & Vergnano (1952) in Rhodesia and Aberdeenshire respectively.

The absorption of nickel and other elements by plants, depends not on the total amount present in the soil, but on the concentration of the element 'available' in either an exchangeable or ionic form. This may vary considerably from soil to soil depending on a number of factors, including pH, Eh, drainage, presence of complexing agents in the soil, antagonistic effects of other ions and the nature or absence of clay minerals. Information on the form and availability of a particular heavy metal is therefore of importance in assessing its effect upon vegetation.

From the literature, therefore, the following points may be inferred:

1. The general infertility and toxicity of serpentine soils and the uniqueness of its vegetation are due to the chemical composition of the parent material.

2. Factors influencing the characteristics of serpentine flora include not only the high levels of nickel, chromium and cobalt, but also other chemical and physical soil characteristics.

3. Because the morphological, physical and chemical characteristics of serpentine soils differ from one part of the world to another, these factors must be assessed in accounting for individual floras.

4. Serpentine regions are typified by extensive regions with sparse populations of stunted vegetation, containing several species restricted exclusively to areas with "disjunctive distributions and boreal affinities" (Whittaker, 1954).

The ultrabasic areas described in this work possess many of the characteristics outlined above. The hyperaccumulating ability of the genera Homalium and Hybanthus has been detailed previously (Brooks et al., 1977). Because of the interest generated by the discovery of these hyperaccumulators, a plant-soil survey was carried out for three of the most widespread of these plants in order to investigate some of the edaphic factors governing their distribution. The concentrations of the major nutrient elements in relation to nickel, cobalt and chromium were studied, along with the suitability of these plants for biogeochemical prospecting. In this investigation, we studied three endemic species and their associated soils from the Grand Massif du Sud. The following aspects were considered:

(i) Morphological, physical and chemical characteristics of the soils.

(ii) Chemical composition of the plant leaves.

(iii) Concentration of nickel readily available for plant uptake.

(iv) Significant plant-soil and plant-plant inter-element relationships.

3.2 PHYSICAL ENVIRONMENT OF THE NEW CALEDONIAN ULTRABASIC AREAS

3.2.1 Location, topography, climate

New Caledonia, a narrow and elongated island some 400 km long by 40 km wide, lies between latitudes 18°S and 22°S on the margin of the Coral Sea, roughly midway between Australia and the Fijian Islands.

Most of the Island is mountainous, plains being restricted to the valleys and to piedmont slopes found only on the Western side. Large masses of peridotite and serpentinite are distributed along the full length of New Caledonia and occupy about one-third of the area of the Island; the southern 'Grand Massif du Sud' with an area of about 5,500 sq. km. is the largest single ultramafic complex in the World, approached in size only by those of Cuba and Celebes. Figure 3.2.1 shows the location of these areas along with the position of the three sampling areas, namely, Rivière Bleue, Mont Koghi and the Plaine des Lacs.

The climate of New Caledonia, which is strongly influenced by the ocean, may be divided into four seasons : December to March, a hot humid period with high rainfall; April to May, cool with moderate rainfall; June to August, the coolest period of the year (19 - 20°C), with moderate rainfall; and September to November, the driest part of the year. Annual rainfall varies from 1000 mm in coastal areas to over 4000 mm in the highest parts of the country. On the ultrabasic massifs the rainfall averages 1800 mm. Temperatures range from a mean of 26°C in February to 19° in August.

The climate of the ultrabasic areas in the South is typically tropical but climatic irregularities play an important part in the geomorphology and vegetation of the area. The temperature and humidity variations are extremely favourable for laterization.

3.2.2 Geology and Soils

New Caledonia is of great geological interest because of the occurrence of huge ultrabasic masses, of glaucophane schists, and of extensive outcrops of basalt. The ultramafic complexes are composed principally of large masses of peridotite and serpentinite (Lillie and Brothers, 1970). The Southern massif is not an undifferentiated zone, and consists of chromiferous dunite, harzburgite, wehrlite and pyroxenite as well as basic rocks such as olivine, gabbro, allivalite and norite. Figure 3.2.2 shows the

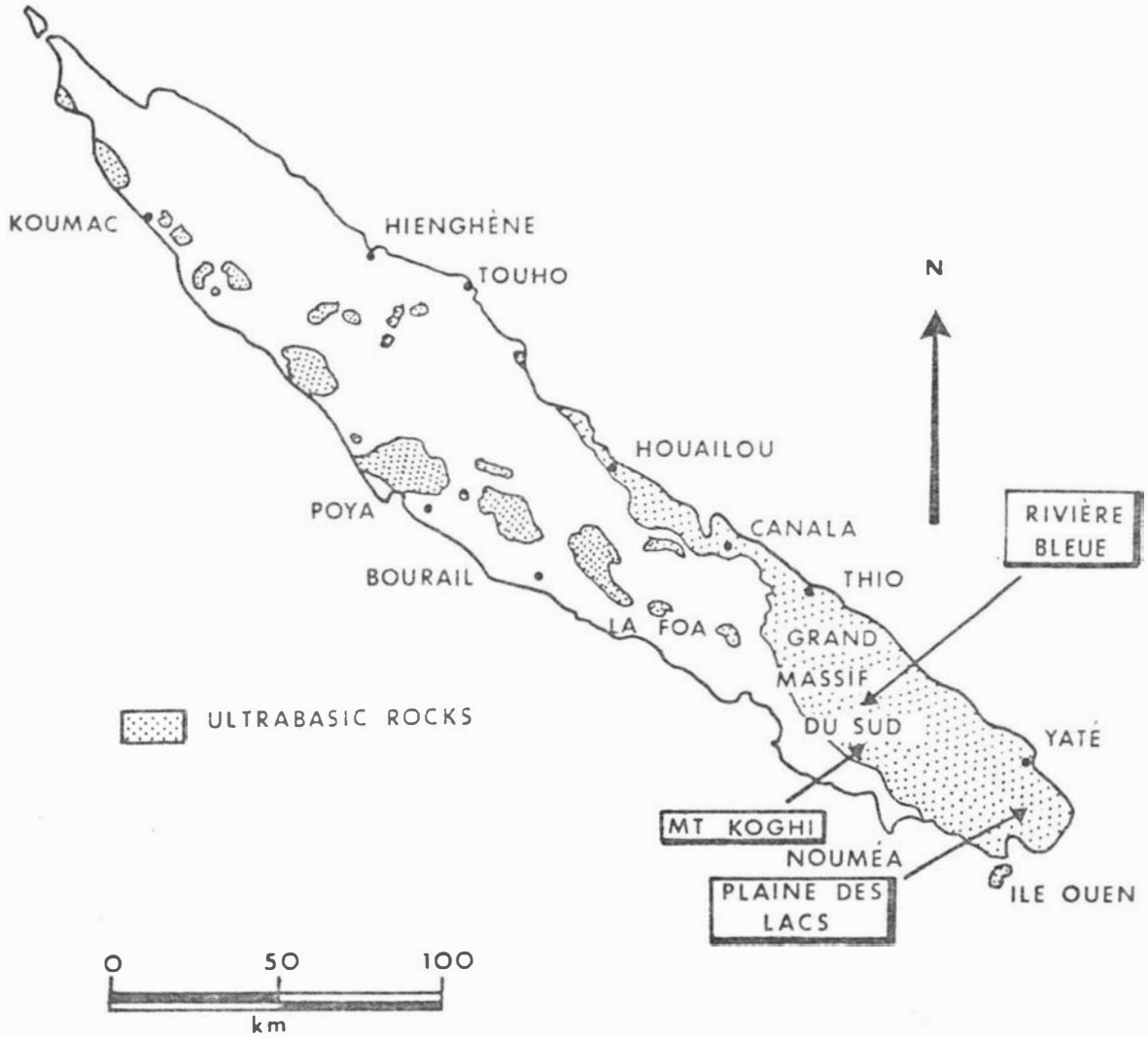


Figure 3·2·1: Map of New Caledonia showing sample areas.

geology of New Caledonia.

Peridotites constitute the principal rock formation in the "terrains miniers" (Mining areas). The widespread deposits of nickel and subsidiary chromite found on the lateritic surfaces of the ultrabasic rocks are of prime economic importance. The lateritic (ferruginous) soils are characterised by a thin organic layer over a reddish leached layer, which in turn is underlaid by a still deeper red layer. Hydrolysis and oxidation have been intense. The soils are granular in nature and concretions of iron oxides vary in size from 1mm to 100mm in diameter. The silica content of these ferrallitic soils is extremely low, owing to the dissolution of silicic acid formed during laterization. During weathering, silica and magnesium are dissolved and carried down from the mountains. Iron, cobalt and chromium concentrate in the upper parts of the profile. Nickel first concentrates in the whole lateritic profile and then migrates slowly down the weathered profile (Battzer et al., 1976). All the soils in the regions studied are poorly developed and contain large amounts of iron and chromium. Table 3.2.1 gives a brief description of the soils in each of the sample areas. The soils are typically low in exchangeable bases. In summation, these soils then, are characterized morphologically by their colour, lack of distinction in soil horizons, high clay content, friable structure and granular nature. These factors are common to other tropical serpentine soils that have been subjected to the pedological process of laterization.

3.2.3 Vegetation

Jaffre' et al. (1971) and Jaffre' (1973) have described the vegetation of several ultrabasic areas, known to the locals as "Maquis des terrains miniers" (scrub-covered mining areas). The vegetation is characteristically shrubby and fairly open on the whole, but woody formations are well represented on iron rich soils, on favourable sites : e.g. talwegs, banks of rivers, sources of springs, etc. The Rivière Bleue area (see map), where Homalium guillainii and Hybanthus austro-caledonicus were sampled, resembles rainforest more typically associated with tropical vegetation.

The unique flora is adapted to survive under strong nutrient imbalances and high cobalt, chromium, nickel and manganese concentrations. In heavy rainfall areas where laterization has been

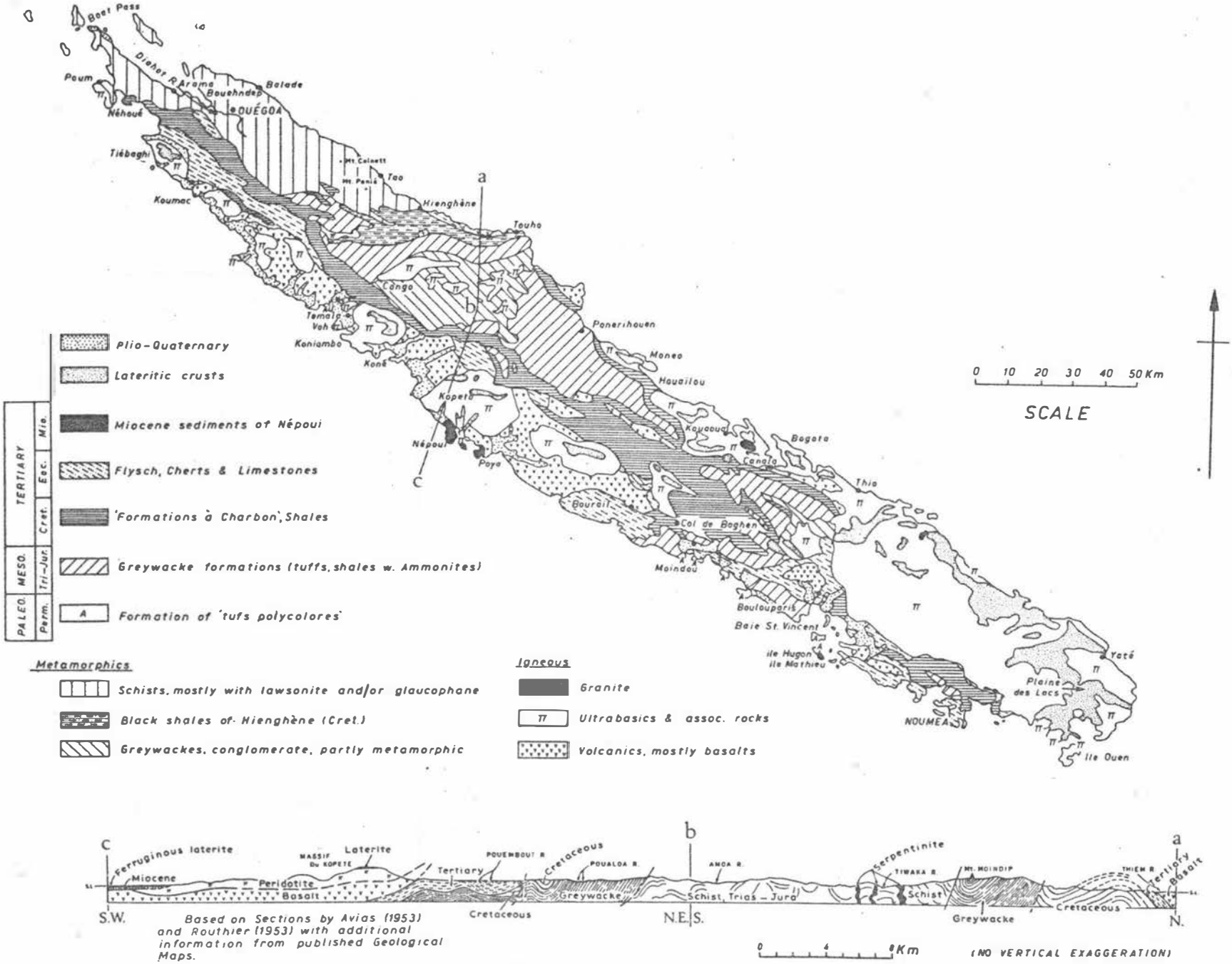


Figure 3·2·2: Geological map of New Caledonia.

severe, e.g. Plaine des Lacs, with the formation of hydromorphic, indurated, residual iron rich soils, open herbacious formations are dominant, often characterized by the species Fristania guillainii.

Table 3.2.1. Description of Plants and their Sampling Localities.

SITE	No. of Specimens.	Species	Location	Soil Type	Distribution
1	47	Hybanthus austrocaledonicus	Mont Koghi	Very humic iron rich soils over shattered peridotite.	Dense rainforest -various substrates mainly peridotites in South of New Caledonia.
2	25	Homalium kanaliense	Plaine des Lacs	Temporary hydromorphic - high percentage of iron oxide concentrations. -lateritic.	Scattered scrub formations - clear areas.
3	25	Homalium kanaliense	Rivière de la Madeleine - Plaine des Lacs.	Residual, moderately humic alluvial iron rich soils.	Scrub and semi-forested formations
4	25	Homalium kanaliense	Hill slope above Plaine des Lacs.	Partially weathered laterites. - highly granular.	Scattered scrub.
5	25 50	Hybanthus austrocaledonicus Homalium guillainii	Rivière Bleue	Residual ferruginous alluvia derived from peridotites.	Dense rain forest mainly over peridotites.

3.3 COLLECTION LOCALITIES AND SAMPLING PROCEDURE

Three hyperaccumulators of nickel were chosen for study: Homalium guillainii, Homalium kanaliense and Hybanthus austrocaledonicus. These species were well represented on several sites of differing soil structure. The site number, number sampled, location and soil type are summarized in Table 3.2.1. The location of the three sample areas (Rivière Bleue, Mont Koghi and Plaine des Lacs) in the southern part of the island are indicated on Figure 3.2.1.

Mont Koghi - site 1

At Mont Koghi (rainfall 2000 mm, altitude 600 m) the vegetation cover consists of dense rain forest overlying very humic (29% loss of weight after ashing dried soils at 500°C) ferruginous soils derived from shattered peridotites. The terrain is steep and boulder-strewn. Hybanthus austrocaledonicus was reasonably well represented in the area and attained heights from 1 to 4 m. The leaves of this species are approximately 100 mm x 40 mm.

Plaine des Lacs - sites 2, 3, and 4

Homalium kanaliense (a small shrub 0.5 to 1.5. m in height) was sampled on each of these sites within the Plaine des Lacs area. Site 2 was typified by the very gravelly nature of the upper soil horizons. These were composed of hard, irregular iron oxide concretions 10 mm to 100 mm in diameter. The soils were poorly differentiated and lacking in humus. The vegetation was very open and herbaceous. Other species found with Homalium kanaliense were Fristania guillainii, Lophoschoenus stagnalis, L. comosus, Xanthostemon aurantiacum, Grevillea gillevrayi, Phyllanthus aeneus and Movria artensis.

Riviere Bleue - site 5

Hybanthus austrocaledonicus and Homalium guillainii were found on this site near the Rivière Bleue. The soils have been derived from peridotites and associated rocks and are lateritic in nature. The upper horizons are characteristically deep red-brown in colour and consist of alluvial clay. They are loose and porous in texture. Vegetation is dense with Agathis lanceolata a dominant species.

Samples were taken from various positions on the shrubs and

as far as possible were taken from specimens of similar size. Leaves were stored in plastic bags and were washed upon return to the laboratory.

Soil samples were taken from the base of each plant as close as possible to the root system. (i.e. about 20 cm depth) The soils were dried at 80°C, sieved to remove particles larger then 2 mm, and stored in paper bags. The number of samples obtained for each species is recorded in Table 3.2.1.

3.4 ANALYTICAL TECHNIQUES

3.4.1 Soil fractionation

A preliminary study was carried out on representative soil samples from each site to gain some information on the distribution of nickel and other elements in different soil fractions. Five fractions were collected and the percentage passing through each sieve size is given in Table 3.4.1. Total sample size was approximately 100g. The mean of three determinations from each site is shown.

Table 3.4.1 Physical fractionation of soils showing percentage of total passing through various mesh sizes.

SITE	+500µm	250-500µm	125-250µm	63-125µm	- 63µm
1	76.40	15.00	6.10	1.70	0.70
2	91.90	6.00	1.30	0.40	0.40
3	69.60	19.80	6.70	2.30	1.60
4	80.10	12.20	4.80	1.80	1.10
5	54.10	22.70	14.70	5.70	2.80

These results reflected the physical nature of the soils from the five collection localities. The large fraction of + 500µm reflects the granular nature of the soils from these areas. The high percentage of iron oxide concretions in the soil from site 2 was largely responsible for the small fraction of fine material. The alluvial character of the soils from the Rivière Bleue (site 5) resulted in a higher percentage of the total passing through the

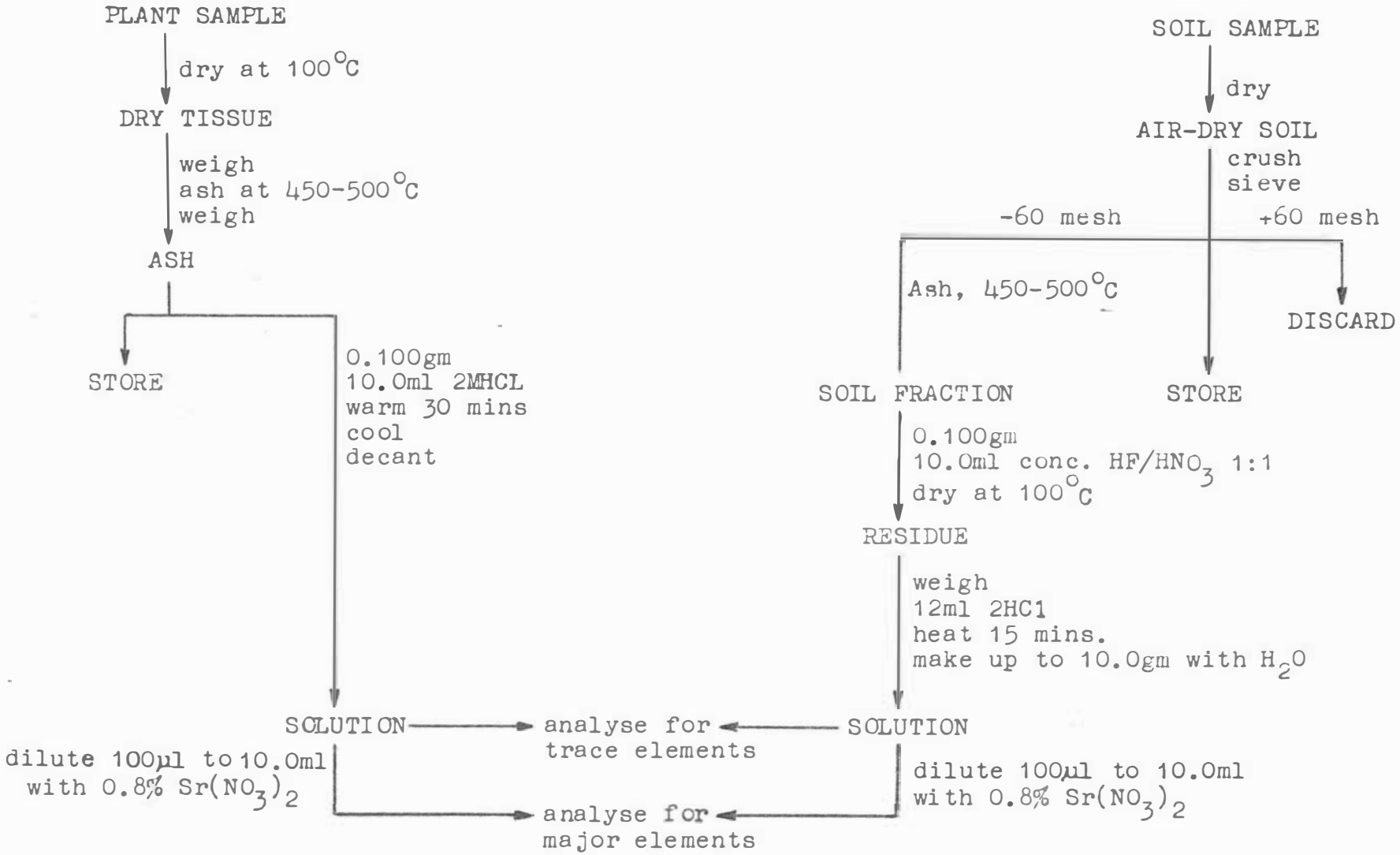


Fig. 3.4.1. Flow Diagram for dissolution and analysis of geochemical and plant samples.

smaller sieve sizes.

3.4.2 Ashing procedure and sample dissolution

The organic material in samples of - 2mm material was decomposed by ashing in a muffle furnace at 500°C for several hours. The ashed soil was then ground to about -150 mesh size (100 μm diameter).

Enough plant material was ashed for eight hours to obtain at least 0.1g of ash. Fresh leaf material, to be used in later work, was freeze-dried and stored in air-tight vessels in a refrigerator. Weight loss on ashing was recorded for each specimen.

Small samples of ignited soil (0.10g) were weighed accurately into 50 ml squat polypropylene beakers and digested with 5-10 ml of a 1 : 1 mixture of concentrated hydrofluoric and nitric acids. The beakers were suspended in a water bath and the solution taken to dryness. The residues were redissolved in approximately 10 ml of 2M hydrochloric prepared from redistilled (5M) acid. The procedure results in virtually complete dissolution of soils, with the remaining metals in the residue being traces of titanium, iron and chromium as shown by emission spectroscopy (Quin, 1974). The chromium probably comes from chromite which is virtually insoluble.

A more serious problem was the loss of calcium, arising not from incomplete dissolution of the sample, but rather from precipitation as insoluble fluoride. This problem could be circumvented by the addition of a small amount (0.5 ml) of high boiling point perchloric acid to the digestion mixture. This had the effect of keeping calcium in solution until all the fluoride had evaporated.

The dissolved residues were washed into 10ml graduated polypropylene vials, made up to the 10ml mark with 2M hydrochloric acid and stoppered prior to analysis.

Plant samples (0.1g of ash) were dissolved directly into 2M hydrochloric acid and warmed for about 30 minutes. These solutions were made up to 10ml in graduated polypropylene vials. For well-ashed material this method of dissolution was quite adequate. The 100 times dilution that this procedure gave was satisfactory to bring all the trace elements into the analytical range desired, but further dilution was necessary for nickel, calcium, magnesium and potassium. Giron (1973) found that dry ashing at 550°C gave more reproducible results than wet ashing for calcium, copper, iron,

manganese, magnesium, potassium, sodium and zinc in plants. Figure 4.3.1 shows in the form of a flow diagram, the general procedure adopted for plant and soil analysis.

3.4.3 Analytical methods

(i) Atomic absorption spectrophotometry

The Varian Techtron model A.A.5 atomic absorption spectrophotometer was used for determining various elements in the plant and soil digests. The conditions of operation are given in Table 3.4.2. Standards were made from analytical-grade (Anala R) chemicals. Impurities at the concentration levels used, were insignificant. Combination standards containing all the trace elements to be analysed were employed and these were diluted from a 1000 µg/g stock solution. The same standards were employed for analyses. Standards and sample dilutions for calcium and magnesium were made with 2M hydrochloric acid containing 0.8% $Sr(NO_3)_2$. The latter reagent was added because phosphates, sulphates and silicates are known to cause depression of the absorption signal. The addition of a spectral buffer in the form of very pure $Sr(NO_3)_2$ helped to overcome these effects (Dinnin, 1960; Elwell and Gidley, 1967). Calcium forms refractory aluminates, so a relatively hot flame was used to help overcome this effect. Alkali metals can also cause interferences in the determination of calcium and magnesium, owing to ionization effects (David, 1959) but these were largely overcome by using mixed standards of the metals.

Light scattering of the incident radiation from the hollow cathode lamp by solid particles, and non-atomic absorption by molecular species in the flame were both corrected for by using a hydrogen continuum lamp. Such problems only occurred at shorter wavelengths (< 250nm).

The most sensitive absorption lines were generally employed except for nickel where the less sensitive 341.4 nm line was used. This was due to the poor linearity of the calibration curve obtained at 232.0 nm. (Mavrodineanu, 1970). For all other elements, good calibration curves were obtained.

Previous work (Lee, 1974) using the standard diabase W-1 has shown the accuracy and reproducibility of the above methods to be satisfactory for this project.

Table 3.4.2 INSTRUMENTAL CONDITIONS.

Element	Wavelength (nm)	Lamp Current (mA)	Slit Width (μm)	Sensitivity + (ppm)	Flame Character	Standards (ppm in 2M HC1)
Ni	232.0	5	170	0.05	Air-C_2H_2	2,5,10,20,40
	341.5	8	200	0.26	Oxidizing	Comb*
Cr	357.9	5	100	0.055	Air-C_2H_2 reducing	1,2,5,10 Comb.
Co	240.7	5	150	0.053	Air-C_2H_2 Oxidizing	1,2,5,10 Comb
Cu	324.7	3	100	0.04	Air-C_2H_2 Oxidizing	1,2,5,10 Comb
Zn	213.9	5	250	0.009	Air-C_2H_2 Oxidizing	5,10,20,40 Comb.
Mn	279.5	5	200	0.021	Air-C_2H_2 Oxidizing	5,10,20,40 Comb.
Fe	248.3	15	250	0.062	Air-C_2H_2 Oxidizing	2,5,10,20,40
Ca	422.7	5	300	0.013	Air-C_2H_2 reducing	2,5,10,20,40 with 0.8% $Sr(NO_3)_2$
Mg	285.2	5	200	0.003	Air-C_2H_2 reducing	2,5,10,20,40 with 0.8% $Sr(NO_3)_2$

+From "Hollow Cathode lamp data" Varian Techtron, 1973
*Combination standard containing Ni Cr Co Cu Zn Mn Fe Pb Cd.

(ii) Colorimetry

Phosphorus in plants and soils was determined by forming a phosphomolybdate complex similar to that in the molybdenum blue method given by Stanton (1966). The absorbance at 882 nm was measured with Unicam SP 1800 u.v. spectrophotometer. Determinations were carried out on the original hydrochloric acid digests after neutralization with sodium hydroxide. Orthophosphate and molybdate ions condense in acidic solution to give molybdophosphoric acid which may be selectively reduced by ascorbic acid and antimony potassium tartrate to molybdenum blue. The intensity of the blue colour is proportional to the amount of phosphate initially incorporated in the heteropoly acid. It is assumed that after ashing and acid digestion all the phosphorus is present as phosphate.

(iii) Flame Photometry

Potassium was analysed in the acid digests of the plant leaves and soils using a Gallenkamp flame photometer or employing the emission mode of the A.A.5 atomic absorption spectrophotometer.

(iv) Statistical treatment of data

A computer was used to calculate arithmetic means, standard deviations and Pearson product moment correlation coefficients (r) for data. Geometric means were calculated separately for chromium and cobalt as these were shown to be log-normally distributed in some soils. Most elements however were normally distributed in plants and soils.

3.5. PLANT AND SOIL ELEMENTAL CONCENTRATIONS

The mean concentrations of various elements in the plants and associated soils of *Hybanthus austro-caledonicus*, *Homalium guillainii* and *Homalium kanaliense* are summarised in Table 3.5.1. Preliminary calculations showed the geometric and arithmetic means for the plants and soils of *Hybanthus austro-caledonicus* to be similar for all elements except chromium and cobalt. The geometric means for these two elements were significantly smaller than the arithmetic means, indicating a log-normal distribution. The geometric means for the concentrations of cobalt and chromium in the various plants and their associated soils are therefore given in Table 3.5.2.

Nickel concentrations in all three species (even on a dry weight basis) were as high or higher than the nickel in the soils. It is notable that nickel must be classified as a major element in these plants and ranks higher in concentration than the principal nutrient elements calcium, potassium, magnesium or phosphorus. Another notable feature is the ability of these plants to accumulate adequate amounts of potassium (~ 0.5 -1.0%) in spite of the deficiency, due to excessive leaching, of this element in the soil. The low levels of calcium and magnesium in the soils of the Plaine des Lacs sites compared to those in the other two areas, reflects the degree of laterization and the more excessive leaching of these elements from the soils of this area. The very high percentage of iron in the soils at site 2 is a reflection of the extensive iron oxide concretions in the area. The high iron content of all areas is typical of lateritic soils. Overall, the high cobalt, chromium, nickel and iron levels in the soils and plants of these areas reflects the ultrabasic nature of the substrate.

Figure 3.5.1 shows graphically the concentration distribution of nickel in the three plant species. The large absolute range for nickel in Homalium guillainii is noteworthy.

Table 3.5.1. Arithmetic means and standard deviations for plant and soil data.

Element	Concentrations. Plant (Drywt)	Plant (ashwt)	Associated Soil
Site 1.	Hybanthus	austrocaledonicus (N=47)	(Mont Koghi)
Ni%	1.24 ± 0.36	12.9 ± 3.2	0.59 ± 0.15
Ca%	0.95 ± 0.23	9.8 ± 1.8	0.68 ± 0.28
Mg%	0.71 ± 0.15	7.5 ± 1.9	5.75 ± 2.20
K%	0.75 ± 0.28	7.7 ± 2.6	0.05 ± 0.09
Mn µg/g	226 ± 92	2356 ± 895	6107 ± 1742
P µg/g	557 ± 84	5855 ± 846	329 ± 136
Cu µg/g	4.8 ± 1.7	50 ± 16	56 ± 40
Fe%	0.022 ± 0.016	0.23 ± 0.17	25.8 ± 5.6
Zn µg/g	51 ± 14	532 ± 141	166 ± 34
Humus %	-	-	23.9 ± 8.0
*X Ni µg/g	-	-	1087 ± 247
Site 5	Hybanthus	austrocaledonicus (N=25)	(Rivière Bleue)
Ni%	1.60 ± 0.37	19.15 ± 3.20	0.71 ± 0.04
Ca%	0.66 ± 0.16	7.98 ± 1.44	0.71 ± 0.24
Mg%	0.58 ± 0.11	7.11 ± 1.52	1.9 ± 0.36
K%	0.65 ± 0.23	7.78 ± 2.33	0.02 ± 0.004
Mn µg/g	178 ± 83	2202 ± 1091	6023 ± 689
P µg/g	647 ± 100	7832 ± 1268	386 ± 138
Cu µg/g	3.9 ± 1.4	46 ± 16	62 ± 27
Fe%	0.022 ± 0.019	0.26 ± 0.23	25.8 ± 1.8
Zn µg/g	64 ± 31	783 ± 386	241 ± 14
Humus %	-	-	-
*X Ni µg/g	-	-	1123 ± 197

* Extractable in ammonium oxalate buffer.

Table 3.5.1 cont...

Element	Concentration		
	Plant(Dry wt)	Plant (Ash wt)	Associated Soil
Site 2.	Homalium	kanaliense (N=25)	(Plaine de Lacs)
Ni%	0.49 ± 0.19	6.28 ± 2.24	0.54 ± 0.07
Ca%	0.85 ± 0.29	10.85 ± 3.00	0.20 ± 0.06
Mg%	0.29 ± 0.07	3.77 ± 0.97	0.10 ± 0.03
K%	0.99 ± 0.25	12.74 ± 2.7	0.006 ± 0.002
Mn µg/g	797 ± 330	10270 ± 4039	6153 ± 2429
P µg/g	263 ± 78	3380 ± 1014	236 ± 47
Cu µg/g	12 ± 6	105 ± 72	117 ± 67
Fe%	0.019 ± 0.007	0.25 ± 0.10	40.7 ± 2.0
Zn µg/g	204 ± 58	2628 ± 721	333 ± 32
Humus %	-	-	15 ± 3
X Ni µg/g	-	-	430 ± 326
Site 3	Homalium	kanaliense	(Plaine de Lacs)
Ni%	0.47 ± 0.12	6.92 ± 1.53	0.39 ± 0.07
Ca%	0.61 ± 0.16	9.02 ± 2.15	0.18 ± 0.09
Mg%	0.38 ± 0.07	5.66 ± 1.10	0.17 ± 0.07
K%	0.73 ± 0.15	10.82 ± 2.62	0.009 ± 0.003
Mn µg/g	379 ± 198	5638 ± 3070	4929 ± 2443
P µg/g	298 ± 105	4408 ± 1593	155 ± 17
Cu µg/g	8.8 ± 4.6	128 ± 60	47 ± 8
Fe%	0.051 ± 0.03	0.75 ± 0.46	32.8 ± 4.5
Zn µg/g	198 ± 47	2910 ± 553	263 ± 36
Humus%	-	-	22 ± 7
X Ni µg/g	-	-	243 ± 103

Table 3.5.1 Cont...

Element	Concentration		
	Plant (Dry wt)	Plant (Ash wt)	Associated Soil
Site 4	Homalium	kanaliense (N=25)	(Plaine de Lacs.)
Ni%	0.37 ± 0.16	3.49 ± 1.16	0.62 ± 0.11
Ca%	0.80 ± 0.27	7.60 ± 1.99	0.57 ± 0.07
Mg%	0.55 ± 0.16	5.34 ± 1.56	1.51 ± 1.14
K%	0.60 ± 0.11	5.97 ± 1.72	0.01 ± 0.005
Mn µg/g	162 ± 130	1631 ± 1395	3062 ± 662
P µg/g	320 ± 54	3152 ± 823	169 ± 68
Cu µg/g	8.7 ± 2.7	84 ± 26	80 ± 27
Fe%	0.01 ± 0.009	0.11 ± 0.10	33.5 ± 4.1
Zn µg/g	235 ± 70	2274 ± 676	339 ± 63
Humus %	-	-	19.3 ± 7.0
X Ni µg/g	-	-	728 ± 148
Site 5	Homalium	guillainii (N=25)	(Rivière Bleue.)
Ni%	0.81 ± 0.36	8.95 ± 3.65	0.67 ± 0.08
Ca%	1.06 ± 0.31	11.70 ± 2.40	0.97 ± 0.63
Mg%	0.45 ± 0.13	5.0 ± 1.2	3.55 ± 2.27
K%	0.47 ± 0.13	5.44 ± 1.88	0.011 ± 0.002
Mn µg/g	88 ± 33	1004 ± 427	5527 ± 1341
P µg/g	536 ± 104	6210 ± 1907	367 ± 94
Cu µg/g	10 ± 3.5	116 ± 47	57 ± 33
Fe%	0.297 ± 0.270	0.33 ± 0.27	23.1 ± 3.8
Zn µg/g	91 ± 39	1031 ± 460	218 ± 28
Humus %	-	-	23 ± 5
X Ni µg/g	-	-	977 ± 187

Table 3.5.2. Geometric means and standard deviation ranges for Cr and Co in Plants and Soils.

Species	SITE	No.	Cr (µg/g) Plant (Dry)	Cr (µg/g) Plant (Ash)	Cr (µg/g) Soil	Co (µg/g) Plant (Dry)	Co (µg/g) Plant (Ash)	Co (µg/g) Soil
Hybanthus austrocaledonicus	1	47	20 (9.5-38.4)	182 (111-365)	3236 (2254-4644)	25 (13.5-51.2)	334 (186-580)	810 (588-1114)
Hybanthus austrocaledonicus	5	25	35.5 (17.5-71.7)	429 (213-862)	4116 (2543-6662)	27.7 (18-44.5)	346 (217-552)	887 (781-1007)
Homalium kanaliense	2	25	7.8 (5 7-10.6)	100 (73-138)	10587 (9239-12131)	65 (25-180)	768 (299-1975)	669 (511-876)
H. kanaliense	3	25	13.7 (8.3-22.3)	201 (122-331)	7741 (4430-13529)	66 (29-146)	981 (438-2193)	749 (495-1134)
H. kanaliense	4	25	5.7 (4.6-7.1)	55 (46-65)	6613 (5437-8042)	145 (77-273)	1406 (735-2686)	879 (713-1083)
H. guillainii	5	50	8 (5.5-13.8)	99 (64-155)	3570 (2973-4286)	12 (7.5-18.2)	132 (84-207)	798 (621-1025)

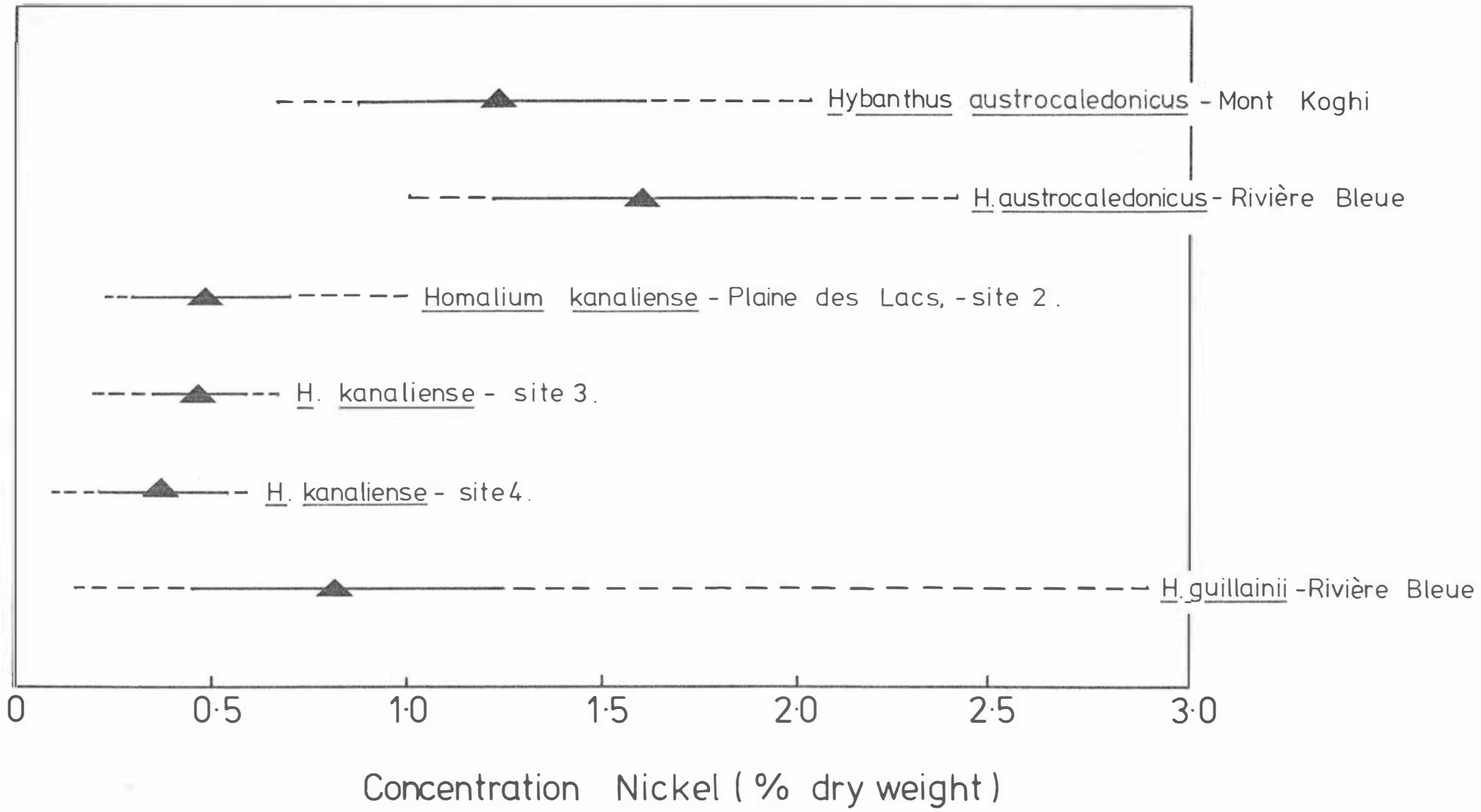


Figure 3·5·1: Concentration of nickel in H. austrocaledonicus, Homalium kanaliense and H. guillainii. ▲- mean, —— - standard deviation, - - - - absolute range.

3.6 EVALUATION OF SOLUBLE NICKEL USING DIFFERENT EXTRACTANTS.

It is well known that plants reflect to some extent elemental concentrations in the associated soils. However poor correlations are often obtained between total elemental content in the soil and the concentrations in the plant. Better results are often obtained when exchangeable levels in soils are assessed and correlated with the total concentration in the leaf material.

A preliminary project was undertaken in order to determine the most suitable extractant for assessing 'available' or exchangeable nickel in the soils associated with each of the plant species. Various solvents and buffers have been used in an attempt to establish this quantity. Commonly used reagents have been ammonium acetate (Halstead, Finn and Maclean, 1969; Misra and Pande, 1974), acetic acid (Mitchell, 1945; Menezes de Sequeira, 1969; Lyon, 1969; Spence, 1957; Misra and Pande, 1974) 0.1M hydrochloric acid (Misra and Pande, 1974) and E.D.T.A., citric acid and oxalic acid (Pande and Misra, 1975).

Extractions on 1g soil samples (-60 mesh, air dried) were undertaken using the following reagents : ammonium oxalate-oxalic acid buffer (commonly called Grigg's reagent (Grigg, 1953)), pH 5.5 , 0.1M ammonium acetate, 0.1M calcium chloride, 0.1N hydrochloric acid and 0.1M tri-sodium citrate - citric acid buffer, pH 5.4. Soil samples, with extractant, were shaken for 1 hour and left to equilibrate overnight before centrifugation and analysis by atomic absorption spectrophotometry. The quantity of nickel extracted by each reagent is shown in Table 3.6.1. For comparison purposes,

Table 3.6.1. Extractable nickel from soils associated with *H. austrocaledonicus*.

Extractant	Nickel (µg/g)
Ammonium oxalate buffer.	1087 ± 247
0.1M Ammonium acetate.	30 ± 6
0.1M Calcium chloride.	55 ± 11
0.1M Hydrochloric acid.	600 ± 120
Sodium citrate buffer.	381 ± 50

the concentrations of other trace elements extracted by ammonium oxalate were : cobalt, 348 ± 110 µg/g; chromium 27 ± 9 µg/g, copper, 5.0 ± 1.9 µg/g and zinc, 15 ± 5.5 µg/g. The very low level of available chromium in these soils is a notable feature. (The soils supporting H. austrocaledonicus contained a mean value of 3236 µg/g Cr). Chromium in chromite form is insoluble and the available chromium is probably related to that in chlorites. These low levels of availability for chromium have been noted in other serpentine soils (Menezes de Sequeira, 1969; Lee, 1974). All the plant accumulators of nickel that were investigated were found to have a low uptake of chromium. Cobalt on the otherhand is readily available in the soil, with 348 µg/g extracted from a total of 810 µg/g. However the leaves of H. austrocaledonicus were found to contain only 25 µg/g on a dry weight basis.

The quantity of nickel extracted by each reagent was correlated statistically with the total level in the corresponding plant sample. Correlations in decreasing order were; ammonium oxalate buffer > 0.1M ammonium acetate > 0.1M hydrochloric acid > 0.1M calcium chloride. No significant correlation coefficient was obtained using the sodium citrate buffer. From this study the ammonium oxalate buffer was selected for extraction of each soil sample. The level obtained was assumed to approximate that available to the plant. The mean extractable concentration of nickel for each soil site is shown in Table 3.5.1.

However, although exchangeable amounts of any cation may be estimated, it is almost impossible to determine what quantity is actually available to the plant, as this varies with species, physical nature of the soil, climate, microbial activity and many other factors (Schütte, 1964). Leaf analysis probably best approximates available levels in the soil, although it does not indicate directly the plant's physiological status or requirements.

From the stability diagrams in Figures 3.6.1 , 3.6.2 , and 3.6.3 it may be seen why the ammonium oxalate buffer was more effective (than the sodium citrate buffer) in extracting nickel from the soils supporting H. caledonicus, although the nickel citrate is more stable than a nickel oxalate complex (log K of 6.1 and 4.9 respectively, Martell and Calvin (1952).) At pH 5.5 the oxalate complex is more stable than that of citrate, which becomes more effective as a chelator at higher pH values. At pH 5.5, iron

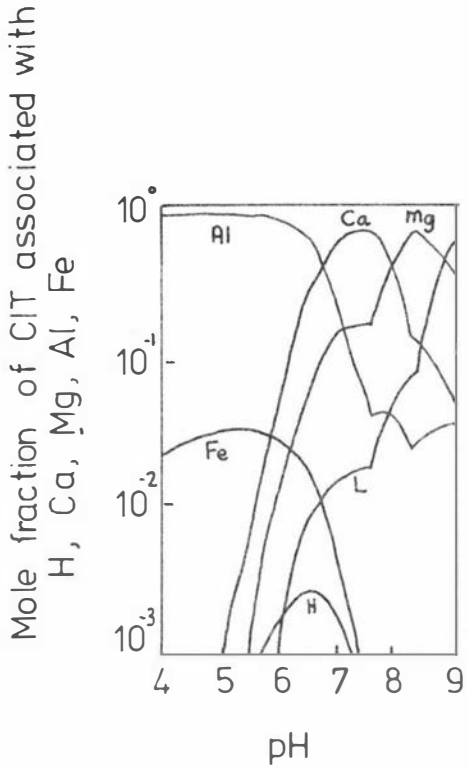


Figure 3·6·1:

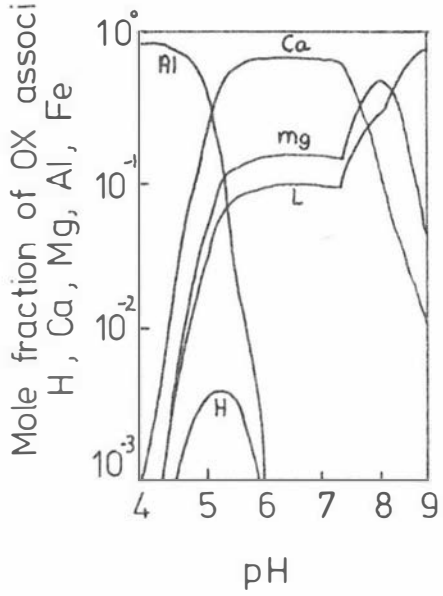


Figure 3·6·2:

Stability diagram for CIT and OX in equilibrium with H^{+}, Ca^{2+}, Mg^{2+}, Al^{3+}, Fe^{3+} in soil solution. (Norvell, 1972)

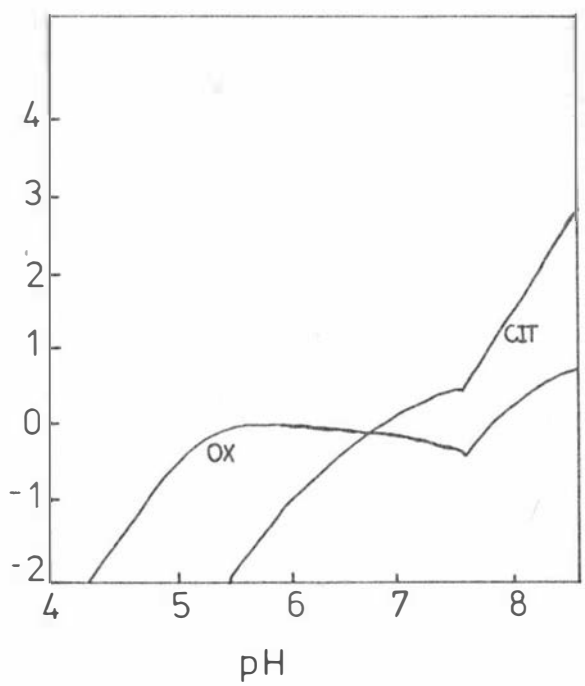


Figure 3·6·3: Comparison of Ni^{2+} chelating ability with OX and CIT in soil solution.
Equilibrium between H^{+}, Ca^{2+}, Mg^{2+}, Al^{2+}, Fe^{3+} is assumed. (Norvell, 1972)

and aluminium compete strongly for citrate ions. The concentration of iron in the soils sampled is approximately 40 times that of nickel. The stability diagram for oxalate is presented in Figure 3.6.2. This diagram is similar to that for citrate, except that iron oxalate chelates are not sufficiently stable to appear in the diagram.

3.7 INTERELEMENTAL RELATIONSHIPS IN PLANTS AND SOILS

3.7.1 Soils

Interelemental relationships for the soils at each of the sites are shown graphically in Figure 3.7.1. Values are based on correlation coefficients denoting relationships significant at the 99.99% level of probability ($P \leq 0.0001$). These levels are said to be highly significant. (Brookes, Betteley and Loxston, 1966). Only those relationships with at least this level of significance are shown in the Figure. Positive or negative correlations are indicated by soil and dashed lines, respectively.

The correlation coefficients for soils reveal some of the trends expected for ultrabasic areas. Nickel is correlated positively with members of the iron family (Co, Cr, Fe, Mn) in the soils of Mont Koghi and Rivière Bleue. However, nickel in the soils from the Plaine des Lacs shows weaker correlations with other members of the iron family. This is because these soils reflect the typical lateritic separation of elements such as nickel, iron and cobalt, owing to heavy leaching (See Figure 3.7.1.(a)) At site 2 nickel is negatively correlated with chromium. (Figure 3.7.1.(b)) This area is the most heavily leached of all the sites investigated. Extractable nickel (ammonium oxalate buffer) is positively correlated to total nickel only in the soils of Mont Koghi and Rivière Bleue. The percentage of total nickel extracted is a lot less in the Plaine des Lacs soils. This again reflects the heavy leaching with the most soluble nickel fraction being carried further down the weathered profile. Only 278 µg/g are extracted from the soils at site 4 compared to 1087 µg/g from the soils at Mont Koghi (site 1). Both soils had similar total nickel concentrations. There is a notable strong negative correlation between potassium and iron in the Plaine des Lacs soils. Potassium in these soils is especially deficient compared with the other sites,

whilst iron is present in extremely large amounts.

3.7.2 Plant-soil elemental relationships.

Highly significant correlation coefficients for plants and soil relationships are shown graphically in Figure 3.7.2. Correlations were computed from data expressed on a dry weight basis. Distributions were considered to be generally normal.

In contrast to soils, there are few correlations between pairs of elemental concentrations in plants and soils, and plants alone, except for element pairs in the plants and soils of Homalium guillainii sites. In some cases correlations merely reflect relationships already evident in the substrate e.g. in H. austrocaledonicus the zinc content of the plant is inversely related to the magnesium content (negative r) and reflects a relationship already existing in the soil. It is interesting to note that in the soils supporting Homalium guillainii, chromium is strongly correlated positively with nickel, cobalt, manganese and iron whilst in the plant it is negatively correlated with these elements in the soil. Chromium uptake is very well restricted by this plant: only 8 µg/g is found in the leaves, compared with 35.5 µg/g in the dried leaves of H. austro-caledonicus growing on similar sites.

Highly significant plant-soil relationships for H. kanaliense are fairly numerous. However, the nickel content of dried leaves is related only to the manganese (site 3) and extractable nickel in the soil. In the plant itself nickel is positively correlated with copper and zinc. The nickel content in the plants is not significantly related to the total concentration in the soils for any of the sites and correlations with extractable nickel are generally weak except for site 2.

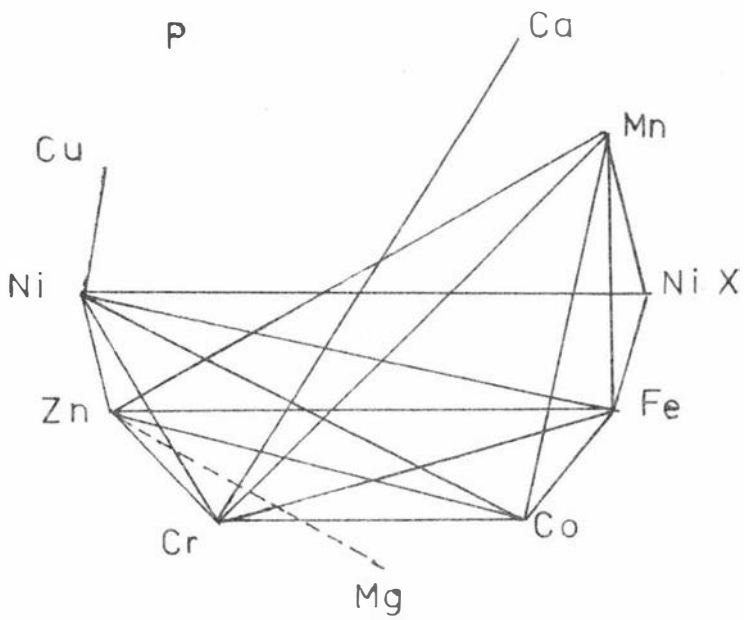
Chromium and iron in the leaves of H. kanaliense are strongly related ($r = 0.95$ for 72 sites) and this significant correlation also occurs in the soil.

3.8 Discussion.

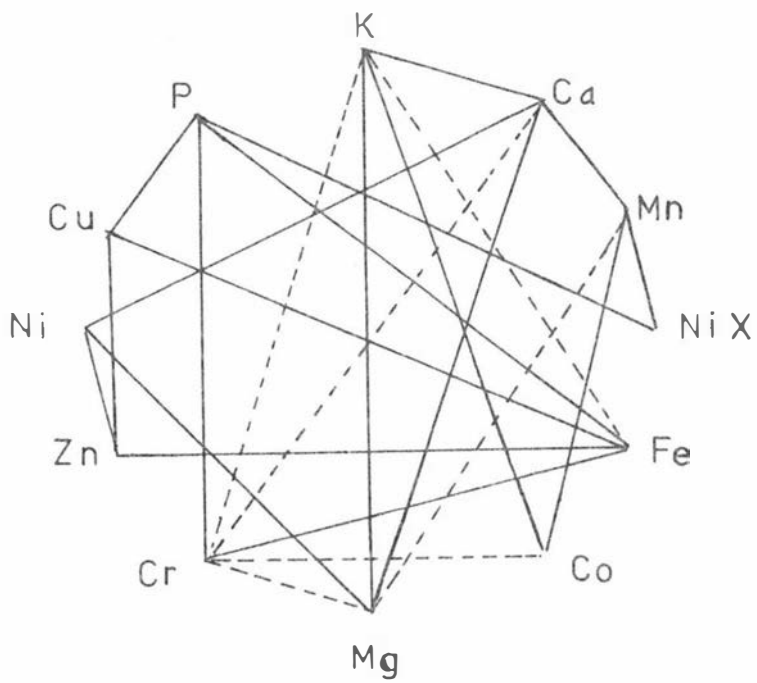
The physical and elemental characteristics of the soils sampled in this project, basically reflect those typical of ultrabasic areas in other parts of the world. The biggest difference in these soils from those of ultrabasic origin in more temperate climates, is the effect of laterization, which has resulted in further depletion of already low potassium, phosphorus and calcium levels owing to

SITE

Mont Koghi
(49)



Plaine des Lacs
(3 sites)
(75)



Rivière Bleue
(75)

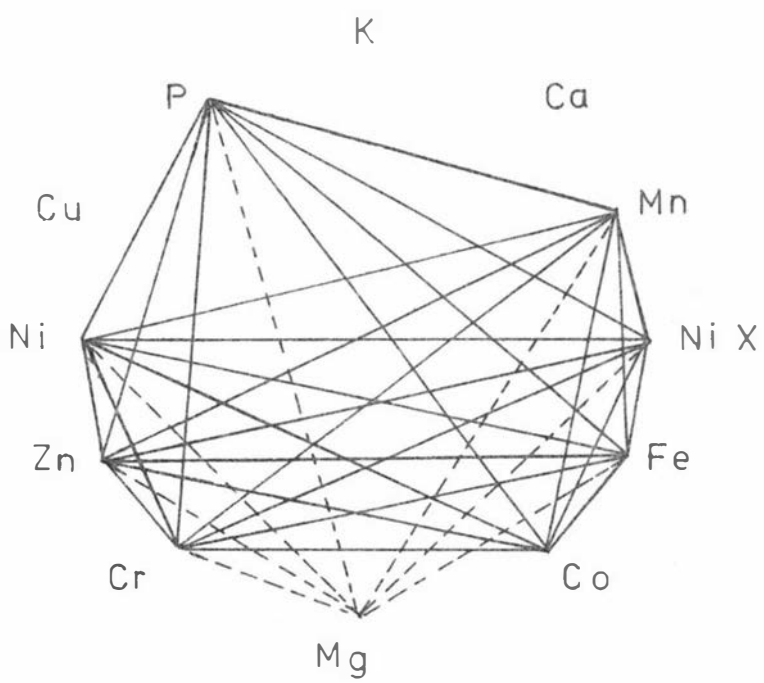


Figure 3.7.1 Elemental relationships in soils. $P < 0.0001$.
Positive —— Negative ------

excessive leaching. Potassium is particularly deficient (0.01%) in the soil. The three species however were able to accumulate adequate amounts of this element (~0.75%) despite the disadvantages of antagonism to uptake caused by several elements. Similarly, phosphorus uptake is affected by iron and chromium in the soil and the low content in the soil (187 µg/g). Phosphorus in Homalium kanaliense is negatively correlated with iron and chromium in the soil.

In serpentine soils, the principal factor is often excess levels of magnesium which result in lower uptake of nutrients such as calcium, potassium and phosphorous (Lee et al., 1973; Walker, 1954). The successful serpentine species are those which are able to adapt to this imbalance of nutrients in the substrate. However, in the soils of the five sites investigated, calcium uptake is apparently unaffected by unfavourable mineralogical edaphic factors. Calcium is related inversely only to the humus content. This shows a significant departure from trends in other serpentine areas and on brown hyper-magnesium soils of New Caledonia (Jaffre', 1976), where high magnesium levels depress calcium uptake.

The extremely high chromium and iron concentrations in those soils are, as previously mentioned, a characteristic feature. The three plants investigated are, however, able to restrict their uptake of these elements, although to some extent this is due to the depressed availability in the soil. This is particularly evident in the case of chromium. The uptake of cobalt is also restricted even though there was found to be a substantial pool of available ions. Homalium kanaliense seems able to extract larger quantities of cobalt from the soil than either H. guillainii or Hybanthus austrocaledonicus although the concentration of this element was similar at all soil sites.

The behaviour of these plants towards iron, cobalt and chromium is in marked contrast to that of nickel, which seems to be accummulated in unrestricted quanties. Many other species growing on nickeliferous substrates are able to tolerate the high levels in the soil by excluding the element from its system at its roots. It is possible that, for these species, the root membranes are impermeable to both cationic and/or complexed nickel.

The lack of interelement relationships between nickel and other elements in the plants and soils is perhaps the most

significant factor revealed, and raises the possibility of an accumulation mechanism dependent on organic constituents. Williams (1959) has stated, "there is much justification for considering a biological system in terms of an equilibrium between cations and their complexes." The chemistry of nickel in several hyper-accumulating plants is examined in more detail in the following chapter.

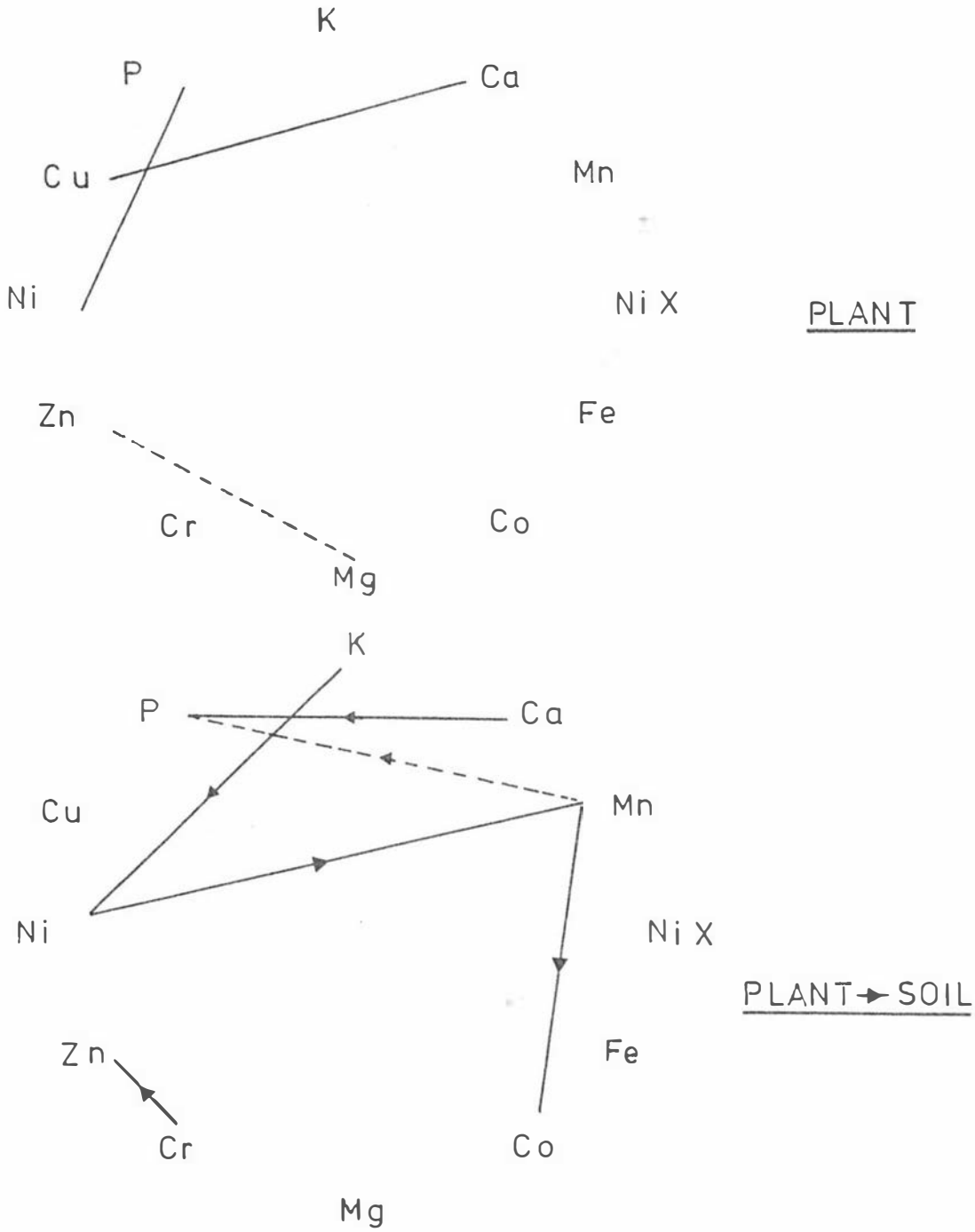
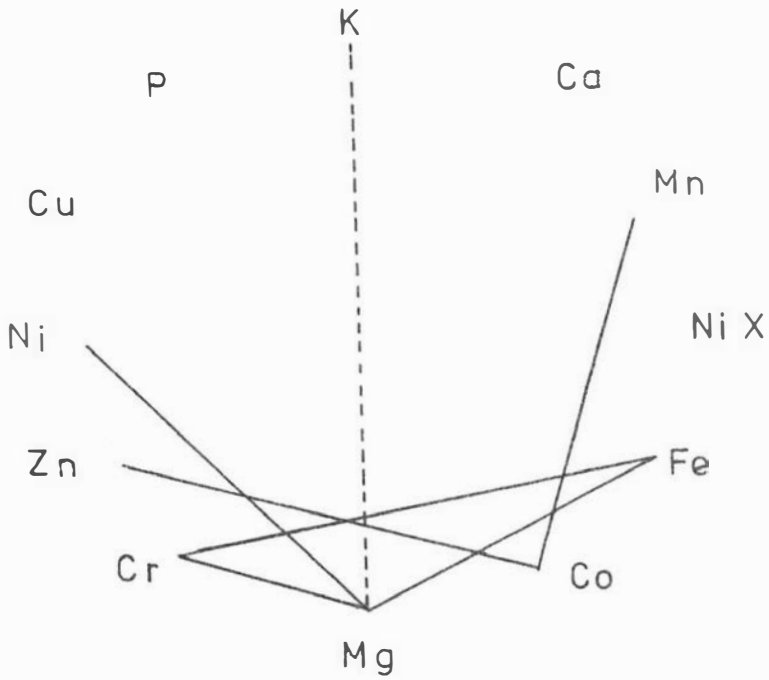


Figure 3.7.2 Relationships between elements in plants and soils of H.austrocaledonicus

—— Positive ------ Negative

$P \leq 0.0001$



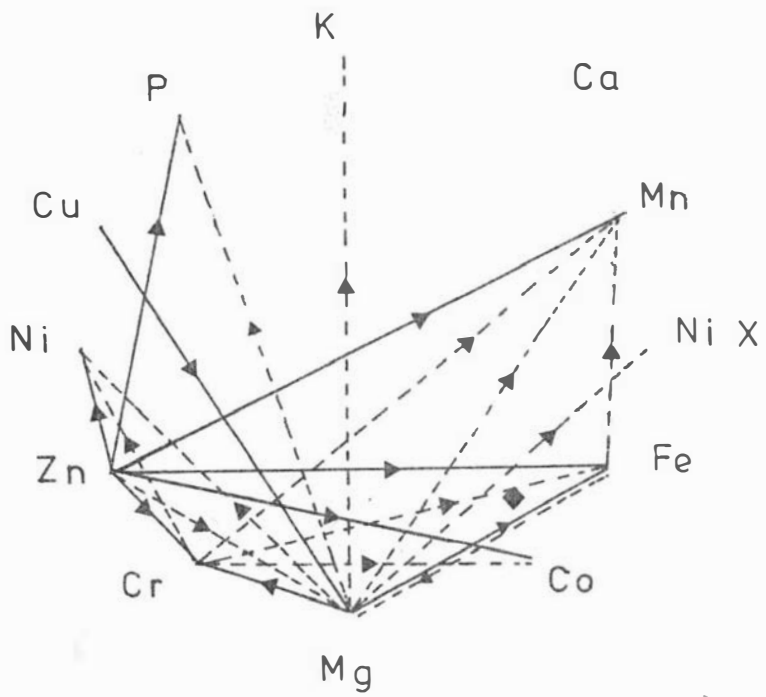


Figure 3·7·3· Relationships between elements in plants and soils of H. guillainii.

— positive ---negative

$\rho \leq 0.0001$

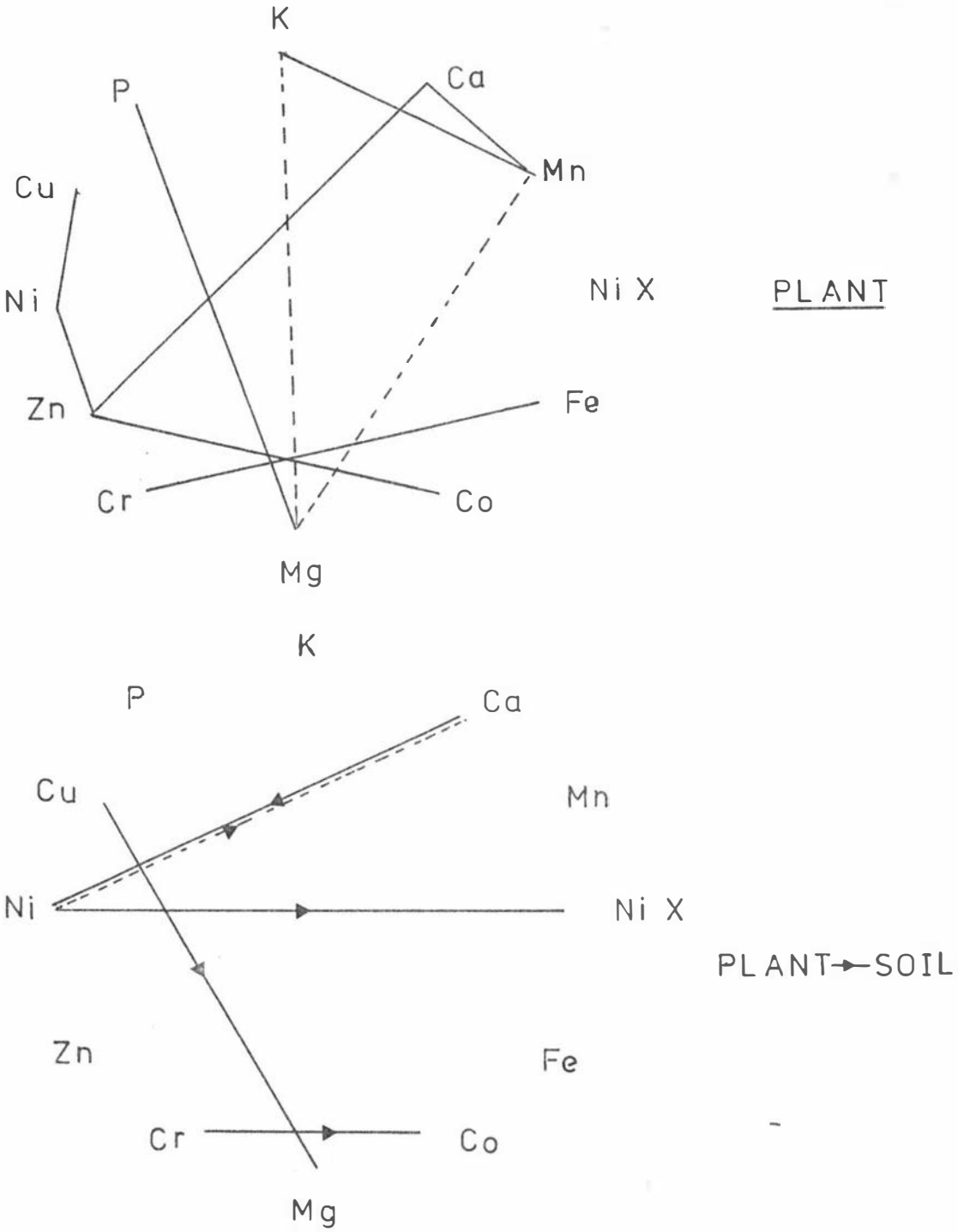


Figure 3·7·4: Relationships between elements in plants and soils of H. kanaliense.

—— positive --- negative

$\rho \leq 0{\cdot}0001$

.4.

Phytochemical Studies on Nickel in some Hyperaccumulating Plants

4.1 INTRODUCTION

In the foregoing chapter a statistical approach was adopted for the study of biogeochemical processes involved with the accumulation of nickel and other elements in three hyperaccumulating plants. Although the judicious application of statistics to observed data can, in some cases, lead to the detection of relationships among trace elements in plants and soils, more meaningful information relies heavily on an experimental approach.

As noted previously, a specific mechanism for the selective uptake and translocation of nickel must be hypothesised in order to account for the strong differentiation of nickel uptake among species growing on the same nickeliferous substrate. The toxic nature of ionic nickel in plants is well known and has been widely documented (Haselhoff, 1893: Cotton, 1930; Brenchley, 1938; Hunter and Vergnano, 1953; Kashin, 1968; Chaney, 1963; Halstead, Finn and Maclean, 1969). Given this, therefore, it is likely that a buffer system is present which would account for the tolerance these species have towards the presence of large quantities of nickel in their system. Two aspects must be considered in accounting for the hyperaccumulation of nickel. Firstly, the selective absorption via the root system, which deals with entry of some ionic species into the cell or tissue by any mechanism, and secondly, the active transport in the plant involving a mechanism dependent on metabolism which moves the ion into cells or through tissues against a concentration gradient and results in accumulation. The uptake of minerals and active transport has been covered in detail by Robertson (1958, 1968),

Price (1970) and Gauch (1972).

As early as the beginning of the century, Pfeffer (1900) recognized that organisms could transport substances across a membrane even in the absence of a gradient favouring transport, and concluded that substances might combine with certain cellular constituents during this transport. This conclusion is very similar to the carrier concept as proposed by Honert (1936) and later supported by Jacobson and Overstreet (1947), Hutner (1948) and Roberts *et al.* (1949). The carriers were regarded as ion-binding and ion-releasing compounds. It was suggested that cations might be bound in plants in the form of chelated complexes; proteins, amino acids, and organic acids could form chelated compounds, particularly with polyvalent cations such as calcium, magnesium and the transition elements which are present in plants. Rosenberg (1948) noted that for cation-binding complexes to be involved in absorption there would have to be a region at the external surface of the cytoplasm that was relatively impermeable to free ions but permeable to the complexed form. Jacobson *et al.* (1950) suggested that ion absorption depended on metabolically produced chelating compounds, and visualized the reaction as

$$HR + M^{+} \rightleftharpoons MR + H^{+}$$

for cations, where HR represents the chelating compound. Overstreet and Jacobson (1952) described the carriers as having, or needing, the following characteristics: an ability to undergo chemical alteration in the course of their carrier function, ability to function as chelating agents, and possession of a degree of instability *in vitro*. They are intermediate metabolic products or closely related substances.

Robertson (1968) in dealing with combination and complex formation commented that 'the chemistry of metal organic complexes requires further study with more detailed knowledge of the stability constants of compounds which complex with metals: further, little is known about the specific compounds into which these ions enter.' Since then, sporadic research has been done and various suggestions made. In the main the nature of carriers for most metals is still unknown.

Stewart (1963) stated that naturally occurring chelates of heavy metals are probably important sources of mineral elements in the soil and most likely are the primary form in which they are absorbed and

translocated in plants. The existence and functioning of carriers would explain many of the experimental findings with regard to absorption of various cations, and interrelationships between cations.

In a biological system there is a wide variety of ligands which are capable of complex formation with a small number of cations. These include porphyrins, amino-acids, peptides, proteins, purines, ribo flavines, nucleotides, nucleic acids, sugars and tri- and di-carboxylic acids, to name a few of the naturally occurring compounds in plants. There is no general order for the affinity of a series of ligands with all cations, but, at a given pH where an excess of several ligands exists, different metals are restricted to the formation of particular complexes of definite ligand types (Williams, 1953). Copper and zinc ions are likely to be co-ordinated to sulfur and nitrogen at physiological pH, but not to oxygen. Cobalt, ferrous iron and nickel ions are likely to be associated with mixed oxygen-nitrogen donors such as amino acids. Manganese, magnesium and calcium ions are possibly co-ordinated to oxygen donors.

It is interesting to note that the order of metal ion activity in producing toxicity, Ni > Cu > Co > Cr > Zn > Mo > Mn (Hunter and Vergnano, 1953) agrees well with the order of stability of metallic organic complexes as given by the Irving-Williams rule (Irving and Williams, 1953).

One example of the biochemical importance of chelating compounds is that at the physiological pH of plants many metals would be likely to precipitate as insoluble phosphates and hydroxides if they did not form complexes (Schmid and Gerloff, 1961; Stewart, 1963). Some mechanisms in plants involving chelates are storage, oxidation - reduction reactions, translocation and the transmission of energy (Stewart, 1963).

From the introductory work of this project the unusual nickel accumulating capacity of several New Caledonian species has been established. The presence of such high concentrations raises interesting questions in plant chemistry and is a major factor stimulating research into the chemical form of transition elements in plants. Although something is known about the great number of compounds in which metals occur in plants there is relatively little information about the form in which these metals are translocated. Much of the existing information has come from electro-phoretic experiments using radioactive tracers because of the generally low physiological levels

of these elements. Unidentified anionic complexes of nickel and copper occur in xylem exudates of plants such as tomato, cucumber and peanut (Tiffin, 1971). Anionic forms of copper and zinc have been found in ryegrass extracts (Bremner and Knight, 1970), and much of the zinc in extracts of Ricinus communis is bound in anionic organic complexes, possibly containing phosphorus (Van Goor and Wiersma, 1976). Tiffin (1970, 1972) found that iron (III) in several plant exudates showed the same electro-phoretic behaviour as anionic iron (III) - citrate complexes, but suggested (Tiffin, 1972; Thompson and Tiffin, 1974) that amino acids act as carriers for copper and nickel, as these elements show a particular affinity for nitrogen-containing ligands. Earlier work (Tiffin and Brown, 1962) on electrophoretic separations on the exudates of soybeans, indicate that most of the iron was present as chelates of malic acid and malonic acid. The iron occurred in these forms even though it had been supplied in the nutrient solution as a synthetic chelate.

Schmid and Gerloff (1961) made studies on the nature of iron in the xylem exudate of tobacco and concluded it was in an organic complex form. However, chromatographic separations suggested that it was not complexed with amino acids, organic acids, or ascorbic acid but rather with a compound of higher molecular weight. These differences in the two studies appear difficult to reconcile but it may be that the principal chelating agent for iron is different among species or even within the same plant. This reasoning seems acceptable when the wide quantitative variation of various compounds capable of chelating with iron are considered.

Organic acids appear to be the most favoured ligand involved with metal absorption and translocation. Bennet-Clark (1933) found large quantities of aluminium malate to be present in some succulent plants. Calcium oxalate crystals have been isolated from plants (Al-Rais, Myers and Watson, 1971) grown under normal conditions but magnesium, barium and strontium may substitute for calcium to a certain degree. Bradfield (1976) implicated the involvement of citric and malic acids in the mobility of calcium in xylem sap of apple shoots. Approximately 50% of the calcium was complexed with these acids. Lyon et al. (1969) identified three anionic complexes of chromium in Leptospermum scoparium of which the major one was trioxalato chromium (III). Gauch and Dugger (1953) have emphasized sugar-borate complexing in sugar transport but proof of this was

dependent on the isolation of boron-containing compounds. Lee and Aronoff (1967) however noted that association constants of borates with hydroxyacids (e.g. citrate) and phenolic acids are equal or several orders of magnitude higher than sugar-borate constants. This raises doubts about translocation of sugar-borate in the xylem.

Using ^{60}Co tracers and sephadex gel chromatography Wilson and Nicholas (1967) showed that 90% of the tracer in legume and wheat species was not associated with cobalamin nor was inorganic cobalt. Unidentified low molecular weight complexes from *Trifolium subterraneum*, *Triteum durum* and *Glycine max* all behaved similarly on sephadex.

Gomah and Davies (1974) identified the active ligands chelating zinc in some water extracts of dried *Vaccinium* leaves to be illagic and catechin using sephadex gel and thin-layer chromatography techniques.

Some information is available concerning the form of metals in various accumulating and metal tolerant plants. Gregory and Bradshaw (1965) studied the heavy metal tolerance in populations of *Agrostis tenuis* and concluded that the mechanism of tolerance of some species may be correlated with their capacity to immobilise metals in conjunction with an organic buffer system. The mechanism of tolerance has been studied in a zinc tolerant clone (Wyn Jones, Sutcliffe and Marshall, 1971). A zinc binding complex of the cell wall was released by enzymatic digestion and purified by column and thin layer chromatography. The complex was partially characterized as containing both sugar and amino acid residues. Tolerance was found to be under genetic control. Peterson (1969) has found that the ^{65}Zn in the pectate extract of *Agrostis tenuis* was higher in zinc-tolerant than in the non-tolerant plants and suggests that the mechanism of tolerance is the deactivation of zinc by cation binding sites in the cell wall.

Work by Reilly (1969, 1970) on copper accumulating species has suggested the presence of copper - protein complexes. Farago *et al.* (1975), using paper chromatography with ammonium oxalate extracts of *Hybanthus floribundus*, showed the presence of a nickel compound with R_F similar to that of nickel pectinate but also noted the possibility that nickel was associated with amino acids. However, no association between nickel and amino acids was found by Lee (1974) and Kelly *et al.* (1975) in studies of *Hybanthus* species from New Caledonia.

Severne (1972) studied the composition of nickel complexes in H. floribundus and although he was not able to isolate a pure complex, he deduced that nickel was in the form of a complex of low molecular weight. Recently Pelosi et al. (1974, 1976), studied aqueous extracts of Alyssum bertolonii Desv., a nickel accumulator from Italy, and results indicated that nickel was associated with organic acids such as malic and malonic acids.

In the present project, studies were carried out on the nature of nickel complexes in several species of accumulator plants from New Caledonia, all of which normally contain nickel concentrations in their leaves and latex, well in excess of 1000 µg/g (dry weight basis). These levels allowed the isolation, purification and identification of nickel complexes to be performed.

4.2 PLANT SPECIES INVESTIGATED

The chemical status of nickel in the following species from New Caledonia was investigated: Sebertia acuminata Desv., Hybanthus austrocaledonicus Schinz et Guillaumin, H. caledonicus (Turcz.) Cretz., Homalium francii Guillaumin, Homalium guillainii Briq., Homalium kanaliense Briq. and Psychotria douarrei (G. Beauvisage) Däniker. Descriptions of these species, and the hyperaccumulation of nickel by them, has been described in sections 2.2 , 2.3 and 2.4. The hyperaccumulator, Pearsonia metallifera, a grass from near the Great Dyke, Rhodesia, (Wild, 1974) was also investigated. The dry leaf material of this plant contained 1.06% nickel.

On arrival, fresh leaf material from New Caledonia, was washed and freeze -dried if not for immediate use. The tapped latex from S. acuminata was stored, as obtained, under refrigerated conditions.

4.3 INTRA-PLANT DISTRIBUTION OF NICKEL AND OTHER ELEMENTS

Table 4.3.1 reports the concentrations of various elements in different organs of Homalium kanaliense and Hybanthus austrocaledonicus on an ash weight basis:

Table 4.3.1. Elemental concentrations in various organs of Homalium kanaliense and Hybanthus austro-caledonicus (% ash).

	Co	Cr	K	Ni	P	Ca	Mg	Ca/Mg
Homalium kanaliense								
Flowers	0.0202	0.006	39.750	2.93	1.120	13.050	6.00	2.18
Mature Leaves	0.0420	0.0120	36.000	7.48	0.560	13.950	3.60	3.87
Bark	0.0060	0.0060	6.450	1.01	0.150	3.450	0.90	3.83
Soil	0.0540	2.9640	0.005	0.49	0.003	0.001	0.10	0.01
Hybanthus austro-caledonicus								
Flowers	0.0087	0.0165	46.500	0.90	3.750	8.400	12.90	0.65
Old leaves	0.0480	0.0795	17.250	24.00	0.840	10.500	13.80	0.76
Mature leaves	0.0270	0.0360	19.800	22.50	1.140	9.600	14.25	0.67
Twigs (2-year)	0.0042	0.0142	16.200	6.75	0.530	7.650	3.00	2.55
Bark	0.0165	0.0165	-	21.00	0.510	6.900	2.25	3.06
Thick roots	0.0042	0.0285	5.400	6.67	0.320	6.750	2.10	3.21
Fine roots	0.0081	0.1080	5.400	18.00	0.510	2.700	3.45	0.78
Soil	0.0620	2.1280	0.005	0.80	0.003	0.080	1.68	0.05

The ability of these plants to restrict chromium uptake is notable. Nickel however is concentrated in the aerial parts of the plants with older leaves containing the highest level. Nickel does not appear to accumulate in the flowers to any great extent. The ability to obtain adequate amounts of potassium from a deficient soil is noteworthy. Another feature is the difference in the calcium : magnesium ratio between Homalium kanaliense leaves and those of Hybanthus austrocaledonicus, these being 3.87 and 0.67 respectively. This is probably a reflection of the concentration differences between the two respective supporting soils. Calcium has been severely leached from the associated soil of H. kanaliense.

4.4 PRELIMINARY STUDIES ON NICKEL IN LEAF TISSUE

The findings summarized in the following paragraphs are based on results obtained from an initial study of nickel in Homalium kanaliense, and given in a former report (Lee, 1974). These results are pertinent in the elucidation of the form of nickel in the hyper-accumulators being studied in this project.

Initial studies involved solvent extraction schemes and differential centrifugation on freeze-dried and fresh leaf material from the species Homalium kanaliense, Hybanthus austrocaledonicus and Psychotria douarrei. These studies were carried out to investigate possible preferential binding sites of nickel within the plant tissue.

4.4.1 Solvent extraction

A solvent extraction scheme based on that given by Bowen, Cawse and Thick (1962) was adopted to study the solubility of nickel in different solvents.

Approximately 2g of leaf material was macerated with 10 ml of 95% ethanol for 5 minutes in a bottom-drive homogenizer. The resulting slurry was centrifuged and the residue washed with portions of ethanol. The filtered ethanol extracts, containing lipids, pigments and other small neutral molecules, were combined and set aside for later analysis. The residue was then extracted with two 10 ml aliquots of distilled H_2O, filtered and washed. The water extract contained readily soluble polar compounds of low molecular weight, such as organic acids.

The remaining residue was extracted three times with 5 ml

portions of 0.2M hydrochloric acid. The acid fractions were combined and equal volume of acetone was added to precipitate any extracted proteins and pectates. The supernatant and gelatinous precipitate were referred to as the hydrochloric acid fraction and acetone insoluble fraction respectively. The residue was similarly extracted with 0.5M perchloric acid at 80°C and the filtrate precipitated with acetone. This procedure removed most of the remaining polar compounds and other more tightly bound groups, such as cellulose, lignin and other structural groups. The liquid and precipitate were referred to as the perchloric acid fraction and nucleic acid fraction.

The remaining residue was boiled with 2 M sodium hydroxide for 10 minutes, which degraded most of the remaining proteins and polysaccharides. The two final fractions were referred to as the soda fraction and the residue.

The fractions were analysed by atomic absorption spectrophotometry for their nickel content. The results are summarized in Table 4.4.1. as a percentage of total nickel in the leaf.

Table 4.4.1. Distribution of nickel in various extracts (%)

Species	Ethanol	H_2O	HCl	Acetone insoluble	$HClO_4$	Nucleic acid	NaOH	Residue
H. kanaliense	0.65	55.92	26.82	0.30	6.26	0.07	3.84	8.04
H. austro-caledonicus	0.19	53.20	40.81	0.17	4.23	0.03	0.87	0.50
P. douarrei	0.42	64.0	27.58	0.40	4.20	0.04	1.80	0.56

Over 80% of the nickel content was easily extracted with water and dilute acid. This indicated the high solubility, polarity and exchangeability of the nickel compound. Only minimal quantities were extracted by 95% ethanol which produced a fraction containing amino acids, non-polar pigments and lipids. The low amounts of nickel in other fractions indicated perhaps that much of the nickel in the leaves of these hyperaccumulators was located more or less in the vacuole system and not tightly bound to structural components. Homalium kanaliense differed from the other two species in having a larger proportion of nickel remaining in the final residue. This quantity (8.04% of total) may be regarded as absolutely immobile

within the cell. Ernst (1970) obtained similar results from sequential extractions of Indigofera setiflora leaves. Butanol extracted only minimal quantities while water, dilute acid and citric acid extracted 23.4, 34.3 and 34.3 percent respectively. Ernst concluded that metals extractable by water are located in the vacuole system and that generally leaves with high metal contents have also a considerable water extractable fraction.

Severne (1972) found that 61% to 64% of the total nickel content in freeze -dried leaf material of Hybanthus floribundus was consistently extracted by water. In fresh leaf material, however, 92% of the nickel was water extractable. These results compare closely with those obtained in this study. Fresh leaf material of Homalium kanaliense yielded 78.0% of the nickel on extraction with water at $0^{o}C$. The freeze-drying process therefore has the effect of immobilizing some of the nickel within the plant tissue. Differential centrifugation of fresh and freeze-dried leaf tissue showed that freeze-drying processes increased the percentage of nickel associated with the cell wall.

Pelosi, Galoppini and Vergnano-Gambi (1974) found that 77% of the total nickel from leaves of Alyssum bertolonii was readily water soluble. Methanol extracted 30% whilst 95% ethanol and acetone removed 9% and 0% respectively. The high aqueous solubility of nickel in plant species has also been confirmed by Kelly et al. (1975) and Timperley (1971). The results seem to imply that the element is present either as free ions or as highly polar complexes of generally low molecular weight.

4.4.2 Differential centrifugation.

A 1g sample of freeze-dried Homalium kanaliense leaf tissue ($\sim$ 7,000 µg/g Ni) was homogenised for 60 secs in 10 sec bursts with 20 cm^3 of 0.4M sucrose and 0.025 M phosphate buffer at pH 6.8. A Waring blender was used at top speed with short intervals between bursts to prevent excessive temperature rises. The resulting pulp was filtered through a 120 µm nylon mesh and the liquid centrifuged according to the following scheme : 5 min at 112g, 10 min at 1085 g, 12 min at 10,200 g and 75 min at 110,000g. Each residue, at each stage, was taken to dryness, ignited at $500^{o}C$, redissolved in 2M hydrochloric acid and analysed for nickel by atomic absorption spectrophotometry. The final supernatant was analysed directly. The results for macerated

leaves of H. kanaliense are summarized in Table 4.4.2.

Table 4.4.2. Nickel in cell fractions (dry mass) of Homalium kanaliense.

Fraction	g	r.p.m.	min	% Ni
residue		-	-	15.50
cell wall	112	1000	5	25.96
chloroplasts	1085	3000	10	2.12
mitochondria	10,200	13,000	12	0.61
microsomes	110,000	30,000	75	0.1
supernatant		-	-	55.60

When account is taken of the dry mass of the cells and the cell components, the residual nickel is of the same order of magnitude for all cell fractions. There is little or no tendency for nickel to be concentrated in any of the cell fractions. The majority of the nickel is found in the supernatant, reinforcing the observations of Section 4.2.1., i.e. the majority of the nickel present in the leaf material is in the form of a readily soluble polar compound of low molecular weight and is located chiefly in the vacuole system of the cell.

Differential centrifugation of fresh-leaves resulted in an even higher percentage of nickel remaining in the supernatant, and only a small amount in the cell wall residue. This indicated that the high percentage of nickel in the 112 g fraction represented by cell wall material, tended to be an artifact of the freeze-drying process.

Electron microprobe studies (Kelly et al , 1974) on Hybanthus austrocaledonicus, H. caledonicus and H. floribundus showed a very uniform distribution of nickel within leaf tissue. Their results confirmed what was found in the above studies on Homalium kanaliense and Hybanthus austrocaledonicus.

Vergnano-Gambi (1967) however found preferential localization of nickel in the stems of Alyssum bertolonii. The red nickel dimethyl-glyoxime complex showed a very intense colour in the sclerenchymatous areas situated between the vascular bundles.

Ernst (1972) found heavy concentrations of nickel in the cell sap from Dicoma niccolifera. Cobalt and chromium were not detected in the

cell sap of the leaves and lead in only small quantities. It was evident that only part of the various heavy metals were localized in the vacuole system, and other parts of the cell must also be involved in accumulation. Turner (1970) showed that a great part of the zinc and copper was deposited in the cell wall of some European heavy-metal accumulating plants and that this may be a general mechanism of detoxification for various elements.

4.5 EXTRACTION AND ISOLATION OF WATER SOLUBLE NICKEL COMPLEXES FROM LEAF AND LATEX MATERIAL

4.5.1 Water extraction of leaf material

To prevent nickel complexes from appearing as an artifact in the separation process, extraction of macerated fresh or freeze-dried leaf material was carried out with distilled H_2O at 0°C. Approximately 20 g quantities of leaf material from the species listed in 4.2. were extracted twice with 100 cm^3 aliquots of iced distilled water in a bottom-drive homogenizer. The resulting slurry was filtered and the aqueous filtrates combined. This procedure readily extracted between 50% and 70% of the total nickel depending on the species involved. The supernatants were shaken with chloroform / n-butanol (10:1) until no further precipitation occurred at the interface. This extraction removed most of the pigments, lipids and other high molecular weight compounds of low polarity. Negligible amounts of nickel were lost from the aqueous phase in this process. The brownish aqueous solutions were reduced in volume on a rotary evaporator at 35°C. These solutions, approximately 10 cm^3 in volume, were filtered and used directly for gel filtration. Nickel contents ranged from 2,000 µg to 8,000 µg, depending on the species. For total nickel concentrations in the plants, see section 2.3.

The latex from Sebertia acuminata was dissolved in water to approximately twice its volume, filtered through paper and aliquots placed directly on to Sephadex columns.

4.5.2 Gel filtration of water-soluble fraction

Gel filtration on Sephadex columns appeared to be the best method for the separation and isolation of the complexed and ionic nickel from other molecular species present in the extracts. The technique is technically simple to perform and is little affected by

composition of the elutant and temperature. Very labile compounds may be treated with little risk of decomposition. The procedure may be used under very mild conditions. The gels available are chemically very stable and have a low content of ionic groups, although it is impossible to avoid charged groups completely. Various gels are available with varying gel matrices so that the fractionation range may be greatly altered. The commercial gels used in this project were dextrans manufactured under the trade name Sephadex.

Various gels with differing fractionating ranges were tested, resulting in the selection of G-50 for initial separations of leaf and latex extracts and G-10 for final filtrations. G-10 has a working range of molecular weights of 100 to 700.
The nickel complex had previously been shown to be of low molecular weight (Severne, 1972; Lee, 1974). The nickel (II) aqueous complex ($Ni(H_2O)_6^{2+}$) has a molecular weight of 167. Small compounds were increasingly retarded as their size decreased.

Sephadex gels were swollen in distilled water overnight before being packed into 60 cm x 2.5 cm glass columns. Owing to the extreme stability of these gels and the ease with which contaminants may be removed, the columns could be employed for many elutions over a period of time. The columns were washed with 0.1M hydrochloric acid and equilibrated with water. Water was initially used to elute the columns to reduce the possibility of complex formation between the extracted nickel and any added reagents. Dextran -blue dye was employed to check the functioning of the column and to establish the void volume (65 ml for G-10 column).

Small quantities (1 ml) of crude aqueous extracts, containing up to 20 mg of nickel, were applied evenly to the column head and fractions collected with an automatic fraction collector. Approximately 4 ml fractions were obtained at a flow rate of about 0.3 ml/min. Every second fraction was analysed for nickel by atomic absorption spectrophotometry. The nickel-containing fractions were combined and recycled through the column. This effectively increased the column length. The green colour of the nickel fraction intensified with improved separation. The green coloured nickel fraction separated out between the faster moving high molecular weight plant substances and a yellow-brown band of low molecular weight organic acids, amino acids and various salts.

The nickel species were more easily separated from the latex because of the large proportion present and the simple nature of the latex matrix. The bulk of the latex was composed of a single high molecular weight species; probably a polysaccharide. The nickel fraction was the major low molecular weight entity. Plate 4.5.1. shows the separation of the green nickel-containing band from the off-white high molecular weight latex component. Using water as the elutant, Ni $(H_2O)_6^{2+}$ was poorly resolved from the nickel complex. However using a volatile buffer such as 0.1M ammonium acetate separation of complexed nickel and aqueous nickel ions could be achieved. Ammonium acetate could easily be removed under reduced pressure. Figure 4.5.1. shows the elution characteristics of the nickel fraction using 0.1M ammonium acetate buffer. Although the shape and position of the peaks were remarkably consistent for extracts from each of the species, the ratio of free nickel ions to complexed nickel varied considerably from species to species. Generally the higher the nickel concentration in the plant the larger was the free nickel ion fraction. In the latex of Sebertia acuminata the ratio, complexed nickel to aqueous nickel, was approximately 1 : 1; whereas in the leaves of H. kanaliense and H. guillainii this ratio was much greater.

The $Ni(H_2O)_6^{2+}$ could be easily removed from complexed nickel by passing the extracts through a weak cation exchange gel. (Sephadex CM-25). The column depicted on the right in plate 4.5.1 shows this separation. The cationic aqueous species is strongly held on the column whilst anionic and neutral complexes of nickel are eluted. Subsequent work substantiated the anionic nature of the nickel species in all plants studied. Water was again used as the elutant.

The green nickel-containing fractions from the columns were taken to dryness at room temperature.

4.5.3 Purification of nickel chelate

The gel filtration procedure produced a nickel fraction of relatively high purity but attempts to obtain highly crystalline samples were not entirely successful. Pale green nickel containing powders could be easily obtained by precipitation from aqueous solutions using various organic solvents such as ethanol, methanol or acetone. The best results were obtained by slow evaporation from

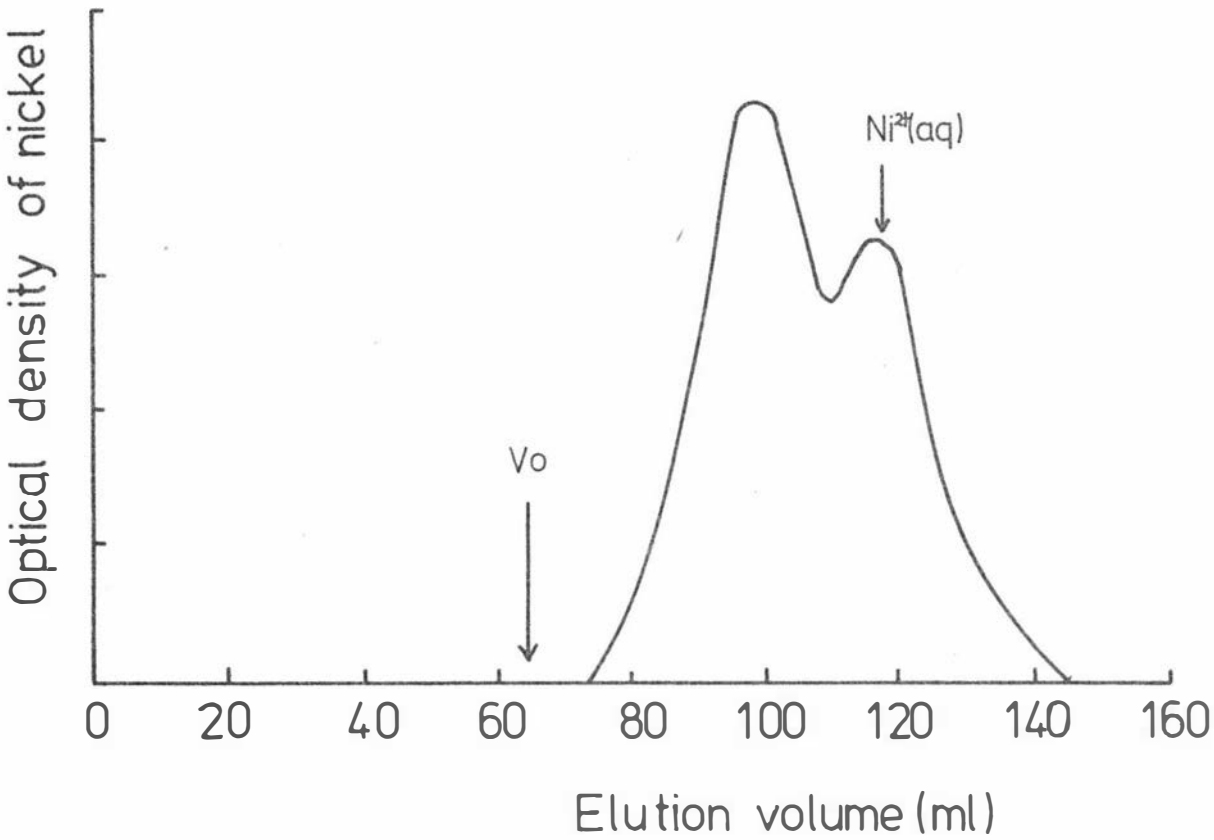


Figure 4·5·1: Elution curve from Sephadex G-10 column showing distribution of nickel from aqueous extract of Hybanthus austrocaledonicus leaves.
Elutant: 0·1 M ammonium acetate; column: 60×2·5 cm.

1 : 1, methanol-water solutions. After several days pale green nodules formed. These were filtered off from the small amount of remaining supernatant and dried in a dessicator for several days.

Microanalysis of the solids showed that the nitrogen content was insignificant and in many cases a nil result was obtained. The possibility of amino acids being involved could therefore be eliminated. The results also gave an indication of purity. The only cations present in measurable quantities were nickel, calcium and magnesium. Only traces of iron, magnesium, sodium, zinc and potassium were found using atomic absorption spectrophotometry. Because of the presence of nickel, oxygen could not be determined. Unfortunately consistent results for the analysis of nickel, calcium and magnesium could not be obtained. Overall elemental composition showed some variation from one preparation to another. Table 4.5.1 shows the composition of preparations from the latex of S. acuminata and the leaves of H. austrocaledonicus. Colorimetric tests for chloride, sulphur and phosphates all proved negative.

Table 4.5.1. Composition of purified nickel extracts (%)

Sample.	Ni	Ca	Mg	C	H	N
Latex	12.6	0.62	0.13	20.03	4.38	Nil
H. austrocaledonicus	9.33	0.66	3.33	21.43	4.16	Nil

An indication of purity and extent of crystallization was obtained from x-ray powder diffraction spectra. A Phillips x-ray powder diffractometer was used to record scintillation peaks corresponding to degrees of 2θ by rotating a thin smear of the powder through 180°. From Bragg's equation, $n\lambda = 2d \sin\theta$, 'd' spacings between adjacent planes in the crystal could be calculated. These are characteristic for specific crystalline compounds. Noise-to-signal ratios were generally low with sharp intense scintillation peaks being recorded. The results indicated a highly crystalline substance, rather than one of an amorphous nature.

Emission spectrographic analysis of the purified nickel extract from the latex produced a photographic plate containing few lines with

the carbon emission line at 2478.57 Å and the various nickel lines, dominated by the 3101.55 Å line forming the basis of the spectrum. Traces of boron (doublet at 2496.78 and 2497.73 Å) and silica were evident. The boron presence is most certainly due to an impurity in the electrode. Magnesium was present at the 2 ppm level.

4.6 CHARACTERIZATION AND IDENTIFICATION OF NICKEL BINDING LIGAND

4.6.1 Preliminary tests

The nickel complex proved to be highly insoluble in a wide range of organic solvents, such as ethanol, methanol, benzene, ethyl acetate, methyl isobutyl ketone, acetone, acetic acid, and toluene. The complex was however, very soluble in water, indicating the highly polar nature of the compound. The microanalytical results showed that nitrogen-containing ligands, such as aminoacids, could be ruled out.

Various spot tests (Feigl, 1966) for specific groups, such as dibasic and polybasic acids, hydroxy acids and simple reducing carbohydrates, while not conclusive, indicated a polyhydroxy acid. The ligand could easily be displaced from the nickel ion using hydrogen sulphide, α-furildioxime or dimethyl-glyoxime. Fehling's test for reducing sugars gave a negative response on the nickel complex as well as solutions from which the nickel had been removed.

4.6.2 Ion - exchange separations

The behaviour of the isolated nickel fraction was investigated on various ion exchange columns. Columns, 10 cm x 1 cm were prepared using the strong anion exchange resin, Dowex -1; Amberlite IR-50, a weak cation exchanger; Amberlite IR-120 (H^+), a strong cation exchange resin, and Sephadex CM-25, a dextran gel incorporating weak carboxylic groups.

Small aliquots of the crude extracts and of purified extracts from the various plant species were eluted through the columns which had been equilibrated with distilled water. Hydrochloric acid (1M) was used to elute strongly held nickel. Fractions were collected and analysed for nickel using atomic absorption spectrophotometry.

The strong cation exchange resin effected the breakdown of the nickel chelate and only one nickel containing fraction was obtained from the column with elution using 1M hydrochloric acid. However,

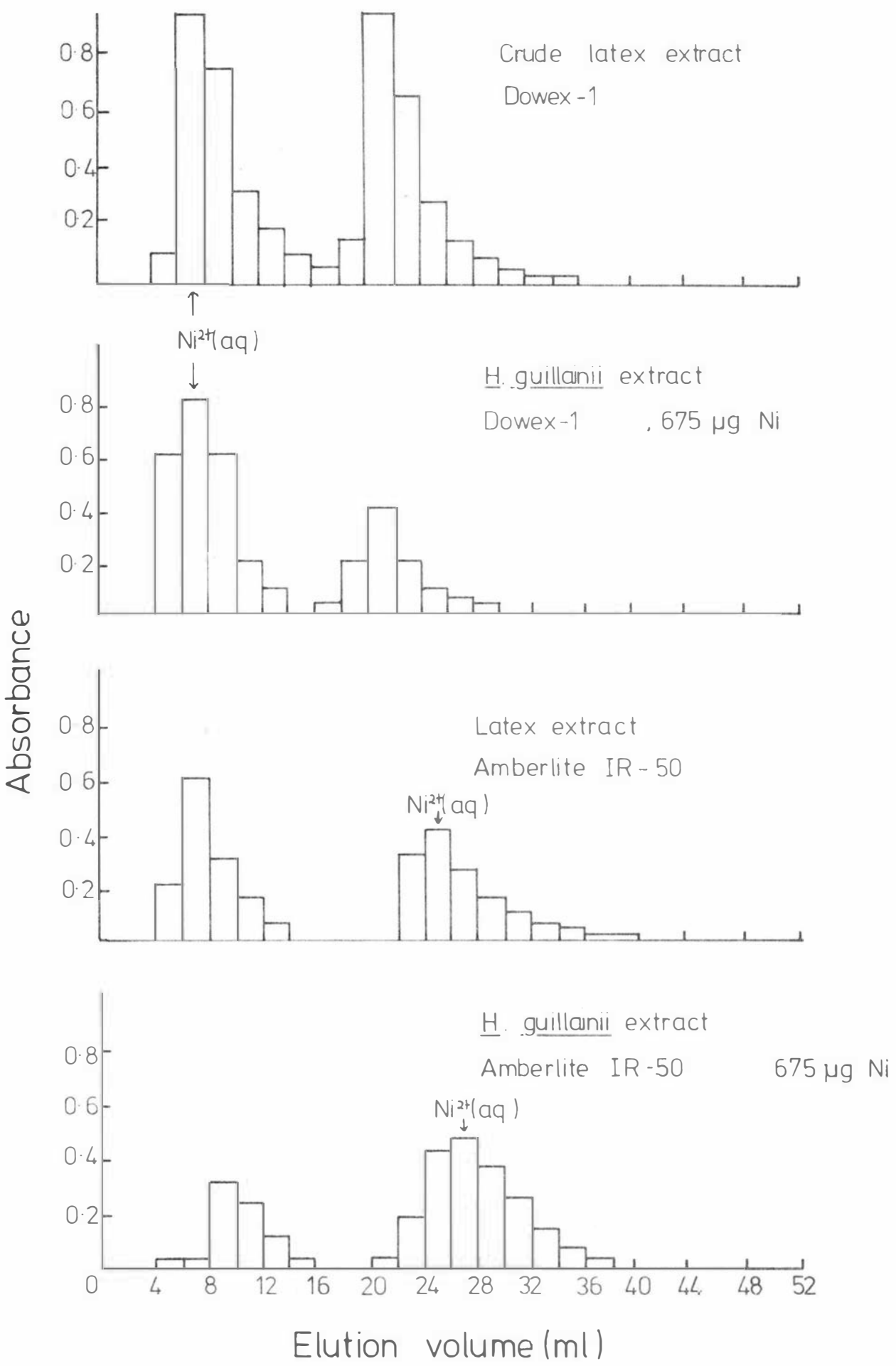


Figure 4·6·1 : Distribution of nickel from ion-exchange column. Fractions correspond to complexed and aqueous nickel.

from the Dowex-1 column and the weak cation exchange columns, two nickel fractions were obtained representing both anionic and cationic nickel. The Amberlite IR-50 column did however appear to exchange some of the complexed nickel as the anionic fraction was not as large as that obtained from the carboxylic acid cation exchange column (CM-25). The distribution of nickel from the columns used is shown in Figure 4.6.1 for extracts from the latex of S. acuminata and the leaves of H. guillainii. Behaviour of the nickel from the other extracts was similar.

4.6.3 U.V. - Visible - I.R. spectrophotometry

Infrared spectra of the purified solids were recorded to identify frequencies characteristic of specific groupings. U.V-visible spectra were recorded and compared with that given by the $Ni\ (H_2O)_6^{2+}$ ion.

Infrared spectra of the nickel complex in both nujol and hexachlorobutadiene mulls showed in every case, carboxylate stretching frequencies at 1590 cm^{-1} (asymmetrical OCO stretching, characteristic of ionized -COOH groupings, shifted from 1700 cm^{-1} in unionized acid) and 1430 cm^{-1} (symmetrical OCO stretching, generally not as strong as former frequency band). Also evident were frequencies characteristic of H_2O bending and OH stretching vibrations. Noteworthy is the splitting of the COO^- stretching band at 1600 cm^{-1}. This possibly indicates the presence of two or more ionized carboxylic acid groups. The broad -OH stretches in the region 3300-2500 cm^{-1} are characteristic of carboxylic acids in general. The strength of this band may also indicate some water of crystallization. Figure 4.6.2. shows the recorder tracing obtained for a nujol mull of the anionic nickel-containing component isolated from the latex of S. acuminata (separated on CM-25 column). The resolution of this spectrum was better than that obtained for nujol mulls of the total purified nickel extract from the latex and leaves of H. austrocaledonicus.

A Shimadzu MPS-5000 with a scanning range of 180nm - 2,500 nm was used to obtain absorption bands from 0.1M solutions of the isolated nickel complex. The spectrum obtained was characteristic of those obtained for nickel in an octahedral environment. The ground state term (^3F) is split in an octahedral field giving rise to the triplet terms with three spin-allowed transitions. These three

transitions are observed in the frequency regions, 7000-13000 cm^{-1}; 11,000-20,000 cm^{-1}; and 20,000-25,000 cm^{-1} (Cotton and Wilkinson, 1966). It is a characteristic feature of the spectra of octahedral nickel (II) complexes, exemplified by that of $Ni(H_2O)_6^{2+}$ that molar absorption coefficients of the bands are low, (1-100).

The spectrum of the isolated anionic component from the plant extracts is shown in Figure 4.6.3 along with that of the nickel (II) aqueous species. The bands are not shifted appreciably from those of the nickel aquo complex and this indicates the co-ordination of oxygen ligands rather than nitrogen. Nitrogen co-ordination is generally associated with shifts of the absorption bands to lower wavelengths. Amine complexes e.g. $Ni(en)_3^{2+}$ characteristically have blue or purple colours due to the shift in the absorption bands towards the red. Noteworthy differences between the two spectra shown in Figure 4.6.3 are; the change in peak shape and shift of the band centred at 700 nm, and the increased absorbance of the nickel complex over that of the aqueous nickel species.

Pestemer et al. (1949) observed that in the absorption spectra of the monobasic carboxylic acid salts of nickel (AcOH, $EtCO_2H$, $PrCO_2H$), the bands of the nickel (II) ions and the anions persist throughout but for the salts of the polybasic acids (oxalic, tartaric and citric), stronger shifts due to complex formation appear in the first and third absorption bands.

The results obtained are consistent with those derived from the infrared spectra.

4.6.4 High voltage paper electrophoresis of purified nickel complex

As electrophoresis separates ionic species according to charge and, to a lesser extent, to molecular size, this technique can give some information about the nickel extracts. High voltage paper electrophoresis has been used by many workers as a convenient tool for the study of metal mobilities and in the separation of biological materials (Effron, 1960: Peterson, 1969; Timperley, 1971; Tiffin, 1967, 1966, 1971; Bremner and Knight, 1970; Van Goor and Wiersma, 1975).

The behaviour of the nickel complex was examined at various pH and the mobility of the nickel compared to that of $Ni(H_2O)_6^{2+}$.

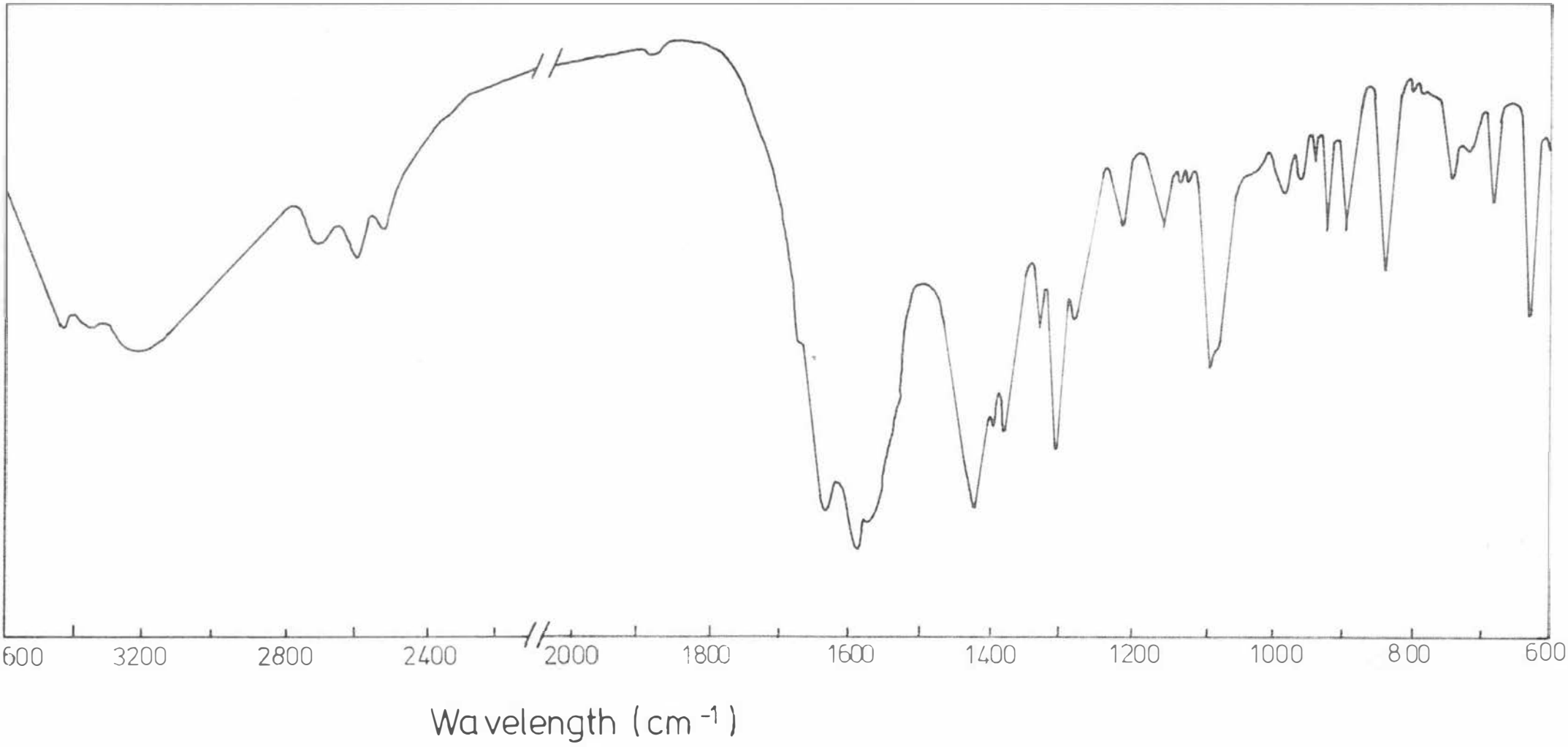


Figure 4.6.2: Infra-red spectrum of nickel complex isolated from latex of S. acuminata

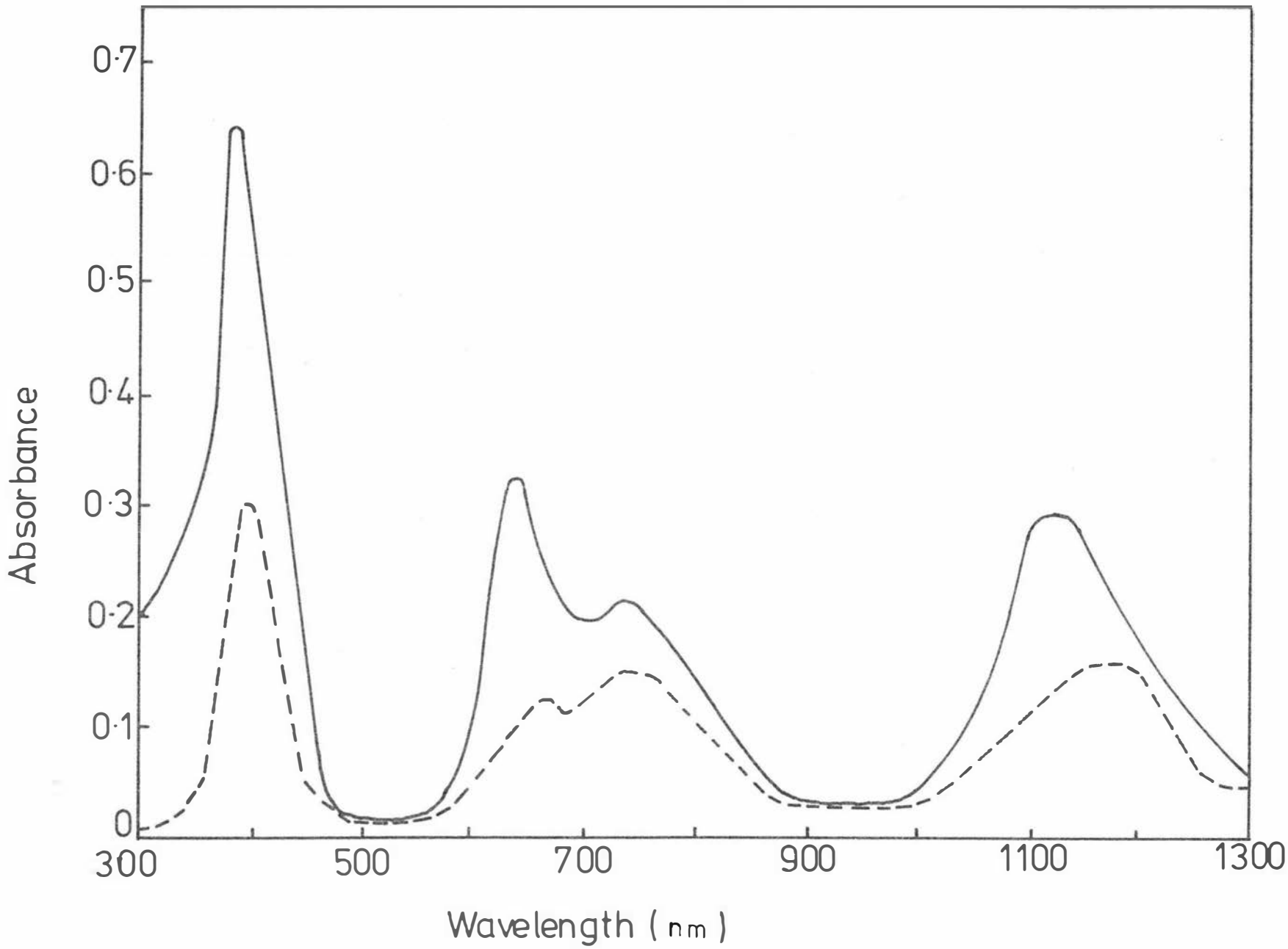


Figure 4·6·3 : Absorption spectra of $Ni(H_2O)_6^{2+}$ (----) and nickel anion from latex (——).

A 'Savant' type apparatus was used, consisting of a glass tank comprising a lower buffer layer overlain with a water-cooled, low flash point, petroleum spirit. The papers (46 cm x 57 cm Whatman No. 1 and 3 MM chromatography paper) were hung vertically from the upper buffer layer through white spirit into the lower buffer layer and a voltage of 3kV applied for 50 minutes. This apparatus was employed at pH 2.1, 3.5 and 6.5. A horizontal commercial electrophoresis table (Pherograph - original, model 64) was used for pH 5.4 and those above 8.0. Table 4.6.1 gives information on the buffer systems employed.

Taole 4.6.1. Buffer systems for use in electrophoresis.

pH	Composition	Ratio
2.1	Formic acid - acetic acid - water	100 : 400 : 4500
3.5	Pyridine - acetic acid - water	25 : 250 : 4010
5.4	0.2M Acetic acid - 0.2M sodium acetate	1 : 9
6.5	Pyridine - acetic acid - water	500 : 20 : 4500
9.0	Boric acid - sodium borate - water	0.62 : 7.63 : 1000

After each run, papers were air dried and the nickel located using α-furil dioxime or dimethylglyoxime sprays. Because of the possibility that some nickel may not have shown up by this method, the papers were cut into 1 cm strips, ashed, and the nickel content analysed by atomic absorption spectrophotometry.

Figure 4.6.4 shows electrophoretic patterns of nickel from nickel extracts of several hyperaccumulators at pH 6.5. Inorganic nickel was the fastest running component and migrated towards the cathode. Both cationic and anionic nickel components were revealed. These results confirmed the presence of the differently charged species as observed from the ion exchange columns. Slower moving cationic species are observed at 3 cm and 8 cm. Essentially the same patterns as those of Figure 4.6.4 were also obtained at the physiological pH of 5.4.

At pH 2.1 all nickel migrated as a compact spot towards the cathode behaving as $Ni(H_2O)_6^{2+}$. This behaviour was also observed at pH 3.5. It appears therefore that for pH below at least 3.5 the equilibrium between the anionic complex and the nickel aquo-complex

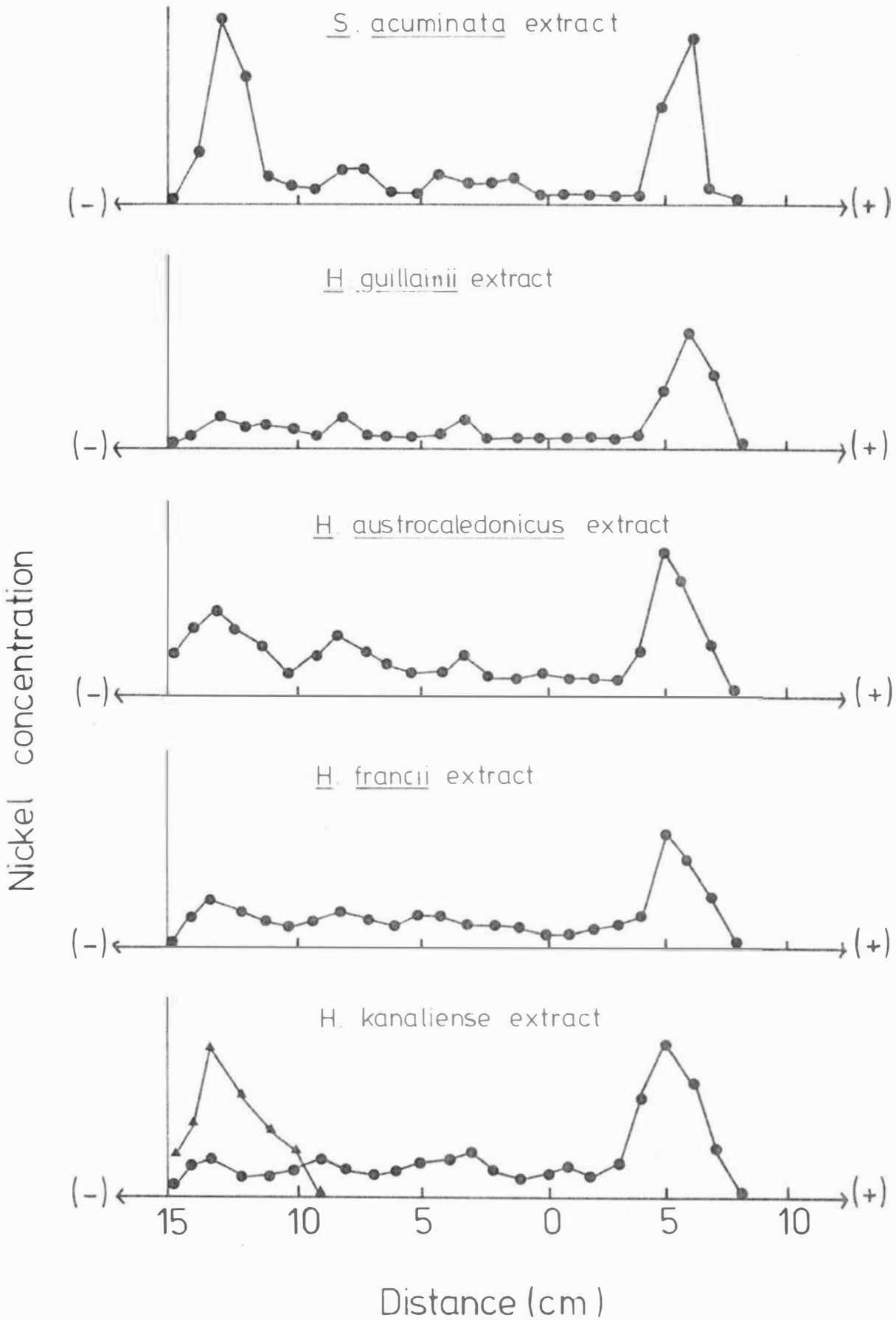


Figure 4·6·4 : Electrophoretic distribution of various extracts (●) compared with $Ni(H_2O)_6^{2+}$ (▲) Pyridine/acetic acid / water at pH 6·5, 3kv, 40 min, Whatman No.1 paper.

strongly favours the latter.

Electrophoresis at pH 9.0 using a borate buffer produced no movement from the origin. Inorganic nickel, however, showed the same behaviour.

The electrophoretic migration rate for the nickel anion at pH 6.5 was identical for all species. Generally it was observed that the greater the total nickel concentration the higher was the proportion of inorganic nickel. Thus, the nickel extract from the latex of S. acuminata, while containing large quantities of anionic nickel, also contained a large quantity of cationic nickel. Homalium decurrens on the other hand, which containes only 650 µg/g of total nickel, produced an extract in which the nickel was almost entirely anionic. It appears that the anodic carrier saturates at certain concentrations of nickel depending on the species and that excess nickel runs cathodically as the inorganic cation.

Previous experiments (Tiffin, 1971) have revealed negatively charged nickel in xylem exudates of tomato, cucumber, corn, carrot , and peanut. The migration rate was the same for all species.

Pickering (1960) observed that on Whatman No. 1 paper the cations lead, zinc, cadmium, copper and nickel were absorbed. This author considered the mechanism to be ion exchange and showed that the exchange capacity of the paper was approximately 4 µg/g. The filter paper itself generally has a certain content of -COOH groups and ion exchange of reversible complexes formed with the paper has a retarding effect on the speed of migration. Such a phenomenon may account for much of the tailing of the anodic moving components observed on the electrophoretograms.

Further support for the above comments was that the anionic complexes in the extracts showed no retention, regardless of the loading. The anionic complexes generally showed higher resolutions on the electrophoretic papers for this reason.

Elution of the anionic complex from preparative electrophoretograms was attempted in early work but this separation proved to be more satisfactorily carried out using a weak carboxylic acid cation exchange resin (Sephadex CM-25) as described previously.

4.6.5 Gas liquid chromatography

(i) Introduction

Gas liquid chromatography provides a quick and easy way of determining the number of compounds in a mixture, the presence or absence of impurities, and, in many cases, primary evidence as to the identity of a compound. The only requirement is some degree of stability at the temperature necessary for the production of the vapour.

Preliminary tests and results indicated that the nickel in the isolated extracts was bound to an organic component showing characteristics of a hydroxy carboxylic acid. Gas liquid chromatography, coupled with mass spectrometry, appealed as the best instrumental technique for unambiguous identification of the ligand. The low volatility of the complex necessitated derivatization of the organic component by silylation. In recent years, trimethylsilyl (TMS) derivatives have found increased use in gas liquid chromatography, largely because they are appropriate for a wide range of functional groups. These are easily formed through the reaction of a trimethylsilylation reagent and an active hydrogen on the group to be silylated. The usefulness of TMS ether derivatives in the gas liquid chromatography of carbohydrates was first demonstrated by Sweeley *et al*. (1963). The preparation and analytical advantages of TMS ester derivatives were described by Horii, Makita and Tamura (1965) for acids of the Krebs cycle, and more recently by Harmon and Doelle (1969), and Pinelli and Colombo (1976). Petersson (1974) has reported the gas-chromatographic analysis of sugars and related hydroxy acids as ester TMS derivatives. The major acids in plants have been determined by gas liquid chromatography of their TMS derivatives by Merkel and Jungh (1973) and Phillips and Jennings (1976). An extensive review of the analysis by g.l.c. of soluble carbohydrates in extracts of plant tissues has been given by Holligan (1971). Dutton (1974) has reviewed the g.l.c. of carbohydrates in more general terms.

(ii) Methods

Approximately 10 mg of solid nickel complex was dissolved in the minimum amount of water and 0.2 ml of trifluoroacetic acid or concentrated hydrochloric acid introduced into the vial. The sample was evaporated to dryness under a stream of dry nitrogen. As trimethylsilylation is adversely affected by moisture complete removal of water

is desirable. TMS reagents react rapidly with water to form hexamethyldisiloxane and the particular silylation reagent by-product, thus reducing their activity.

The residue was taken up in 0.5 ml of dried Analar dimethylsulphoxide, and 0.5 ml of N-(trimethylsilyl)-imidazole introduced through the rubber sealed screw cap of the vial (these had been acetone-leached overnight to remove any organic impurities). The reaction was catalysed by the addition of 0.1 ml of trifluoroacetic acid (TFA). This reagent has also been used by Brobst and Lott (1966) instead of the more often used trimethylchlorosilane (Sweeley et al. 1963). The former workers have found that TFA was useful in catalysing silylation reactions in the presence of small amounts of water which could not be easily removed. Previous silylations on the nickel extract with TMS -imidazole alone proved to be slow and often erratic. The addition of TFA considerably improved the derivatisation.

The silylating mixture was shaken occasionally until dissolution was effected. A successful silylation was indicated by the appearance of an upper bright clear layer identified by Reid et al. (1970) as hexamethyldisiloxane. The TMS derivative has a high affinity for this phase. The nickel and certain by-products are conveniently retained in the bottom DMSO layer. This two phase system thus provided a useful selective process for the removal of interfering substances. The hexamethyldisiloxane phase, on isolation, could conveniently be concentrated when only small amounts of derivative were present, and gave a rapidly eluted and reduced solvent peak compared with pyridine as used by Sweeley et al. (1966).

The silylating reagent, N-trimethyl silylimidazole (TSIM) was selected because of its specificity towards -OH groups and its ability to silylate in the presence of moisture. Silylation was found to be complete within 5 minutes (by comparison of peak areas from successive injections). Derivatives were stored in moisture proof containers under refrigerated conditions.

Samples taken from the upper layer with a micro-syringe could be injected directly on to the gas chromatograph column.

Analyses were conducted using a Pye Series 104 chromatograph with the following instrument parameters : injection sample, 0.1 - 1 µl (depending on sample concentration): column conditions, 200 cm x 3mm glass, 1.5% OV-101 on Chromasorb W, AW-DMCS, 100-120 mesh, isothermally operated at 170°C: flow rate, approx. 45 ml/min nitrogen; flame

ionization detector, hydrogen and oxygen flow rates 40 ml/min and 450 ml/min respectively. The detector temperature was that of the oven at all times. The TMS derivative of glucitol was used as an internal standard.

(iii) Results.

Both standard acids and extracts were fully silylated, even when injected into the g.l.c. 5 minutes after derivatization. No increase of peak area with time was found, and the silyl derivatives were stable for at least 5 days. The chromatogram (Figure 4.6.5) showed the presence of a single TMS derivative with no sign of impurities or partially silylated derivatives.

Table 4.6.2 gives retention times, relative to that of TMS glucitol (11.5 mins), of the silylated ligand from the isolated nickel complex, along with various authentic carboxylic acids representative of different homologous series. The retention times of the TMS ethers of nickel extracts from different species were the same in every ease.

Table 4.6.2. Retention times and relative retention times of the TMS-derivatives of isolated nickel ligand and various standard acids.

Acid	Retention time (min)	Relative retention time (R_T)
Nickel extract	5.75	0.50
malic acid	0.75	0.065
arabinonic acid lactone	2.20	0.19
citric acid	5.75	0.50
iso-citric acid	5.75	0.50
gluconic acid lactone	7.75	0.67
quinic acid	7.90	0.69
galactaric acid mono-lactone	8.6	0.75
glucitol	11.5	1.00
galactaric acid	17.5	1.52

Citric acid and iso-citric acid TMS -ethers also gave the same R_T value as that of the nickel extract. Citric and iso-citric acids could not be separated on either OV-101 or OV-17 columns.

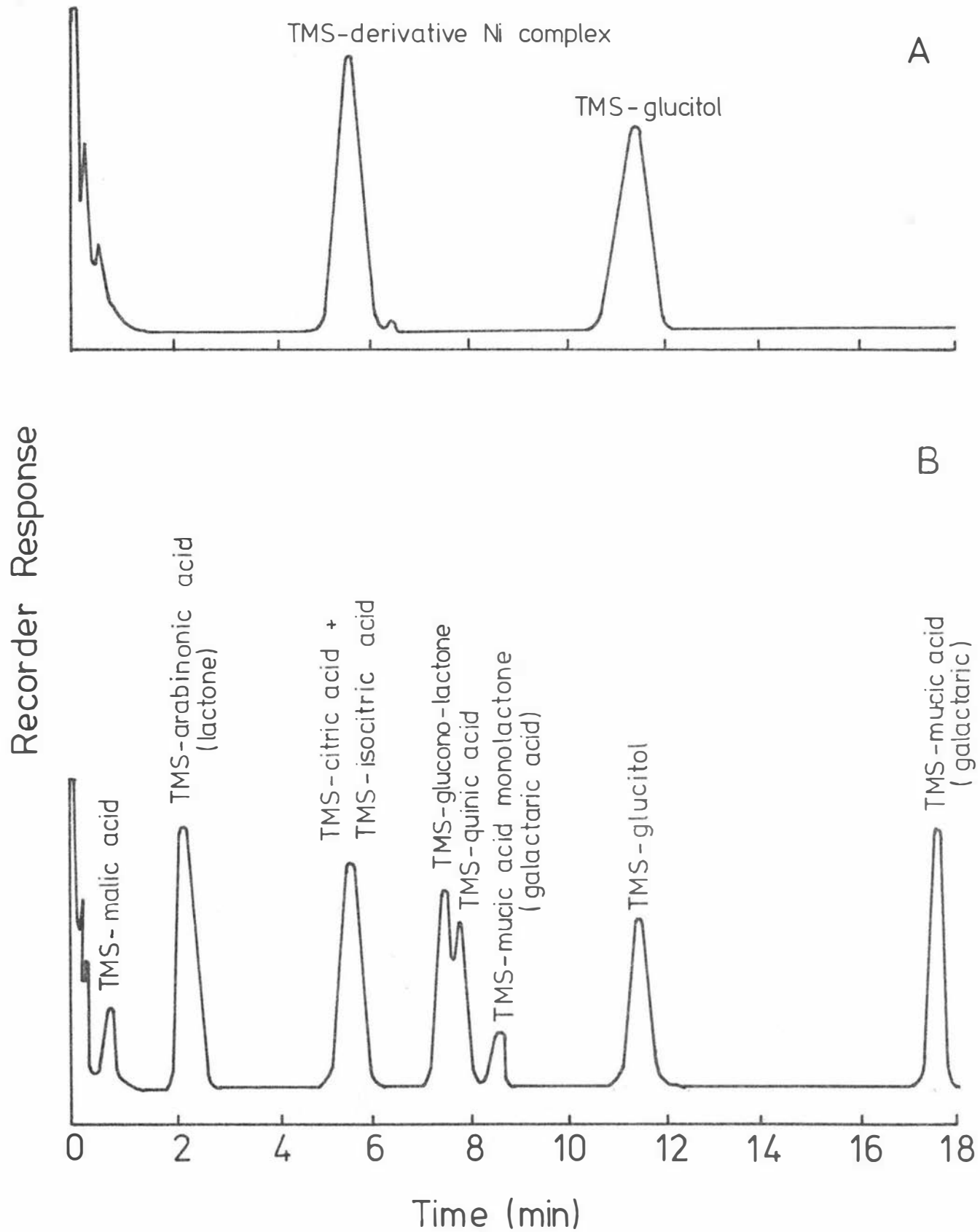


Figure 4·6·5 : Gas liquid chromatograph of silyl derivatives of:

A : Ni extract

B : various standard hydroxy carboxylic acids

1·5% OV-101, 170°C , I.S. TMS-glucitol , 0·1 µl sample.

Co-chromatography of the three silylated together produced a single well-defined peak.

Results indicate that the complexing ligand is either citric or iso-citric acid, or something very similar to these in structure and physical properties, as, for a homologous series, retention time is closely related to the number of OH groups, the presence or absence of side chains, and boiling points. Ideally peak identification should be based on two or more retention times for each compound, using different volatile derivatives or stationary phases. The problem of clearly separating citric and iso-citric acids is not new (Pinelli and Colombo, 1976; Soderstrom, 1962; Phillips and Jennings, 1976). The quantitative aspects of this problem are also compounded by the equilibrium between iso-citric acid and its lactone, the lactone being produced in varying amounts during concentration steps and acid extractions. Phillips and Jennings (1976) have developed a procedure which involves two successive analyses using different stationary phases, one of which separates citric and iso-citric acids, directly as silyl derivatives.

However, for this project positive identification could be more readily obtained by gas chromatography coupled with mass spectrometry.

4.6.6 Mass spectral analysis of g.l.c. peak

The TMS derivatives described above are well suited for identification by mass spectrometry, and various aliphatic carboxylic acids have been studied by a number of researchers (Petersson and Samuelson, 1967; Eglinton *et al.*, 1968; Draffan *et al.*, Petersson, 1970, 1972). Characteristic fragmentation and rearrangement pathways have been obtained and recorded. Mamer *et al.* (1971) have accumulated a library of known spectra of a number of TMS -acid derivatives. Their report includes a summary of the important and characteristic m/e values and intensities of some of these compounds, including the TMS ethers and esters of authentic heterocyclic, aliphatic, aromatic and phenolic acids that frequently occur in biological fluids. Comparison of these spectra with those of unknowns was instrumental in their identification.

In this project the eluted peak of the TMS -derivative of the isolated nickel extract was scanned by an AEI MS 30 mass spectrometer after elution under the g.l.c. conditions previously described and passage through separators to exclude the large excess of carrier gas

which would otherwise interfere in the operation of the instrument. Mass spectrum scans were started near the top of the eluting peak on the ascending side so that by the time the molecular ion was recorded, the source pressure was near its maximum value, enhancing if possible, the intensity of this frequently weak (Petersson, 1970) but very important peak.

Mass spectra were obtained for the tris -(TMS) ester TMS ethers of citric and iso-citric acid and compared with that obtained for the TMS derivative from the nickel containing extract of S. acuminata (Figure 4.6.6). The spectrum of the effluent from the chromatographic column showed the presence of a single TMS derivative with only traces of impurities. The highest mass peak seen in the low resolution spectrum was at m/e = 465, derived from a molecular ion of mass 480. The molecular ion peak is often absent from spectra of hydroxyacid TMS derivatives (Petersson, 1970), the largest detectable fragment being formed by loss of $-CH_3$. Peak heights are measured relative to that of the most abundant ion at m/e 73 (trimethylsiliconium ion). This fragment (along with its analogs at m/e 147 and 221) is ubiquitous in the mass spectra of TMS ethers and esters of hydroxy carboxylic acids and carbohydrates in general, and its structure $(CH_3)_3 Si^+$, and derivation are well known (Eglinton et al. 1968; Diekman et al., 1968; DeJongh et al., 1969). Peaks between m/e 73 and 147 are not shown as they are generally weak and not pertinent for identification and comparison purposes.

Accurate measurement of the masses of several fragments led to a mass of 480 . 185 and molecular formula $C_{18} H_{40} O_7 Si_4$ for the TMS derivative of the isolated complex. This corresponded to a parent compound $C_6 H_8 O_7$. Known compounds of this formula include the saccharic acid monolactones, citric acid and iso-citric acid. G.L.C. of TMS derivatives of monolactones of galactaric acid and glucaric acid, using OV-101, gave retention times considerably different from those of the unknown TMS derivative, which was inseparable from derivatives of citric and iso-citric acids on both OV-101 and OV-17. The mass spectra (Figure 4.6.6) clearly showed the presence of citrate rather than iso-citrate in the isolated nickel complex. The spectra are also characteristically different from those of the TMS -ethers of the saccharic acid monolactones having the same mass for the molecular ion. The spectra for the saccharic acid lactones show strong mass peaks at m/e 333 and m/e 292.

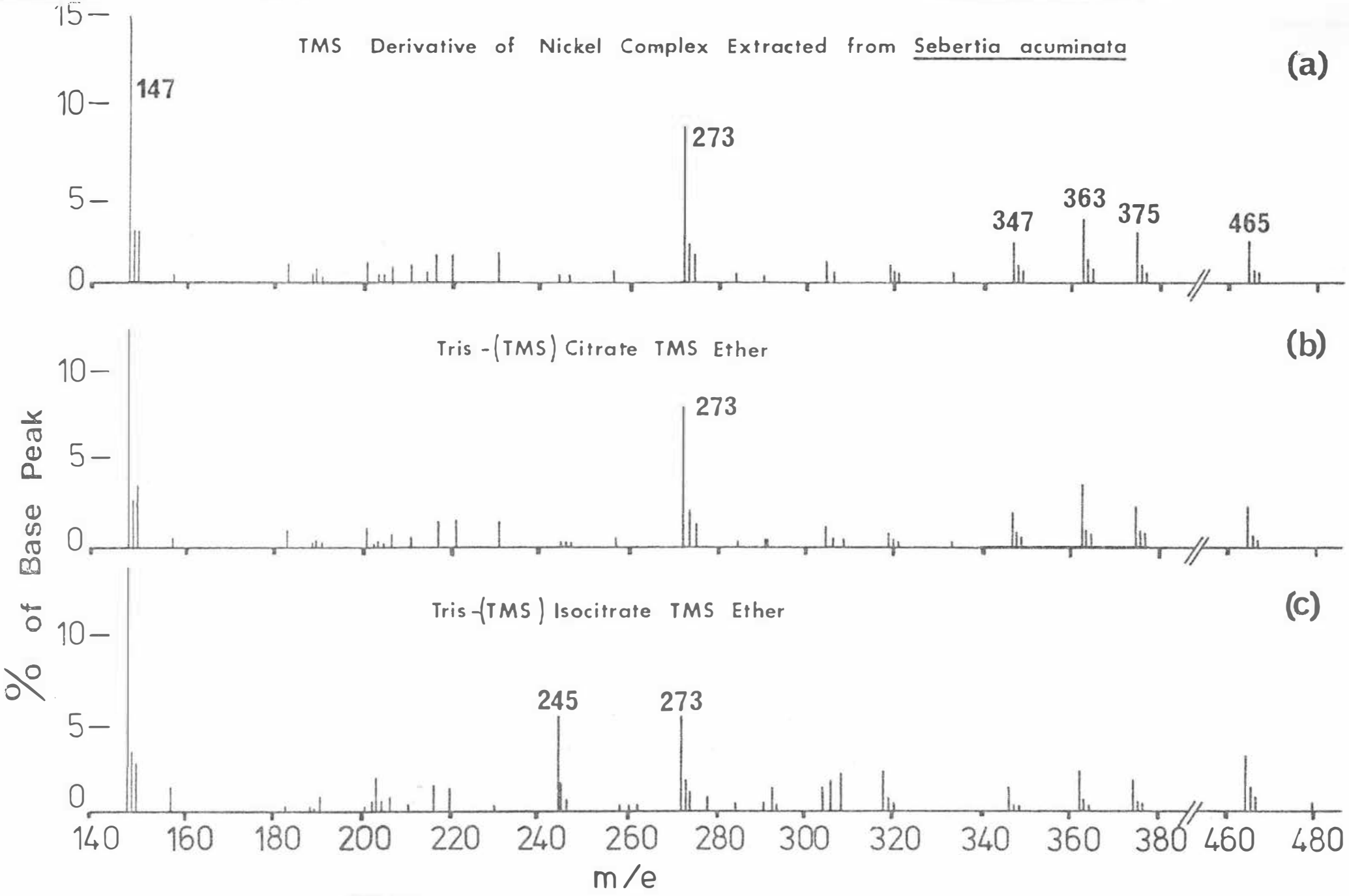


Figure 4·6·6: Mass spectra of TMS derivatives of citric acid, iso-citric acid and isolated nickel complex.

The abundant ion at 292 represents the fragment, $(TMSO)_2$ C- $\overset{+}{C}H(OTMS)$ which is characteristic of a McLafferty-type rearrangement of the molecular ion and involves the loss of -RCHO. This rearrangement requires the structure of a 2,3 - dihydroxy acid (Petersson, 1972).

Table 4.6.3 lists the principal fragmentation and rearrangement ion characteristic peaks in the mass spectra of Tris-(TMS) citrate TMS ethers.

Table 4.6.3. Characteristic fragmentation and rearrangement ions in the mass spectrum of Tris -(TMS) citrate TMS ether.

Mass	Formula	Origin
480.1817	$C_{18} H_{40} O_7 Si_4$	M
465.1582	$C_{17} H_{37} O_7 Si_4$	M - CH_3
393.1205	$C_{14} H_{29} O_7 Si_3$	M - CH_3 -$(CH_3)_2$ Si =CH_2
375.1098	$C_{14} H_{27} O_6 Si_3$	M - CH_3 -$(CH_3)_3$ Si OH
363.1489	$C_{14} H_{31} O_5 Si_3$	M - COO Si$(CH_3)_3$
347.1173	$C_{13} H_{27} O_5 Si_3$	M - CH_3 -CO -$(CH_3)_3$ SiOH
319.1596	$C_{13} H_{31} O_3 Si_3$	M - COOSi $(CH_3)_3$ -CO_2
305.1437	$C_{12} H_{29} O_3 Si_3$	M - 175
273.0988	$C_{11} H_{21} O_4 Si_2$	M - COOSi $(CH_3)_3$ -$(CH_3)_3$SiOH

The major difference between the mass spectra of the TMS ethers of citric acid and iso-citric acid is the abundant ion with mass 245 in the spectrum of the latter. A possible decomposition scheme explaining the formation of the major mass peaks in the mass spectrum for the TMS citric acid derivative is shown in Figure 4.6.7 . Support for the origin of the significant fragmentation and rearrangement ions comes from studies of metastable peaks where

$$m^* \text{(metastable)} = \frac{(M_1^+)^2}{M^+}$$

A metastable peak centred at 205.3 (calc. 205.5) demonstrates the formation of the intense molecular ion at mass 273 from the m/e 363 ion through the loss of trimethylsilanol. Another metastable

(m^* = 302.5: calc. 302.1) supports the formation of the m/e 375 ion from m/e 465 again through the loss of trimethylsilanol.

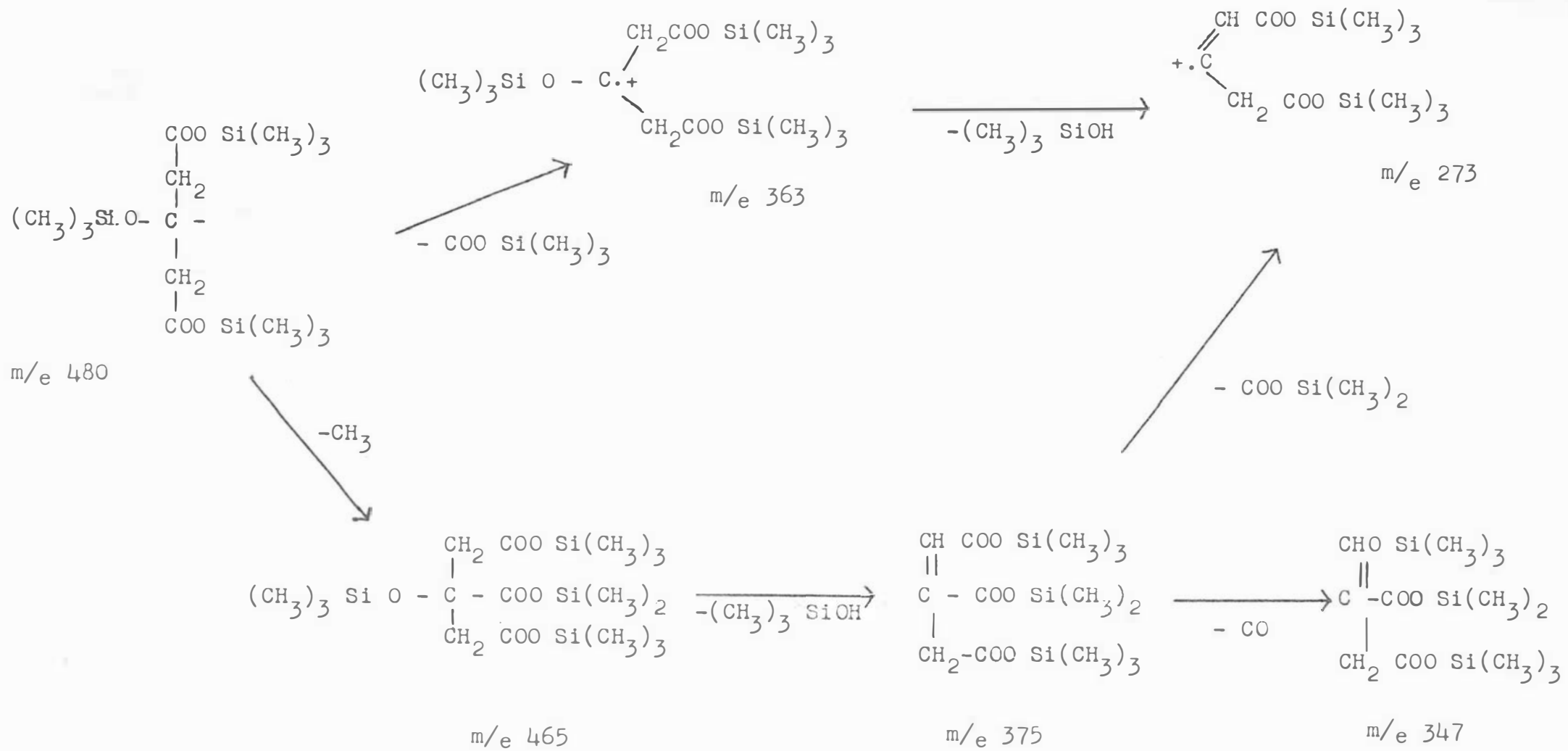


Figure 4.6.7. Decomposition of the molecular ion for TMS -(TMS) Citrate TMS Ether.

4.6.7 Conclusion

Much of the significant chemistry of trace elements and non-essential elements arises from their ability to bind with organic chelating agents which are present at exchange sites. Models for metal chelation are being increasingly used in describing metal relationships in uptake, translocation, enzyme systems and toxicity problems. It is well known that stability constants of micro-element chelates of organic acids suggest possible association in transport (Stewart, 1963; Tiffin, 1972). Such an association is implicit in results of the present study, and the agent responsible in the chelation of nickel in several hyperaccumulators has been identified as citric acid. Purified nickel complexes with citric acid were obtained from Hybanthus austrocaledonicus, H. caledonicus, Homalium guillainii, H. kanaliense, H. francii, S. acuminata and Psychotria douarrei. Identical nickel citrate complexes were verified in all species. The ratio of cationic nickel aqueous species to anionic nickel citrate varied depending on the species and total nickel. Nickel-citric acid chelation was also implicated in the lesser nickel accumulators Homalium austrocaledonicum and H. deccurrens. High voltage electrophoresis showed that practically all the nickel migrated as an anionic species with identical mobilities as those of the hyperaccumulators. It has also been observed that the nickel electrophoretograms of Tiffin (1971) closely resemble those of plant extracts shown in Figure 4.6.7. It appears probable that the nickel carrier in the plants studied by Tiffin is also a citrato-complex, and that translocation of nickel by citrate is not confined to those plants which accumulate high concentrations, but is a more widespread occurrence.

Significant work was carried out by Pelosi et al. (1974, 1976), who studied extracts of Alyssum bertolonii Desv. The first paper reported the separation of water soluble compounds by gel chromatography on Sephadex G-15 into two main fractions. The first fraction contained most of the nickel with no nitrogen, phosphorus, sulfur and halogens. Pelosi et al., (1976) identified malic and malonic acids in the second fraction but the first fraction remained unidentified. Only 3.0% nickel was associated with the second fraction. It is possible that the first fraction obtained by these researchers contains citric acid.[a]

[a] Considerable quantities of citric acid have been found in leaves of Alyssum bertolonii.

In attempting to elucidate the structure of a metal-organic complex in plant material the question always arises as to whether the complex was formed metabolically or whether it is an artifact of the extraction process. One way to approach this problem is to examine the spatial distribution of metal concentrations in the plant and then check to see whether the organic ligand has the same distribution. Electron-probe measurements on leaves of several of the New Caledonian accumulator species showed that nickel was always distributed in a uniform manner without concentration at specific sites, and the above method of approach was clearly inoperable. However, since the nickel-citrate complex was clearly evident in the latex of S. acuminata which was directly separated on Sephadex columns without use of extraction procedures, there is no reason to suppose that the presence of the same complex in extracts from the leaves of this and other species is the result of non-metabolic formation. High voltage electrophoresis of crude water extracts showed the same anionic movement of the nickel as exhibited by the purified nickel-citrate complex and that of model nickel citrate solutions.

The following section examines the behaviour of solutions containing nickel and citrate in various mole ratios, using electrophoresis and u.v. spectrophotometry, and comparisons are made with that of the isolated anionic nickel citrato complex from the plant species.

4.7 SPECTROPHOTOMETRIC AND ELECTROPHORETIC BEHAVIOUR OF MODEL NICKEL-CITRATO COMPLEXES COMPARED WITH THAT SHOWN BY NICKEL CITRATE FRACTION ISOLATED ON SEPHADEX

4.7.1 Introduction

Information on the composition and structure of complexes of nickel with the hydroxy-acids, citric acid in particular, is insufficient and often contradictory. Although the composition and stability of various nickel-citrato complexes has been studied by several workers, notably Bobtelsky and Heitner (1951), Heitner-Wirguin *et al.*, (1958), Li *et al.*, (1959), Campi *et al.*, (1964), Patnaik and Pani (1965), Grigoreva and Tsimbler (1968, 1970), Rautkina (1969) and Field *et al.*, (1975) there remains incomplete agreement as to the formation of 1 :1 or 1 : 2 complexes.

One of the earliest absorptiometric studies of the nickel-citrate system (Bobtelsky and Heitner, 1951) showed that the effect of the citrate ion was to increase the molar absorption coefficient of the nickel ion, but there was essentially no shift of the 393- and 657-nm absorption maxima. This contrasts with the shift toward shorter wavelength, and marked drop in intensity ratio, of the amino acid and amine complexes, with their characteristic blue colour.

Tsitovich and Nikitina (1963) employing ion exchange methods, showed that copper and nickel complex citrate anions are present at low citric acid concentrations (0.01 - 1M solutions). Iron, cobalt and nickel became fixed on a cation exchanger under all investigated citric acid concentrations (0 - 10N).

Using potentiometric and spectrometric procedures, Heitner - Wirguin *et al.*, (1958) in one of the first detailed studies to be reported, provided evidence for the existence of $(Ni\ HC)^{o}$, $(Ni\ C)^{-}$ and $(Ni\ H_{-1}\ C)^{2-}$ species (Although citric acid, C, has an OH group and acts as a tetrabasic acid, H_4C, toward Cu (II) and Fe (III) as shown by Warner and Weber (1953), it has been found that for most metals in the pH region below 9, citric acid acts as H_3C; Li *et al.*, (1959), Campi (1964)). Although Heitner-Wirguin *et al.* asserted that only 1 : 1 complexes were formed, fresh interpretation of their spectrometric results indicates the possible existence of $(Ni\ (HC)_2)^{2-}$ species at pH between 4 and 8 with molar ratios of citrate to nickel equal to or in excess of 2 : 1. Heitner-Wirguin and co-workers plotted absorbance of the nickel ion at 400 nm against pH. A sharp

increase in absorbance was observed up to pH 4. Absorbance then decreased for the 2 : 1 and 3 : 1 solution, but remained the same for 1 : 1 solutions, up to pH 8. It seems likely that the decrease in absorption for the former solutions indicates the formation of a new species which may be postulated as $(Ni\ (HC)_2)^{2-}$. After pH 8 all solutions increased in absorbance, with maxima at pH 11. To explain this, Heitner-Wirguin *et al*. postulate the existence of a $(Ni\ H_{-1}C)^{2-}$ species.

Grigoreva and Tsimbler (1968) isolated several citrate nickel complexes and determined their composition. Both doubly-charged anionic 1 : 1 and 2 : 1 complexes were said to be formed from mixtures of nickel and the sodium salt of citric acid. At different pH, using 1 : 1 and 2 : 1 mole ratios of citrate to nickel, solid compounds corresponding to the following formulae were identified: pH 1 and pH 3 : $Ni\ C_6H_6O_7$; pH 6, $Na\ Ni\ C_6\ H_5\ O_7$ and $Na_2\ Ni\ (C_6H_5O_7)_2$; pH 12.5, $Na\ Ni\ C_6\ H_4\ O_7$. The experimental values for the percentages of sodium and nickel were in good agreement with those calculated. Grigoreva and Tsimbler (1970) used spectrophotometric, anion exchange and electromigration methods to determine that the order of stability of various hydroxy acids complexed with nickel was citric > glutaric > tartaric > malic.

Campi *et al*., (1974) derived the following stability constants for nickel with citric acid; Log $K^{Ni}_{NiH_3C}$ = 1.75, log $K^{Ni}_{NiH_2C}$ = 3.30 and log K^{Ni}_{NiHC} = 5.40. These values are in reasonable agreement with those obtained by Li *et al*., (1959) More recent work (Field *et al*., 1975) report values which are in satisfactory accord with those reported under nearly comparable conditions by Campi *et al*. and Li *et al*. Table 4.7.1 shows metal-citrate stability constants for a number of metal ions. The order of stability of the complexes of different metal ions with citric acid is in agreement with the Irving-Williams rule (Irving and Williams, 1953).

The elemental analysis of the nickel-citrato complex isolated from the latex of *S. acuminata* and the leaves of the other species was consistent with the presence of a dicitrato nickelate (II), the counter-cations being a mixture of $Ni\ (H_2O)_6^{2+}$ and hydrated Mg^{2+} and Ca^{2+}. These results are comparable with the 2 : 1 citrate - nickel species isolated by Grigoreva and Tsimbler (1968) and discussed previously. Rautkina (1969) prepared a compound, formulated as $Na_4\ (Ni\ (C_6H_5O_7)_2)$

Table 4.7.1. Metal citrate equilibrium constants.

Metal ion.	Equilibrium constant log K^{Me}_{HeHc} [a]	Reference
Fe^{3+}	21.17 [b]	Timberlake (1974)
Fe^{3+}	11.40	Timberlake (1974)
Cu^{2+}	5.90	Campi et al. (1964)
Ni^{2+}	5.40	" " " "
Co^{2+}	5.00	" " " "
Zn^{2+}	4.98	" " " "
Fe^{2+}	4.40	Timberlake (1964)
Cd^{2+}	3.75	Campi et al. (1964)
Mg^{2+}	3.40	" " " "
Ca^{2+}	3.55	" " " "
Mn^{2+}	3.67	Sillen and Martell (1964)
Na^{+}	0.69	Walser (1961)
K^{+}	0.43	" "

a $$K^{Me}_{MeHC} = \frac{(Me\ HC^{-})}{(M^{2+})\ (HC^{3-})}$$

b This constant is for the dimer in which Fe^{3+} = citrate = 2 : 2.
All other complexes are for metal: Citrate = 1 : 1.

in the presence of excess tri-sodium citrate. To confirm the existence of a dicitrato nickelate (II) species, in the anionic extract eluted from Sephadex CM-25 column, various mole ratios of citrate to nickel were examined spectrophotometrically and electrophoretically and comparisons made with the plant extract.

4.7.2 Spectrophotometry

(i) Infra-red

Nujol mulls of both the isolated anionic nickel citrate and preparations of sodium citrate and nickel in the ratio of 2 : 1 were scanned in the infra-red. The resulting spectra are shown in Figure 4.7.1. (a) and Figure 4.7.1. (b). The two spectra are comparable, with the splitting of the intense band centred at around 1600 cm^{-1} indicative of more than one ionized carboxylic acid grouping. This is also reflected in the less intense symmetrical OCO^{-} stretch centred at 1430 cm^{-1}. Sharp bands from 1300 cm^{-1} to 1000 cm^{-1} demonstrate the presence of primary OH stretches.

(ii) Visible

Non-complexed Ni $(H_2O)_6^{2+}$ can be removed by passing the extracts through a weak cation exchange column consisting of Sephadex CM-25 gel as described previously. The absorption spectra (340-740 nm) of the resulting solutions correspond to those of a 2 : 1 citrate/nickel solution and differ from those of Ni $(H_2O)_6^{2+}$ and 1 : 1 citrate/nickel solutions. The spectra of 0.1M solutions are shown in Figure 4.7.2. Citric acid in the form of the tri-sodium salt was used as this resulted in citrate/nickel solutions in the pH range 5.5 to 7.0, approximately the physiological status of the nickel extract. The 2 : 1 citrate/nickel solution gave a pH of 6.7 and the pH of latex from S. acuminata was 6.4.

The characteristic dark green of aqueous nickel (II) becomes a bright blue-green as the cation is complexed by the hydroxy acid. There is essentially no shift of the 393 - and 660- maxima, with the addition of increments of citrate up to a 1 : 1 molar solution with nickel, but there is an intensification. This may reach a factor of nearly three at its maximum, but the ratio of the two absorption peaks (393/660) stays almost constant, at around 2.4. However, with the further addition of citrate resulting in a 2 : 1 molar solution of citrate to nickel, there is a shift towards shorter wavelengths, and

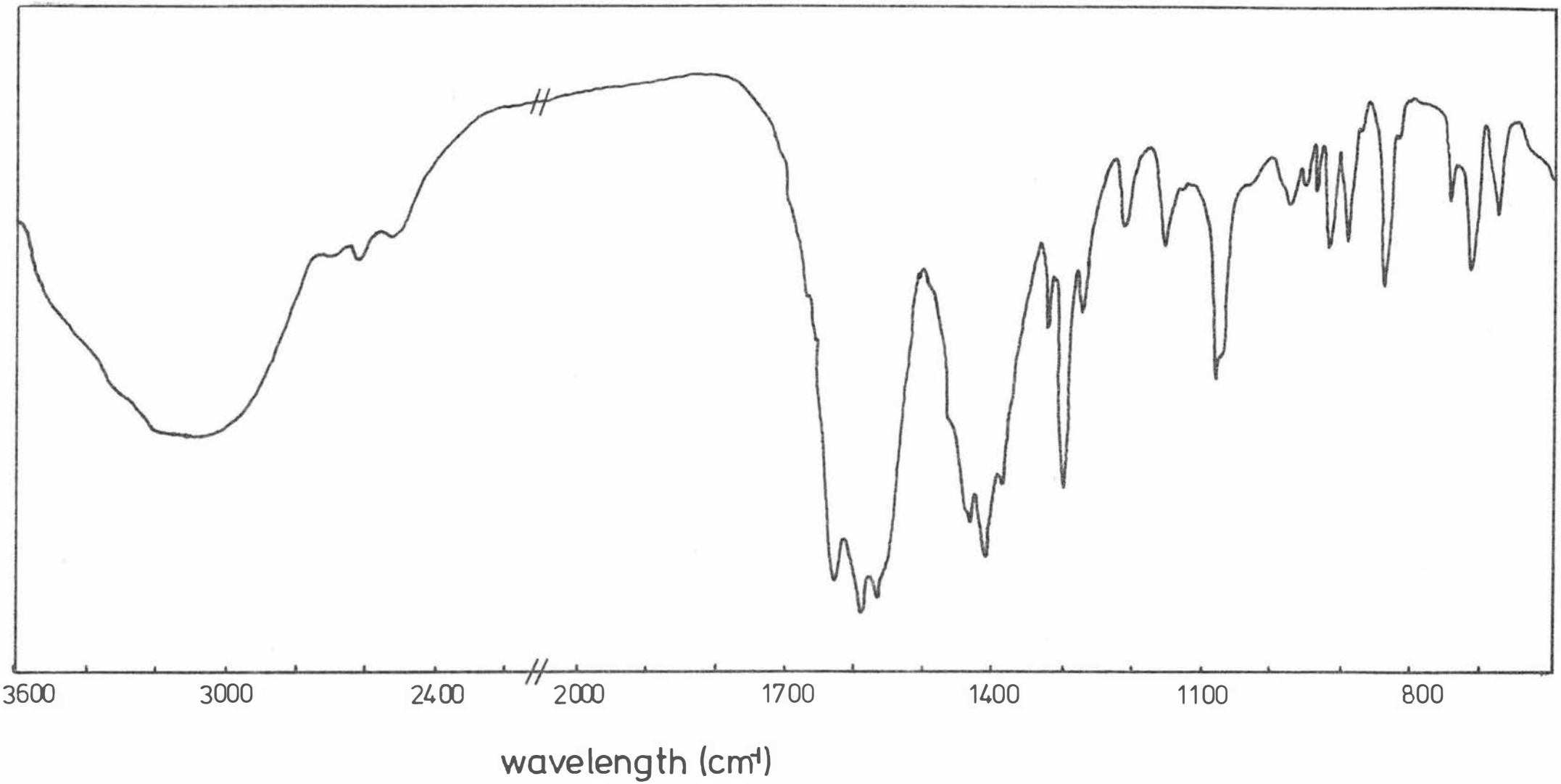


Figure 4·7·1 (a): Infra-red spectra of anionic nickel complex isolated from latex of S. acuminata.

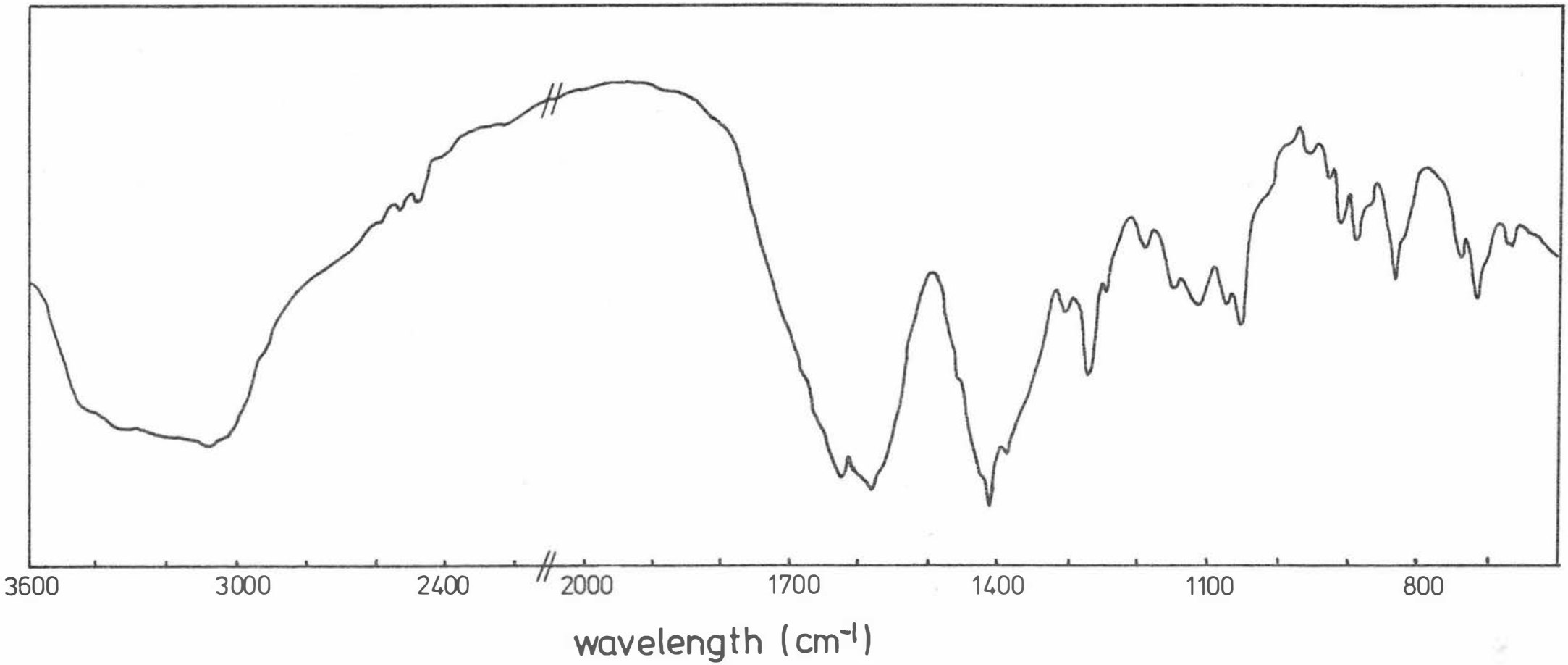


Figure 4·7·1 (b): Infra-red spectra of nickel citrate complex prepared from $NiCl_2$ and Na_3 citrate.

marked drop in intensity ratio, with the solution taking on a blue rather than green hue. This compares favourably with the behaviour observed by Heitner-Wirguin *et al.* (1958) discussed previously. Additional citrate beyond a 2 : 1 mole ratio did not change the absorption characteristics further. It was interesting to note that the wavelength maxima for the 1 : 1 species did not change until almost the 2 : 1 mole ratio was approached and then the change was abrupt. This indicates the stability of the 1 : 1 species.

In the spectral studies, a poorly defined maximum was found when Job's variation method was applied to a series of nickel solutions containing varying mole fractions of citrate ions. Absorbances were read at 640 nm, corrected for Ni(II) aqueous absorption and plotted against increasing concentration of citrate. The maximum corresponded to a solution containing 2 moles of citrate and 1 mole of nickel. In this case, however, Job's variation method is somewhat insensitive as the nickel species are not sufficiently defined from each other (Figure 4.7.2.). The shift in wavelength of species 'B' represents firm evidence of the existence of a 2 : 1 citrate-nickel species.

The spectrophotometric work was carried out using a Shimadzu MPS-5000 spectrophotometer with a scan range of 180 nm to 2,500 nm.

4.7.3 High-voltage electrophoresis

Experiments with high-voltage paper electrophoresis (Figure 4.7.3) show the behaviour of the nickel at pH 6.5 in two of the plant extracts, compared with $Ni(H_2O)_6^{2+}$ and with nickel in a 2 : 1 citrate/nickel solution. The presence of an anionic citrato-complex corresponding to $(Ni(HC)_2)^{2-}$ or $(Ni\ C)^-$ is clearly evident. The movement of the $Ni(H_2O)_6^{2+}$ ion and the anionic nickel is fairly compact, whilst a certain proportion of the nickel from the citrate complex streaks somewhat irregularly towards the anode. This latter movement is more difficult to explain. It is possible that under the electrophoretic conditions there is a decomposition of different species with time, resulting from the fact that an equilibrium situation no longer exists.

The behaviour of solutions containing various mole ratios of citrate to nickel was examined electrophoretically at pH 2.1, 3.5 and 6.5. The electrophoretograms were run for 40 minutes at 3kV, dried, and developed, firstly for citrate using an alcoholic solution of Bromocresol green made slightly alkaline with sodium hydroxide, and then for nickel with dimethylglyoxime. The results are summarized

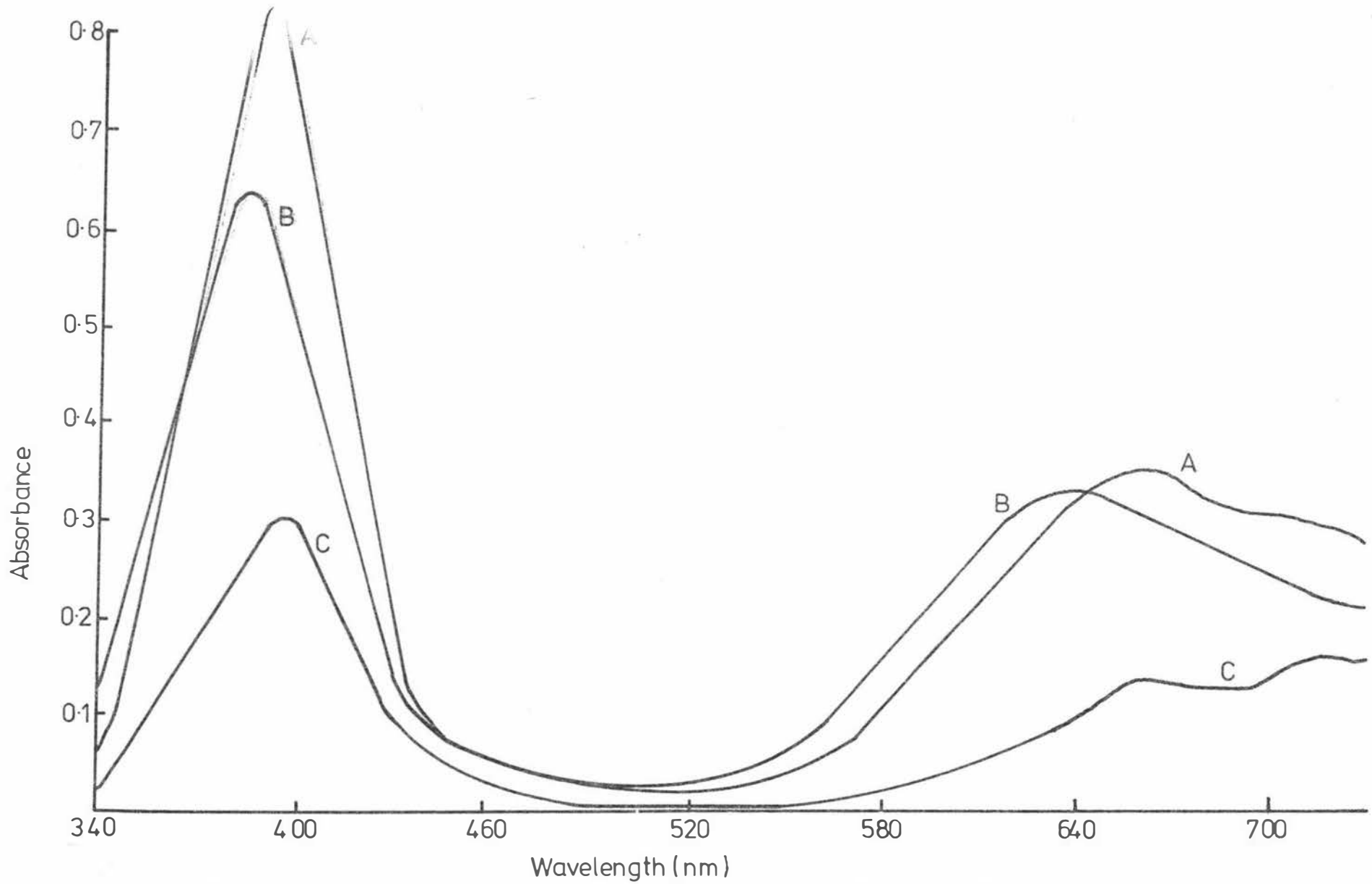


Figure 4·7·2: U.V. spectra of : A: 1:1, 0·1M $NiCl_2$ + 0·1M $Na_3C_6H_5O_7$ B: 1:2, 0·1M $NiCl_2$ + 0·1M $Na_3C_6H_5O_7$
C: 0·1 M $NiCl_2$(aq)

schematically in Figure 4.7.4. The movement of the citrate could easily be explained with reference to the pK values for citric acid determined by the following equilibria present in aqueous solutions:

$$H_3C \rightleftharpoons H_2C^- + H^+: \quad pK_1 = 2.87$$

$$H_2C^- \rightleftharpoons HC^{2-} + H^+; \quad pK_2 = 4.35$$

$$HC^{2-} \rightleftharpoons C^{3-} + H^+; \quad pK_3 = 5.68$$

At pH 2.1 all the citrate was present as the protonated species, H_3C which moved only slightly off the origin. No complexed nickel species were present; all the nickel moving towards the anode as $Ni(H_2O)_6^{2+}$. At pH 3.5 the majority of the citrate moved towards the cathode as H_2C^-. The nickel migrated mainly as $Ni(H_2O)_6^{2+}$ with possibly a species such as $(Ni\ H_2C)^+$. The non-equilibrium conditions prevailing under the electrophoresis resulted in extensive tailing. Both 1 : 1 and 2 : 1 molar solutions of citrate to nickel, as well as nickel citrate isolated from the plant extracts, behaved similarly.

Electrophoresis at pH 6.5 was more interesting and more complex to interpret. Spectral studies had previously indicated that for a 2 : 1 mole ratio of citrate to nickel the existence of $(Ni(HC)_2)^{2-}$ is probable. However only one anionic nickel species could be detected at pH 6.5. Nickel from various citrate/nickel solutions with mole ratios ranging from 0.2 :1 up to 3 : 1 moved identically towards the cathode in each case. Free nickel at the low citrate levels moved the farthest anodically. The amount decreased as a mole ratio of 1 : 1 citrate/nickel was approached. It is expected that at pH 6.5 the following equilibria would be operative in aqueous solutions:

$$Ni\ HC^o \rightleftharpoons NiC^- + H^+$$

$$Ni(HC)_2^{2-} \rightleftharpoons Ni\ HC^o + HC^{2-}$$

It appears that under the non-equilibrium conditions imposed by the electrophoresis the anionic nickel from both 2 : 1 and 1 : 1 citrate/nickel solutions migrates as $(Ni\ C)^-$. At pH 6.5 the major citrate species would be HC^{2-} and C^{3-} as expected from the third pKa for citric acid ($pK_3 = 5.68$). These species were observed running ahead of the $(Ni\ C)^-$ spot on the electrophoretogram (Figure 4.7.4.).

Although the electrophoresis experiments did not substantiate the spectrophotometric results regarding the existence of a $(Ni\ (HC)_2)^{2-}$

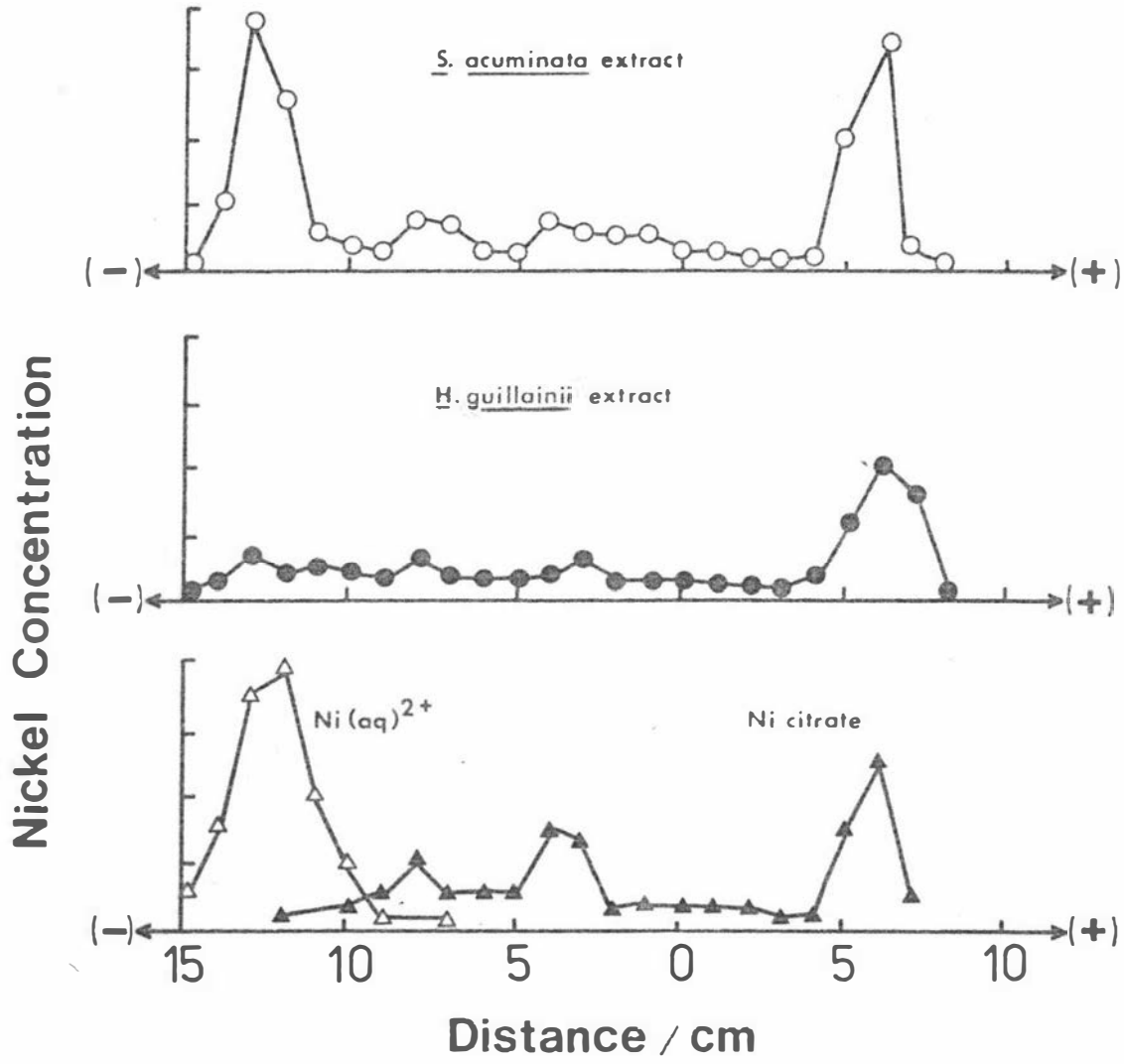


Figure 4·7·3: Electrophoretic distribution of nickel in extracts of S. acuminata (○) and H. guillainii (●), compared with $Ni(H_2O)_6^{2+}$ (△) and a 2:1 citrate:nickel solution (▲).

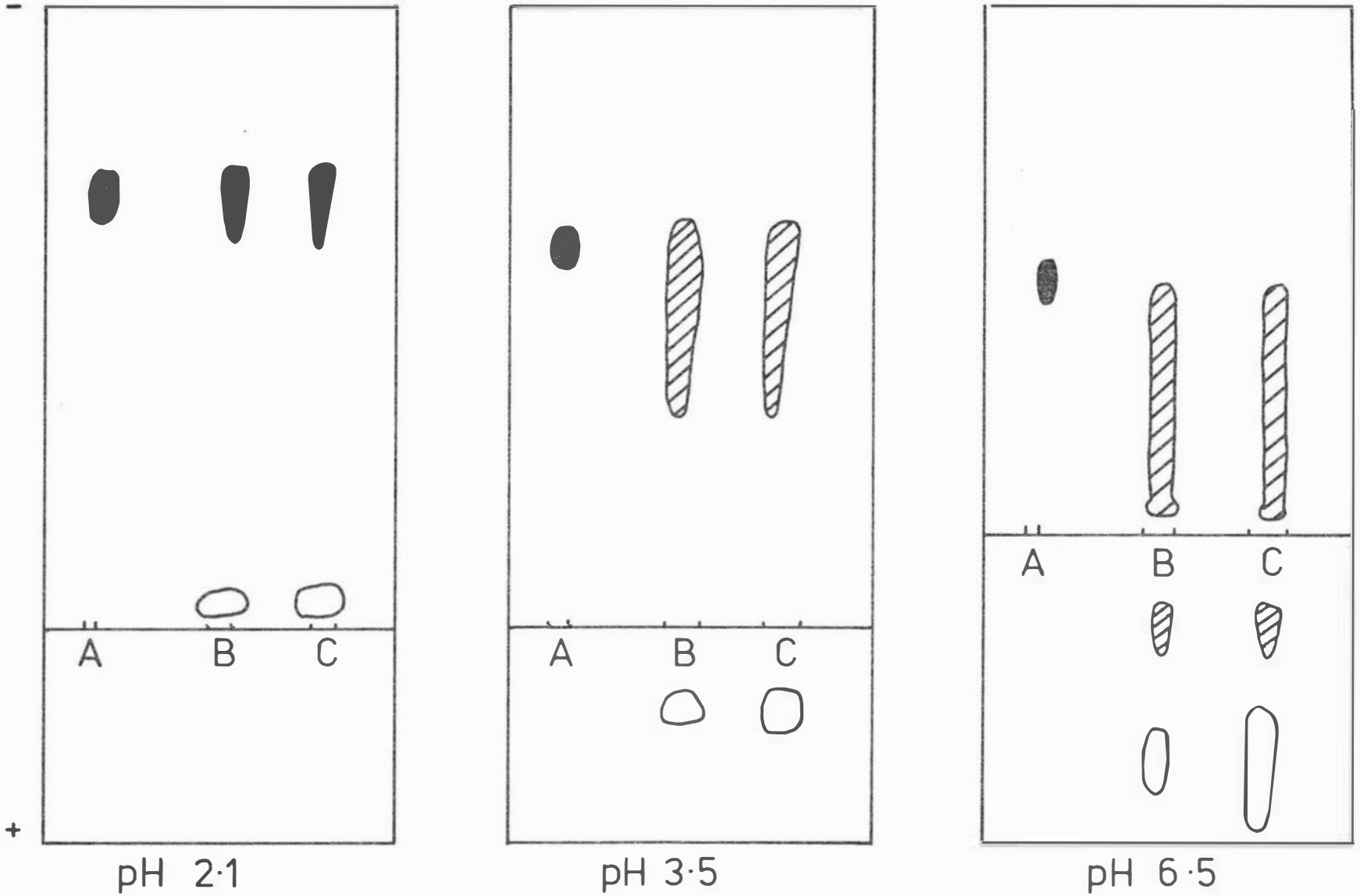


Figure 4·7·4 : High voltage electrophoresis of:

A : $Ni(H_2O)_6^{2+}$ B : Citrate : Ni, 1:1 C : Citrate : Ni, 2:1

■ Nickel only ▨ Nickel and Citrate □ Citrate only

(40 mA, 3kv, Whatman No.1, 40min)

species, the evidence is negative rather than contradictory. The nickel extracts from the New Caledonian accumulating plants behaved in all electrophoretic experiments identically to the 1 : 1 and 2 : 1 citrate/nickel solutions. Separation of the extracts on a weak cation exchange column (Sephadex CM-25) produced a solution behaving spectrophotometrically the same as a 2 : 1 citrate/nickel solution. It appears probable however, that in the plant sap the 1 : 1 species predominates, and that the formation of the 2 : 1 species is an artifact of the separation on the cation exchange column. As $Ni(H_2O)_6^{2+}$ exchanges the carboxylate H^+ ions on the column the concentration of the citrate builds up relative to $Ni(H_2O)_6^{2+}$ enabling the formation of $(Ni(HC)_2)^{2-}$ species.

4.8 ORGANIC ACIDS AND NICKEL CONCENTRATIONS IN VARIOUS ACCUMULATING PLANTS

4.8.1 Introduction

The simple aliphatic acids are of interest because of their ubiquitous nature and their effectiveness as chelators of various metals and solubilizers of mineral matter. The organic acids most effective in forming stable metal chelate complexes are the di- and tri- carboxylic hydroxy types. Johnston (1956) found that calcium chelates most strongly with citric acid (a tricarboxylic acid) less strongly with some dibasic acids such as malic and tartaric, and only slightly with α-hydroxy monobasic acids (e.g. lactic). Among the dibasic acids, hydroxyl derivatives formed the strongest complexes (Campi et al., 1964). The same trend has been observed for nickel and other metal ions (Li et al., 1960, Campi et al., 1964).

Pucher et al., (1938) were among the first to show that the large excess of positive ions over simple inorganic anions in plants was correlated with the quantity of ether-soluble organic acids. Similar observations were made by Ulrich (1941), Pierce and Appleman (1943), Jacobson and Ordin (1954), Poole and Poel (1965) and Van Steveninck (1966). The latter showed that citrate and malate, in particular, increased. Torii and Laties (1966) concluded that organic acid synthesis was increased when cation uptake exceeded anion uptake, because organic acids and cations moved into the vacuoles. Bound calcium and magnesium in tomatoes have been associated by Kirkby and Mengel (1967) with oxalic and uronic acids. In

Valencia leaves, malate, and oxalate in particular, increased with increasing Ca^{2+} in the leaves (Rasmussen and Smith, 1961). Increases in potassium increased oxalate but not malate. Citric acid concentration was unaffected. Brown and Tiffin (1965) found that in soyabeans there was a strong correlation between the concentration of iron and citrate in stem exudate. When iron was increased citrate was increased, and decrease in iron was paralleled by a decrease in citrate. More recent studies (Brown and Chaney, 1971; Tiffin, 1966, 1967, 1970, 1972) have confirmed that several crop plants, including maize, transport enough citrate in the xylem to maintain all the iron chelated as ferric citrate. Trans-aconitic acid played only a very minor role, if any, in the solubility and translocation of iron in maize. Although citrate has a high affinity for a number of diverse metals, and has been taken as a model for metal binding and displacement reactions (Tiffin, 1972), this does not rule out the participation of other organic acids with similar chemical structures and related metabolic functions.

Pierce and Appleman (1943) observed that magnesium content increased with increasing oxalic acid content. Those plants with little or no oxalic acid, had a large proportion of soluble calcium in the sap whereas those with high oxalic acid content had little or no soluble calcium in the sap. Excess inorganic cations were highly correlated with total ether-soluble organic acids.

Excretion products of roots include a variety of simple organic acids. Those identified in the exudates from common cereals (Riviére, 1960, Vančura, 1964) include acetic, oxalic, glycolic, pyruvic, oxaloacetic, succinic, malic, fumaric, tartaric, valeric and citric. A wide variety of organic acids has been found in exudates of the common vegetables (Vančura and Hovadik,1968). The likelihood that organic acids influence plant growth by functioning as carriers of inorganic cations becomes probable when the potential concentrations in soil solutions are considered (Tiffin, 1972).

Differences in the tolerance of plant species to trace element deficiences have been attributed to variations in organic acid production (Hodgson, 1963; Wallace, 1963). Charlanes (1960) concluded that an oat variety known to be resistant to manganese deficiency secreted more organic acids into the soil than susceptible varieties.

The main organic acids found in the majority of plants are malic, citric, succinic, fumaric, iso-citric and cis-aconitic. These acids

represent those most active metabolically in biological systems. They are all involved in the TCA cycle, which concerns the oxidation of carbohydrates and fatty acids and is coupled to the generation of ATP from ADP and inorganic phosphate.

Muir et al. (1964) identified several organic acids, including citric and malic acids in aqueous extracts of pine needles. Hydroxy acids accounted for 85% of the acidity in the extracts. The major and minor acids present in dried leaf tissue of Bryophyllum calycinum were identified or characterized using the combined methods of ion exchange chromatography, paper and thin-layer chromatography, gas chromatography and infra-red spectroscopy (Marriage and Wilson, 1971). Malic, citric and iso-citric acids constituted nearly 90% of the total acid content. Minor acids identified were succinic, fumaric, pyruvic, oxaloacetic α-ketoglutaric, glyoxylic, lactic, oxalic and cis-aconitic. After esterification of the acids in methanol containing cation exchange resin (H^+ form) their amounts were determined by gas liquid chromatography.

Problems of organic acid analysis in biological material have been reviewed by Schramm (1973). Extraction, purification, separation and quantitative estimation are discussed with regard to ion exchange chromatography and colorimetric analysis. In the latter years the use of gas liquid chromatography for the analysis of organic acids has proved to be popular because of its speed and simplicity. As organic acids are not sufficiently volatile to permit their vaporization under the conditions prevailing in the inlet system of a gas chromatograph or mass spectrometer, conversion of their functional groups into less polar ones to improve volatility has been required. The use of various silylating reagents to form trimethylsilyl (TMS) derivatives has proved to be rapid and convenient, and is generally favoured over the more time consuming preparation of methyl derivatives.

Separation of silyl derivatives of plant organic acids on gas liquid chromatograph columns using a variety of liquid phases has been reported by a number of workers, including Clark (1969), Nierhaus and Kinzel (1971), Barta and Osmond (1973), Boland and Garner (1973), Baker (1973), Merkel and Jungh (1973), Pinelli and Colombo (1976) and Phillips and Jennings (1976). Generally only one peak is obtained for the TMS-derivative of any one organic acid, although partial derivatives and more than one peak may be obtained for some acids e.g.

shikimic and quinic, depending on the silylating reagent employed (Barta and Osmond; 1973).

In the work reported in this section citric acid was determined, initially, by a colorimetric technique as preliminary gas chromatography with OV-101 did not separate citric and iso-citric acids as the silyl derivatives. The results for the silylation of these acids with trimethylsilylimidazole were inconsistent. On some occasions, they did not fully silylate whilst on other occasions, their derivatives were not stable and rapidly disappeared over a 2-day period. Phillips and Jennings (1976) noted similar problems and reported a procedure involving two successive analyses using different stationary phases, one of which separates citric and iso-citric acids directly as silyl derivatives.

As citric acid has been shown to bind nickel in all the New Caledonian hyperaccumulating plants and at least two of the strong accumulators, it was thought pertinent to determine the total concentration of citric acid in their leaves and to correlate this quantity, if possible, with the total nickel concentration. Gas liquid chromatographic separation, and identification by mass spectrometry, of the TMS - derivatives of the organic acids occurring in a number of nickel accumulators and non-accumulators has also been achieved.

4.8.2 Citric acid - nickel relationship in various nickel accumulating plants

(i) Analytical methods

Two grams of freeze-dried leaf material were homogenized with 50 ml of 80% ethanol for five minutes using 1 minute bursts to prevent any temperature rise in the supernatant. The residue was filtered and washed with successive portions of water and 50% ethanol. The residue from the filtration was combined with 5g of cation exchange resin (Amberlite IR-120 (H^+)) and the residue, with resin, were extracted with 50 ml distilled water by shaking for 2 hours. The suspension was then filtered through paper and the filtrate combined with the ethanol extract. This method ensured that organic acid salts were extracted. The volume of the solution was reduced to about 20 ml under reduced pressure without exceeding 40°C.

Further cations remaining in the supernatant were removed by passage through a 10 x 50 mm cation exchange column (Amberlite IR-120 (H^+)). The elutant was allowed to drip directly on to a 10 x 100 mm column of Dowex 1-X8 (Formate) 20-50 mesh anion exchange resin. The exchange capacities of these columns were well in excess of the maximum amount of organic acids detected in any of the leaf material. Organic acids were eluted from the Dowex column with 25 ml of 20% formic acid, followed by 25 ml of 50% formic acid and finally washed with distilled water. The eluate was dried under reduced pressure at 40°C to remove formic acid and the residue redissolved in distilled water and made up to 25ml. This solution was used for the determination of citric acid and gas liquid chromatographic identification of other organic acids.

Citric acid was determined in the leaf extracts by the penta-bromoacetone method (Natelson, Pincus, Lugovoy, 1948). The procedure used was similar to that given by Camp and Farmer (1967). One ml of the organic acid extract or citric acid standard was pipetted into a ground glass stoppered test tube and 1 ml of a 1 : 1 solution of 9M sulfuric acid and 10% meta-phosphoric acid added. This solution was placed in an ice bath and 2 ml of 5% potassium permanganate - sodium bromide slowly introduced with shaking. After 10 minutes the mixture was titrated until colourless with 3% hydrogen peroxide. The penta-bromoacetone so formed was then extracted into 4.0 ml of n-hexane by shaking on a vortex mixer for 30 seconds.

One ml of the organic phase was then transferred to a glass stoppered tube and 4.0 ml of borax-buffered 4% (w/w) thiourea solution (pH 9.2) introduced. After further vortex mixing the absorbance of the resulting yellow thiourea complex in the aqueous phase was read at 455 nm against a blank of thiourea buffer solution which had been extracted with n-hexane. A typical standard curve is shown in Figure 4.8.1. The complex is stable at room temperature and the colour was maintained for at least 2 hours. The same reaction is caused by acetoacetic acid and itaconic acid (Schramm, 1973) but since both these acids occur, in the main, in very small amounts in comparison to citric acid, the latter can be determined directly in the mixture of acids in the extracts.

(ii) Results

Table 4.8.1 shows the total citric acid, nickel and titratable

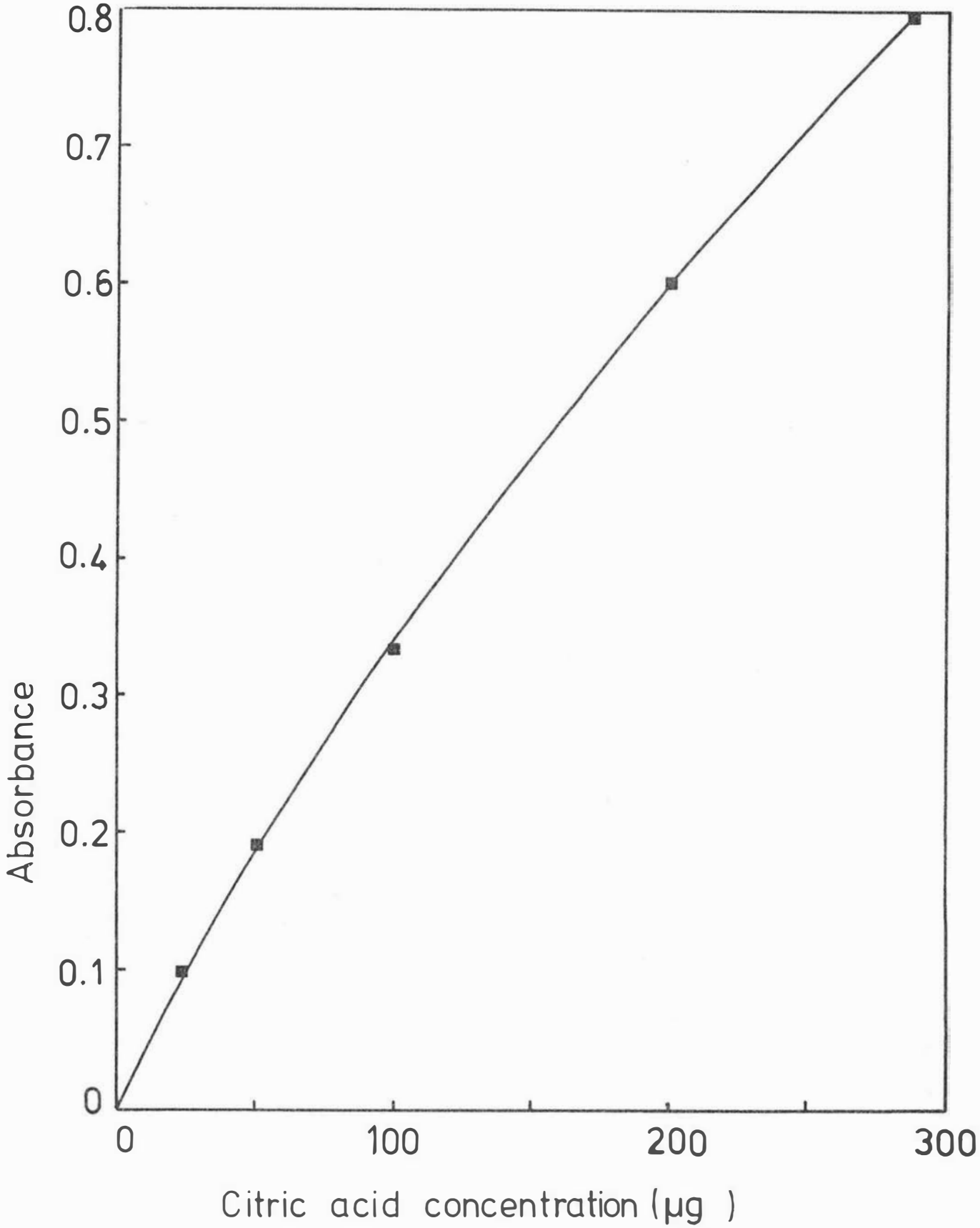


Figure 4·8·1: Standard curve for the determination of citric acid by pentabromoacetone method.

Table 4.8.1. Total titratable (H^+), nickel and citric acid concentrations in various nickel accumulating plants (μ moles g^{-1} dry weight).

Species.	Total $(H^+)_{-1}$ (μ moles g^{-1})	Citric (μ moles g^{-1})	Nickel (μ moles g^{-1})	Citric / Nickel
Hyperaccumulators.				
Hybanthus austrocaledonicus	700	97.0	254.2	0.38
" caledonicus	750	56.8	116.2	0.48
Homalium guillainii	812	68.5	163.2	0.42
" kanaliense	781	77.2	63.5	1.21
" francii	1031	65.4	122.8	0.53
Sebertia acuminata -leaves	1982	57.6	172.8	0.30
" " -latex	-	1302	2847.5	0.45
Psychotria douarrei	875	50.7	228.7	0.22
Geissois pruinosa	359	50.8	114.4	0.44
Strong accumulators.				
Homalium decurrens	687	29.1	8.6	3.4
" austrocaledonicum	1068	29.3	10.6	2.7
" deplanchei (I)	671	26.7	3.5	7.6
" " (III)	750	36.8	2.1	18.1
" le-ratiorum (II)	671	37.3	2.3	16.4
" " (III)	468	37.3	7.6	4.8
" rubrocostatum	468	31.3	19.6	1.59
Others.				
Homalium polystachyum	922	15.6	0.79	82.1
" intermedium	328	6.2	1.27	4.8

continued...

Species	Total H^+ (μ moles g^{-1})	Citric (μ moles g^{-1})	Nickel (μ moles g^{-1})	$\frac{[Citric]}{[Nickel]}$
Homalium mathieuanum	412	16.5	0.25	66
" deplanchei (II)	1156	18.9	0.5	37.8
" le-ratiorum (I)	656	20.4	0.5	40.8
" buxifolium [a]	656	44.1	0.025	1760
Hybanthus floribundus [a]	793	6.6	0.67	9.8
Alyssum bertol.onii [b]	618	13.0	44.1	0.29
Pearsonia metallifera [b]	512	13.0	180.5	0.072

[a] non -ultramafic substrate.

[b] No nickel/citrate association.

(H^+) in the leaves of a number of nickel accumulating plants from New Caledonia. The latter quantity was determined by 0.05M sodium hydroxide titration of the organic acid extracts and approximates the total organic acid concentration. Levels in Hybanthus floribundus (Australia), Alyssum bertol onii (Italy) and Pearsonia metallifera (Rhodesia) are also given. As seen from the table the hyperaccumulators (> 1000 µg/g dry weight) contain substantially higher levels of citric acid than those species of lesser nickel accumulating ability. The extremely high level in the latex of S. acuminata is noteworthy. The mole ratio of citric acid to nickel for the hyperaccumulators is generally less than 1, but increases markedly as the ability of the species to accumulate nickel decreases. The relationship between citric acid and nickel is graphically shown in Figure 4.8.2. The graph shows a very significant relationship ($P \leq 0.001$, $r = 0.855$) between the two sets of data.

(iii) Discussion

In summation it is evident from Table 4.8.1. that the citric acid concentration in the hyperaccumulators is approximately twice that of the strong accumulators which are in turn twice as rich in citric acid as those species of only average nickel content. In all the New Caledonian hyperaccumulators citric acid has been implicated in nickel chelation. Electrophoretic evidence also exists which strongly points to this acid being involved with nickel chelation in the strong accumulators as well.

It is pertinent to note that the data for A. bertol onii and P. metallifera do not fit this relationship. However, no chemical association between citric acid and nickel has been found in these two species. The citric acid level in the leaves of these species (13.0 µ moles g^{-1}) is considerably lower than expected, considering the high nickel concentrations present. P. metallifera, a grass from Rhodesia (Wild, 1974) which accumulates over 1.0% nickel in the dry material, contains only 0.24% citric acid, whereas H. guillainii with a similar nickel concentration in the dry leaves contains 1.3% citric acid.

Pelosi, Fiorentini and Galoppini (1975) have provided some evidence that in A. bertol onii malic and malonic acids may be associated with nickel complexing. Therefore, in view of the trends shown in Figure 4.8.2 and Table 4.8.1 it may well be that for those

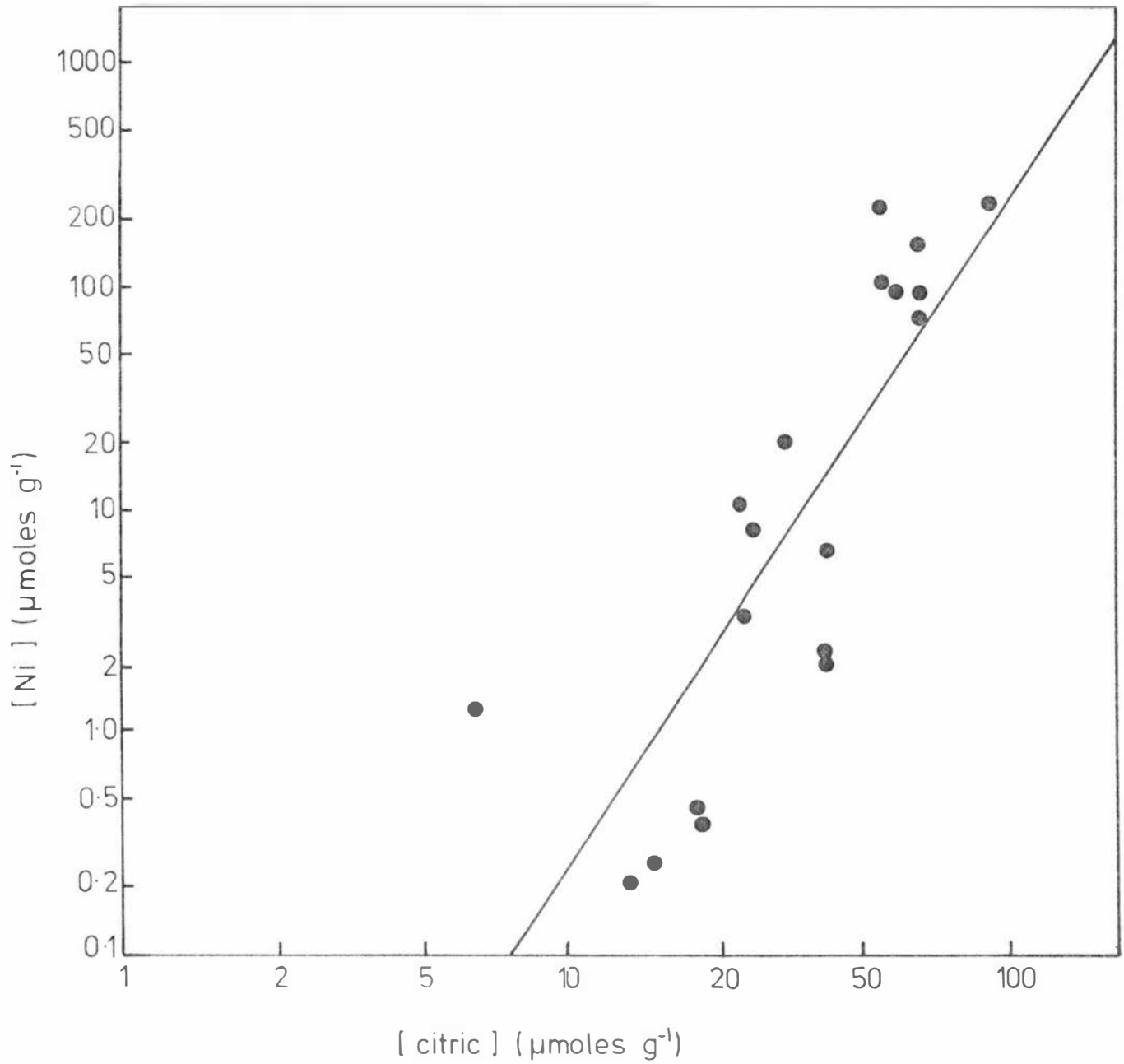


Figure 4·8·2 : Relationship between citric acid and nickel in various New Caledonian Homalium and Hybanthus.

hyperaccumulators of nickel which contain only small concentrations of citric acid, other nickel carriers are involved. These may not necessarily be organic acids.

In view of these findings it was decided to prepare TMS derivatives of the organic acid solutions and separate and identify, by gas liquid chromatography, the major acids present.

4.8.3 Gas liquid chromatography of organic acid extracts from various nickel accumulating species

(i) Methods

Aliquots containing 250 - 500 µ moles acid were taken from the organic acid extracts, the preparation of which has been outlined previously (4.8.2), and reduced to absolute dryness under reduced pressure at 45°C. If silylation was not to be immediate, samples were stored above anhydrous silica gel. The solids were dissolved in 1 ml of anhydrous dimethylsulphoxide, in rubber-capped glass phials, and 0.4 ml of N-trimethylsilylimidazole introduced. This quantity of silylating reagent was well in excess of that required for complete silylation of the most concentrated organic acid fraction (Table 4.8.1). Silylations were catalysed with 0.1 ml of trifluoroacetic acid and reaction mixtures allowed to stand for 30 minutes before injection of samples into the gas chromatograph.

Separations were carried out with a Packard 802 chromatograph fitted with flame ionization detection, digital temperature programme, and a 200 cm x 2.5 mm glass column packed with 3% OV-101 on chromosorb W, AW-DMCS, 100-200 mesh. Samples were injected directly on to the column head with an injection port temperature of 250°C. The gas flow rates were: nitrogen 45 ml min^{-1}, hydrogen 30 ml min^{-1} and air 450 ml min^{-1}. The oven temperature was programmed from 90°C at a rate of 8°C min^{-1}. Sample sizes were 1.0 µl unless stated otherwise. Total time for the analysis was approximately 20 minutes, after which the oven was cooled and allowed to equilibrate at 90° before the next injection.

Mass spectra of the eluted peaks from a similar column were obtained for additional identification, which confirmed those already made from comparisons of retention times with authentic compounds. The mass spectral scans were started near the top of the eluting peak where the source pressure was near its maximum value. The mass spectra, covering m/e values from 50 to 500, were compared, wherever possible, with those of authentic derivatives. The base peak was

generally that given by the trimethylsiliconium ion at m/e 73.

(ii) Results

The degree of separation by gas chromatography of the silylates of various authentic organic acids is illustrated in Figure 4.8.3. No trace of other compounds could be detected in the mass spectrum of each when the spectra were taken near the tops of the peaks. Return to baseline values of the ion current gave assurance that there was little memory of past peaks, or anticipation of peaks to come. The mass spectra of the silyl derivatives of malonic, malic, tartaric, aconitic and citric acids were in good agreement with those reported by Mamer et al., (1971). Polysiloxanes which result from column bleed, and by-products of the silylation reaction, elute much later at terminal programme temperatures, and do not contribute in any detectable way to the spectra. The solvent peaks are dumped before entry into the mass spectrometer. Incomplete silylation was indicated when there was a large discrepancy between the amount of acid in the sample measured in the titration and the amount measured subsequently by G.L.C. It was confirmed by the addition of further silylating agent. This immediately silylated any incompletely reacted citric acid; this being the acid most prone to incomplete silylation.

Figures 4.8.4 to 4.8.7 show typical chromatographs of mixtures of silyl derivatives of the organic acids extracted from leaf material of various plant species. Citric acid was the major acid present in all the hyperaccumulators of nickel except for the two species found outside New Caledonia, Pearsonia metallifera and Alyssum bertollonii. There was good agreement between the citric acid concentrations obtained by the gas chromatograph and those assessed by the pentabromo acetone method. Generally, large quantities of malic acid were found in the hyperaccumulators with the exception of Sebertia acuminata. Small quantities of malonic and aconitic acid could be detected in most of the samples analysed. Quinic acid was also prominent in all the species. The mass spectra indicated that this acid produced two peaks on the gas chromatograph. The silyl derivative of authentic D(-) quinic acid produced only a single peak and it was assumed that in the leaf material two isomers are present. Two peaks for quinic acid were also noted by Barta et al., (1971) using bis-trimethylsilylacetamide as the silylating agent. An unknown acid (B, Figs. 4.8.4 to 4.8.7),

eluted after citric and quinic acids, gave a silyl derivative with molecular ion at m/e 450 and was prominent in all the gas chromatographs of the extracts. Small quantities of unknown A (molecular ion, m/e 364) were also common to all species. Citric acid was the only acid present in the latex of S acuminata except for traces of three or four minor acids (Fig. 4.8.8). Appendix IV lists prominent m/e values for some of the TMS ethers of the known and unknown acids.

(iii) Discussion

Although the gas chromatographs of the organic acid extracts showed a large number of minor peaks which were not identified, the major acids were well resolved and generally could be easily identified on the basis of retention times and comparison of their mass spectra with those of authentic silyl derivatives. In leaves of the nickel accumulators, malic, citric, quinic and unknown B were far in excess of other acids. Malic and citric acids were the major acids of the TCA cycle present, although small quantities of malonic acid were also detected. Among the Homalium species there did not appear to be any large difference between the accumulators and non-accumulators in the number and type of organic acids present. Generally, however, the hyperaccumulators had elevated levels of malic and, in particular, citric acid.

The hyperaccumulator from Rhodesia, Pearsonia metallifera, did not contain any appreciable quantity of citric acid (Figure 4.8.6), the major acids being malonic and malic acids. Moderate amounts of quinic and an unknown (C) were detected. The latter acid was shown by mass spectrometry to be identical to the ligand associated with the nickel from this plant, the partial elucidation of which is outlined in the following section.

4.9 ISOLATION AND IDENTIFICATION OF NICKEL COMPLEX FROM PEARSONIA METALLIFERA

Wild (1965) has described the serpentine endemic, Pearsonia metallifera, a grass from the Great Dyke area of Rhodesia, which accumulates 15.3% nickel in the leaves on an ash weight basis (Wild, 1974). The freeze-dried leaf material used in this study contained 1.06% on a dry weight basis.

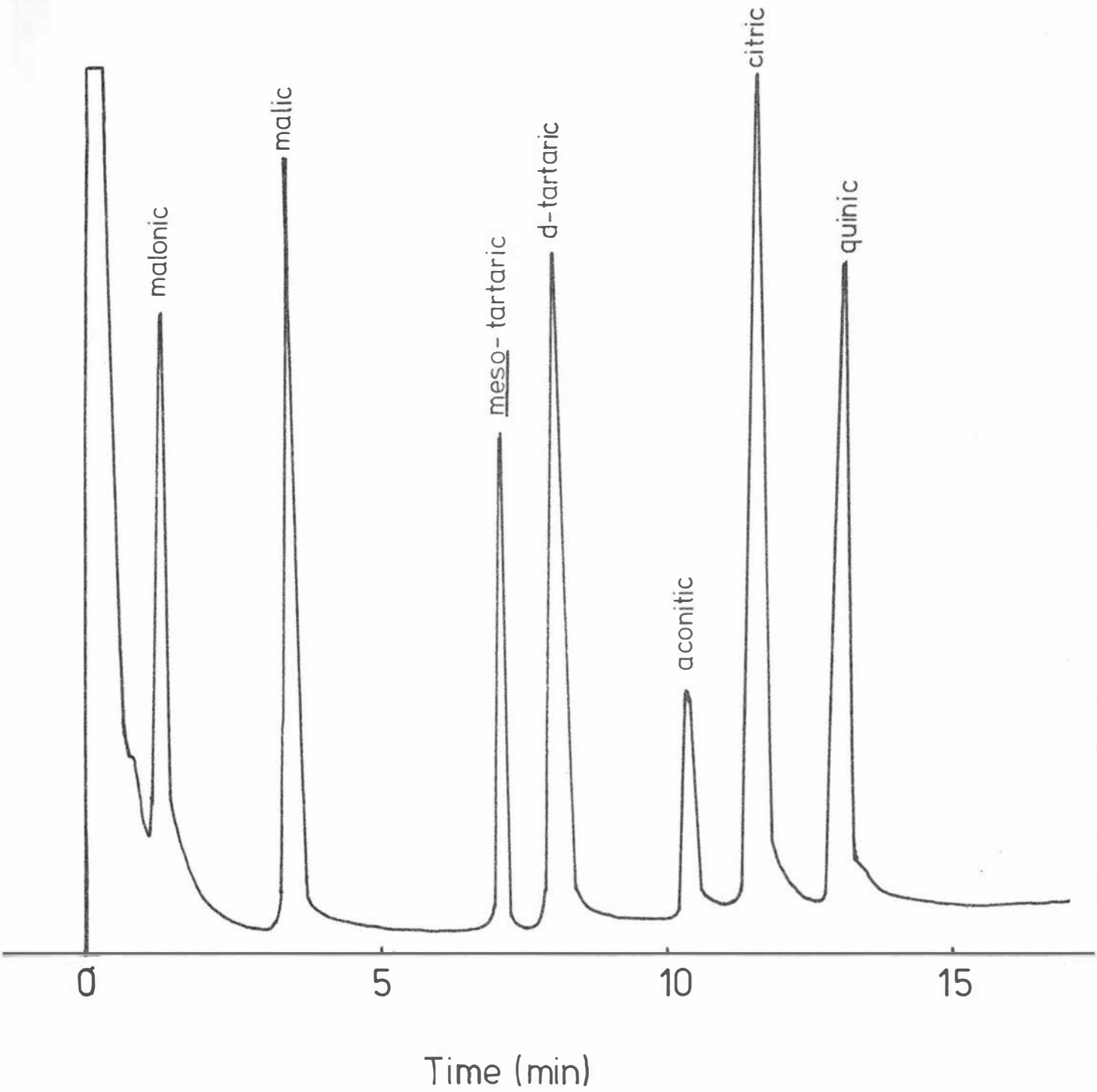


Figure 4·8·3: Gas liquid chromatograph of silyl derivatives of various standard organic acids.
3% OV-101; temp. programme 90°- 240°C × 8°min^{-1}

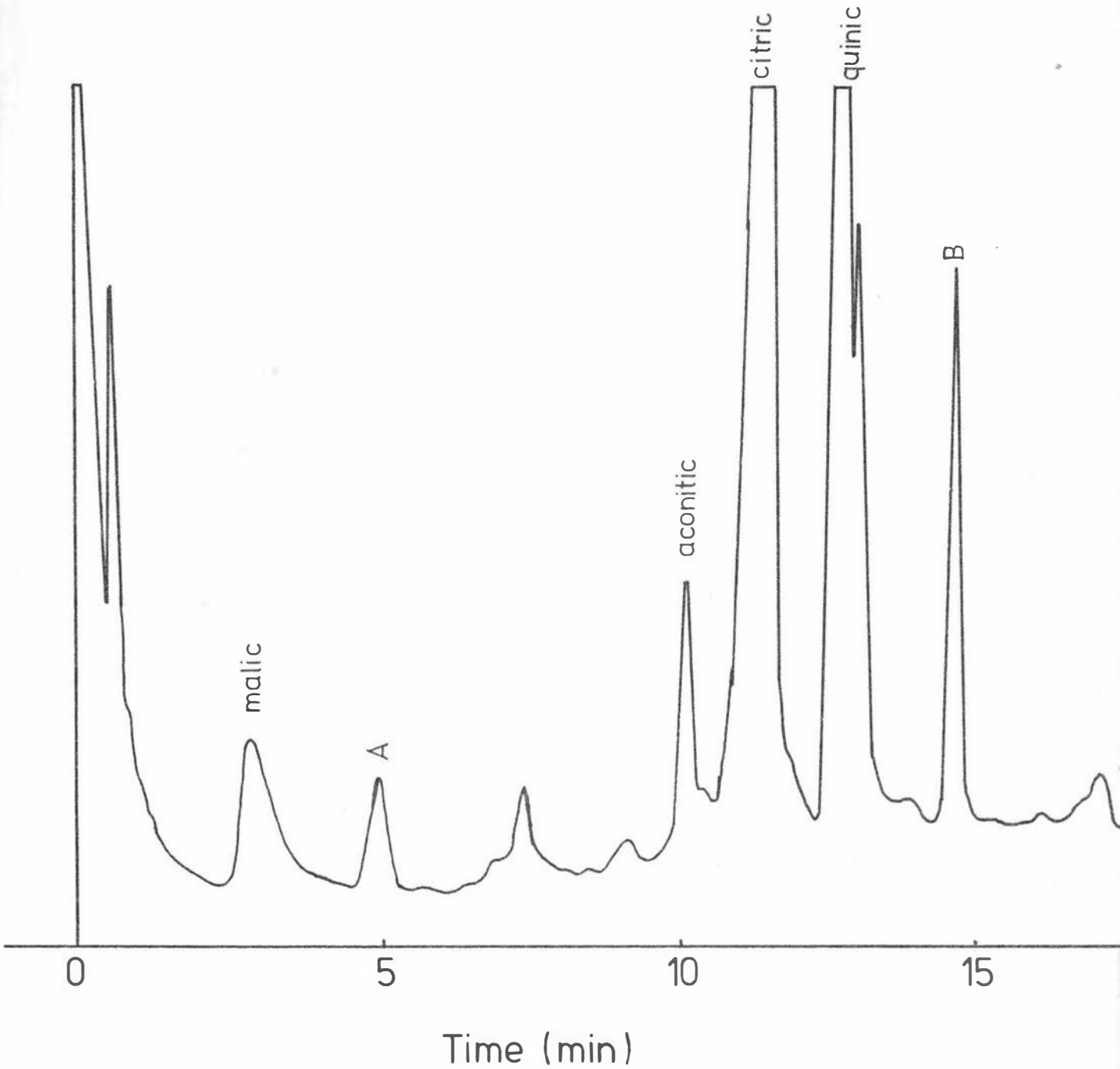


Figure 4·8·4 : Gas liquid chromatograph of silylated acids from leaves of S. acuminata.
temp. programme 90°- 240°C ×8°min^{-1}

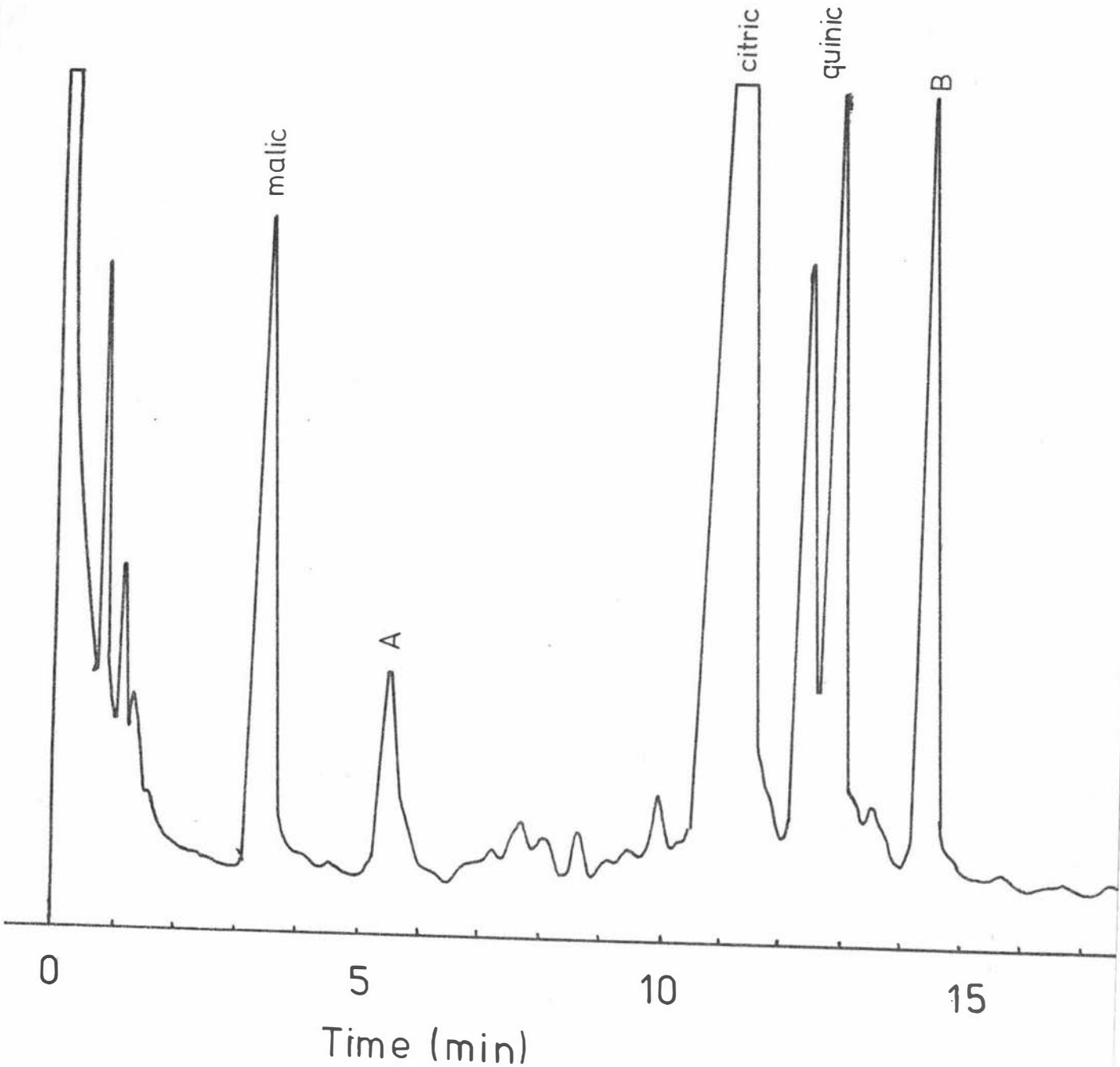


Figure 4·8·5 : Gas liquid chromatograph of silylatedacids from leaves of Homalium kanaliense. temp. programme 90°- 240°C x 8°min^{-1}

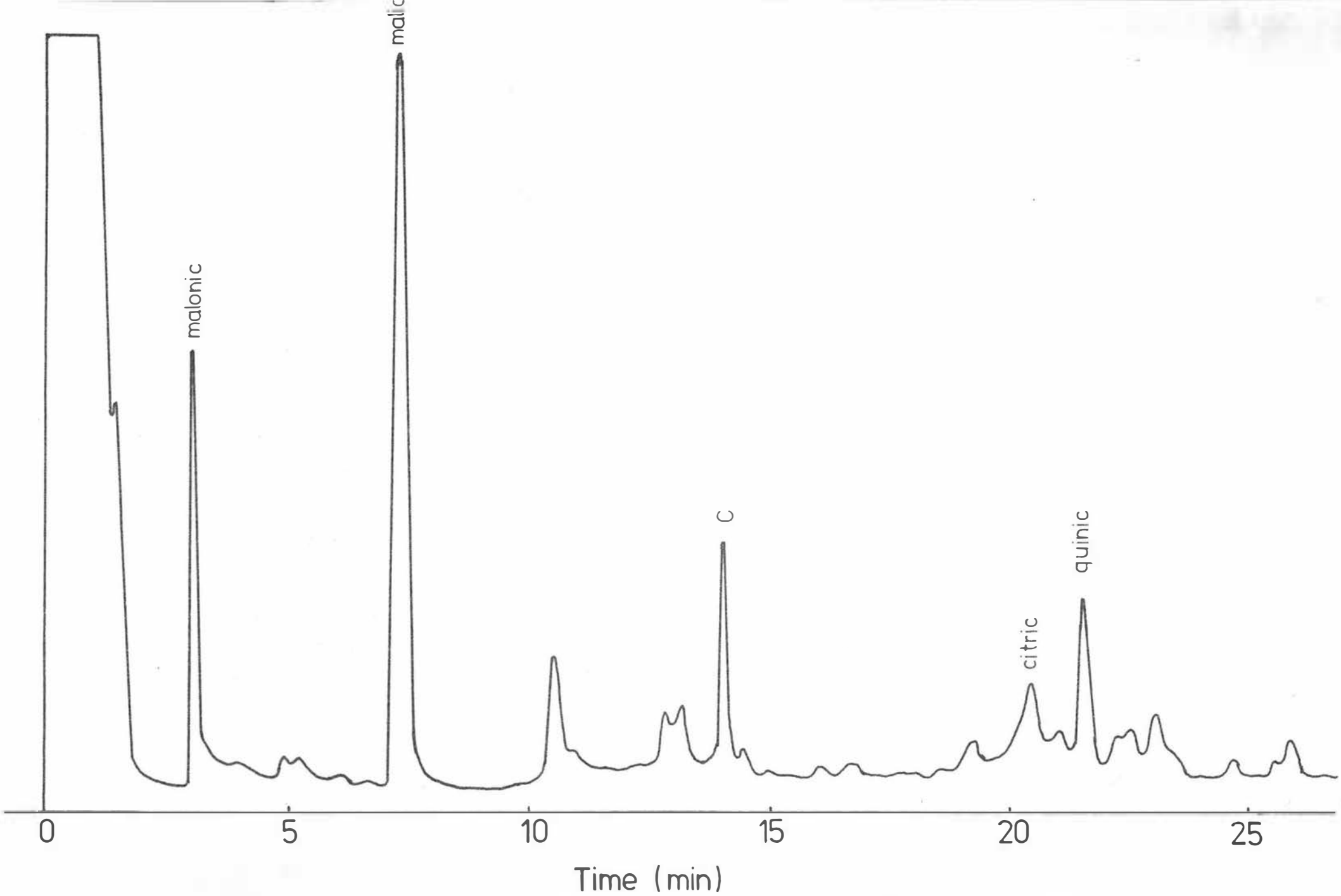


Figure 4·8·6: Gas liquid chromatograph of silylated acids from leaves of Pearsonia metallifera. temp. programme 90 - 240 x 4 min^{-1}

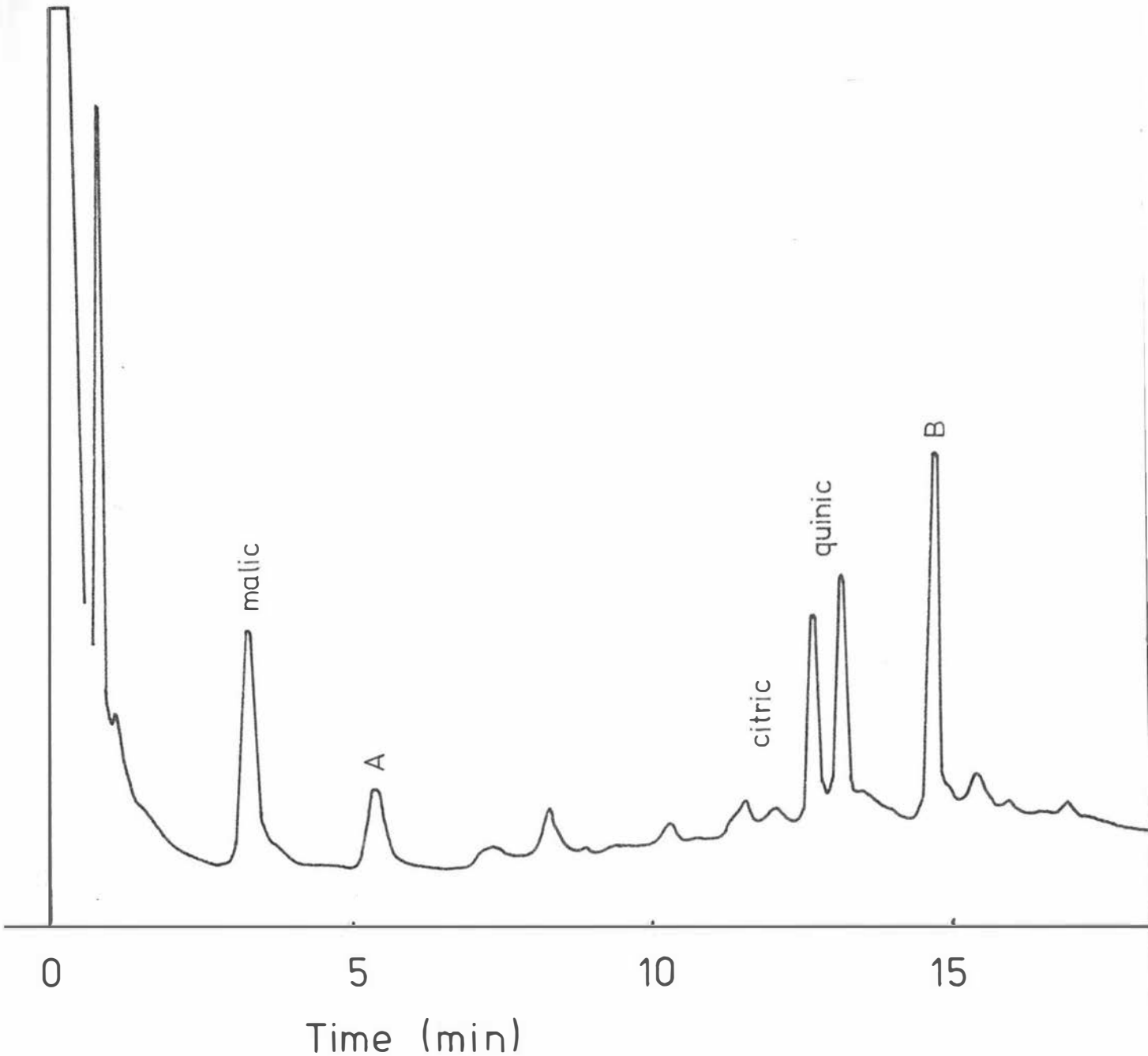


Figure 4·8·7 : Gas liquid chromatograph of silylated acids from leaves of Homalium intermedium

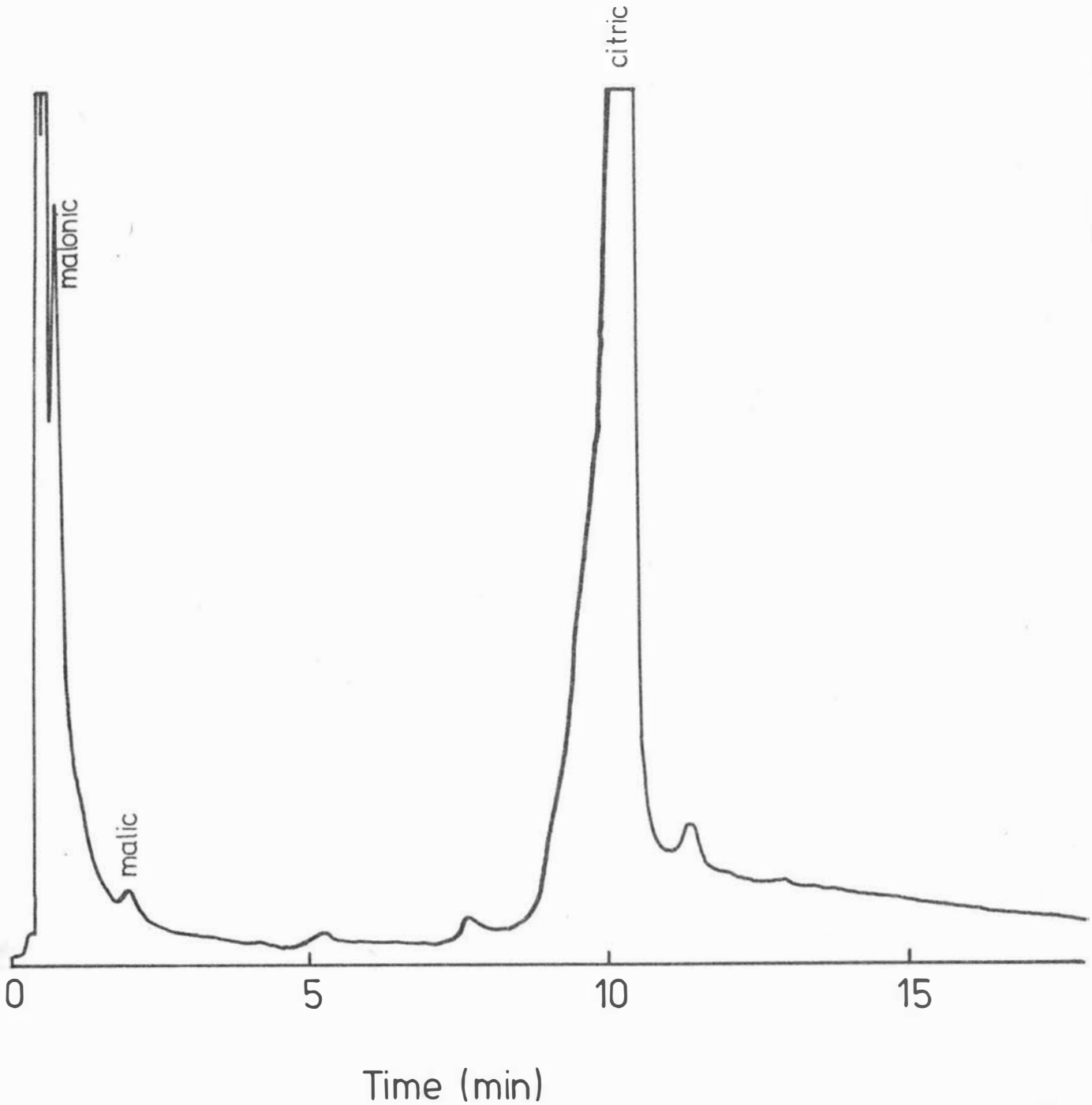


Figure 4·8·8: Gas liquid chromatograph of silylated acids from latex of S. acuminata.

4.9.1. Extraction and isolation of nickel complex

Approximately 10g of freeze-dried leaf material containing a little over 100 mg of nickel was extracted with water as outlined previously (Section 4.5). Aliquots of the crude aqueous extracts were separated on Sephadex and a nickel-containing fraction obtained as detailed for the New Caledonian hyperaccumulators. Approximately 80 mg of a pale green powder was isolated.

4.9.2 High voltage electrophoresis and Infra-red Spectrophotometry.

The behaviour of nickel from a purified nickel extract from P. metallifera was examined by high voltage paper electrophoresis at pH 6.5. The conditions have been previously described (Section 4.6.4). The mobility of the nickel is shown in Figure 4.9.1 and compared with the movement of the Ni $(H_2O)_6^{2+}$ ion. The first notable feature that is observed is the lack of any anionic nickel. This contrasts with the electrophoretic patterns obtained from the New Caledonian hyper-accumulators and the nickel citrate extracts. All the nickel from the P. metallifera extract was seen to move towards the anode with a large amount of tailing. The most mobile of the nickel moved the distance covered by the Ni $(H_2O)_6^{2+}$ ion, and probably represents this species. The rest of the nickel covered lesser distances from the origin indicating a complexed form which is positively charged at pH 6.5.

The infra-red spectrum of a nujol mull of a small amount of the pale green nickel extract is shown in Figure 4.9.2. An intense band centred at 1600 cm^{-1} typical of a COO^- asymmetric stretching mode is a dominant feature of the spectrum. The symmetrical vibration, which appears at approximately 1410 cm^{-1} for the co-ordinated carboxyl groups in nickel citrate, is extremely weak and not as prominent as for nickel citrate. The presence of water and hydroxyl groups can be considered responsible for the absorption peaks in the 3000 - 3500 cm^{-1} region. However, owing to poor resolution of the spectrum obtained, little further structural information can be derived. The crystallinity and quantity of the isolated nickel-containing solid was insufficient to produce infra-red spectra with better resolution.

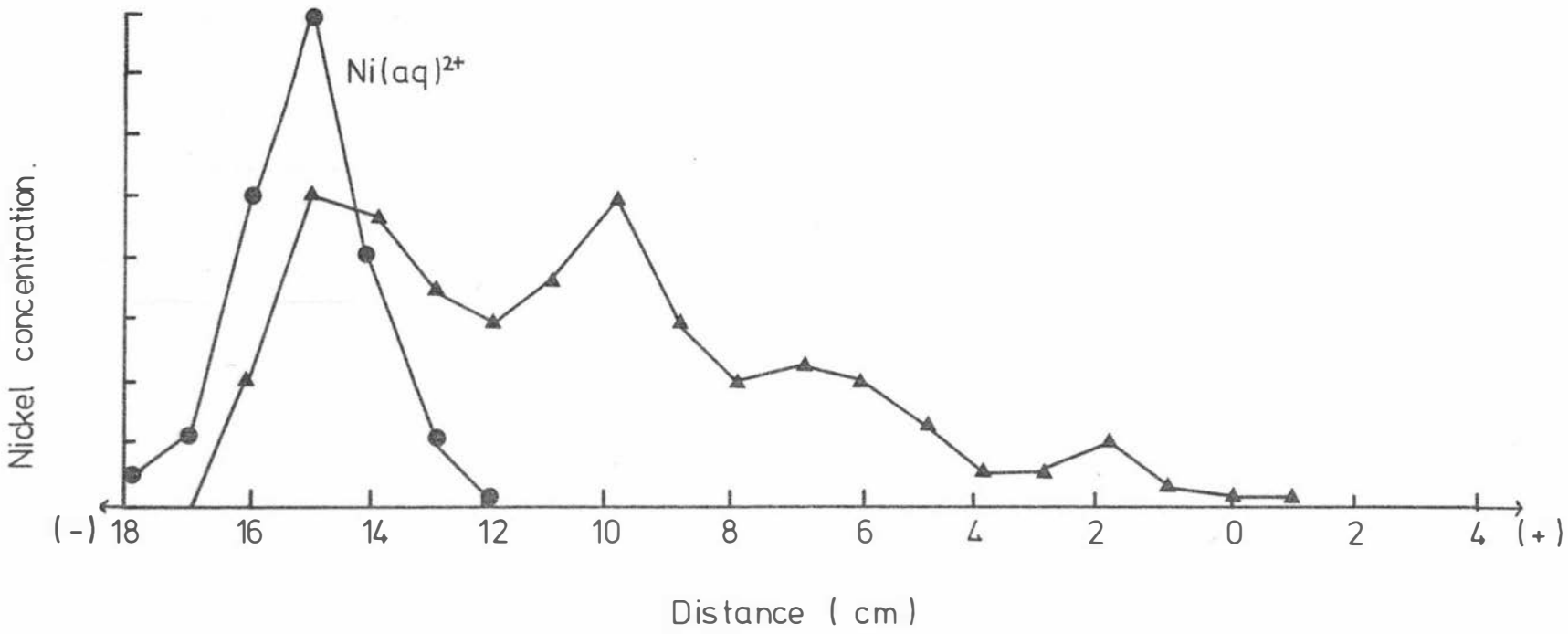


Figure 4·9·1 : High voltage electrophoresis of nickel from Pearsonia metallifera (▲) and nickel aqueous ion $Ni(H_2O)_6^{2+}$ (●) :
Whatman No.1 paper, 3 kv, 45 min, 40 mA, pyridine/acetic acid/water, pH 6·5

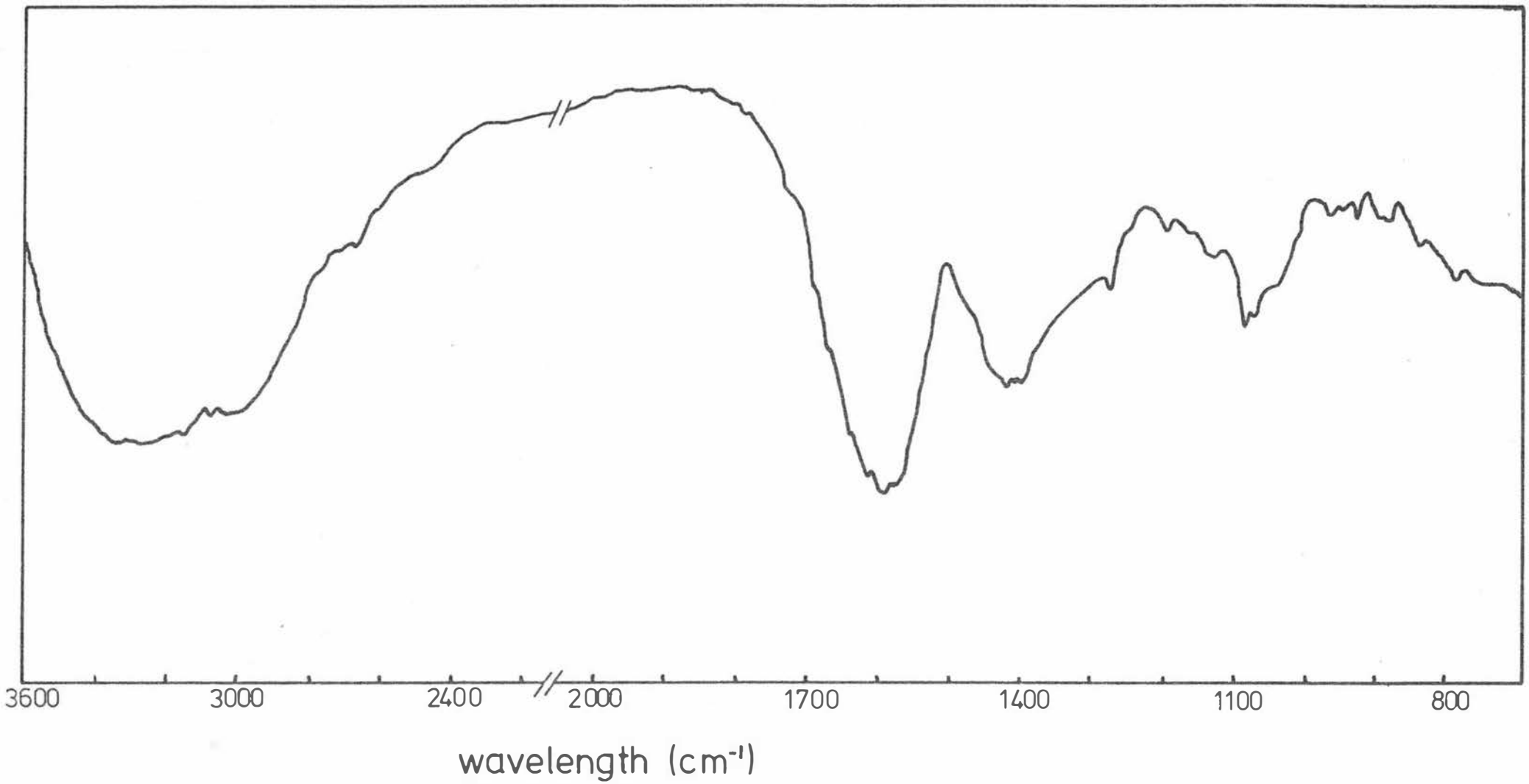


Figure 4·9·2: Infra-red spectrum of nickel extract from Pearsonia metallifera.

4.9.3 Gas liquid chromatography - mass spectrometry

A trimethylsilyl derivative of the organic component from the nickel extract was prepared by silylating a small quantity of the nickel preparation in dimethyl sulfoxide with N-trimethylsilylimidazole and trifluoroacetic acid as described in Section 4.6.5. The silylation mixture was injected in 1 µl aliquots on to the gas chromatograph and the eluted peaks passed through molecular separators into the mass spectrometer. The instrumental parameters were the same as those used for the gas chromatography and mass spectral identification of citric acid. The gas chromatograph of the silylated mixture is shown in Figure 4.9.3. The major peak, eluted at 7.5 minutes, had the same retention time as meso-tartaric acid. However, although having the same molecular ion (m/e 438) the mass spectra of meso-tartaric acid and the TMS derivative of the extract were decidely different. The mass spectrum of the peak C in Figures 4.9.3 and 4.8.6 is shown in Figure 4.9.4. No molecular ion was seen, as is common for many TMS derivatives of hydroxy carboxylic acids. Characteristic features of the spectrum are the loss of formaldehyde from the M-15 ion (m/e 423) and the abundant ions at m/e 306, m/e 205 and m/e 117. The mass spectrum of the minor peak in Figure 4.9.3, labelled D, also gave the same rearrangement and fragmentation pattern with only minor differences in relative intensity of some of the peaks. This peak probably represents an isomeric form of the major peak.

A molecular ion of mass 438 for the TMS derivative corresponds to a parent compound with an empirical formula of $C_4H_6O_6$ or $C_5H_{10}O_5$. Tartaric acid is representative of the first formula whilst the pentoses, pentuloses and tri-hydroxy C-5 monocarboxylic acids represent the latter. Apiose, a naturally occurring branched chain sugar also has the formula $C_5H_{10}O_5$. Accurate mass measurements of the m/e 423 ion gave a value of 423.1864 ± 0.0016 and led to a mass of 438.2099 ± 0.0016 for the molecular ion. This compares well with the calculated mass of 438.2109 for $C_5H_{10}O_5$ with 4 TMS substituent groups ($C_{17}H_{42}O_5Si_4$). The alternative formula $C_4H_6O_6$, which, with 4 TMS groups ($C_{16}H_{38}O_6Si_4$) also produces a molecular ion of m/e 438, may be ruled out on the basis of its accurate mass (438.1745). Accurate mass measurement of other fragments including the M - CH_3 - CH_2O (meas: 393.1743; calc: 393.1768) and M - CH_3 -COO $SiMe_3$ or M - O = CH-CH_2O $SiMe_3$ (meas: 306.1477; calc: 306.1503) ions gives further support for

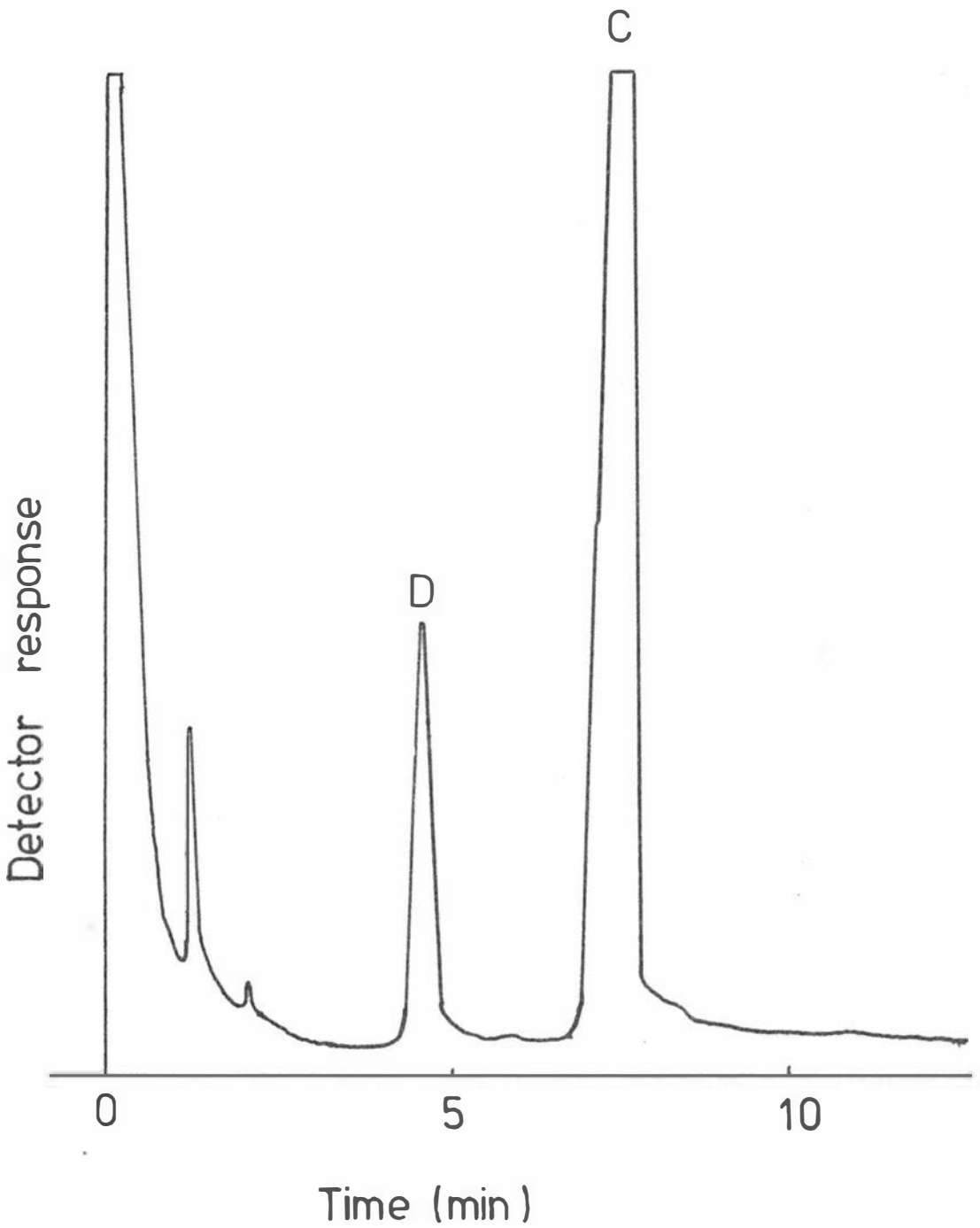


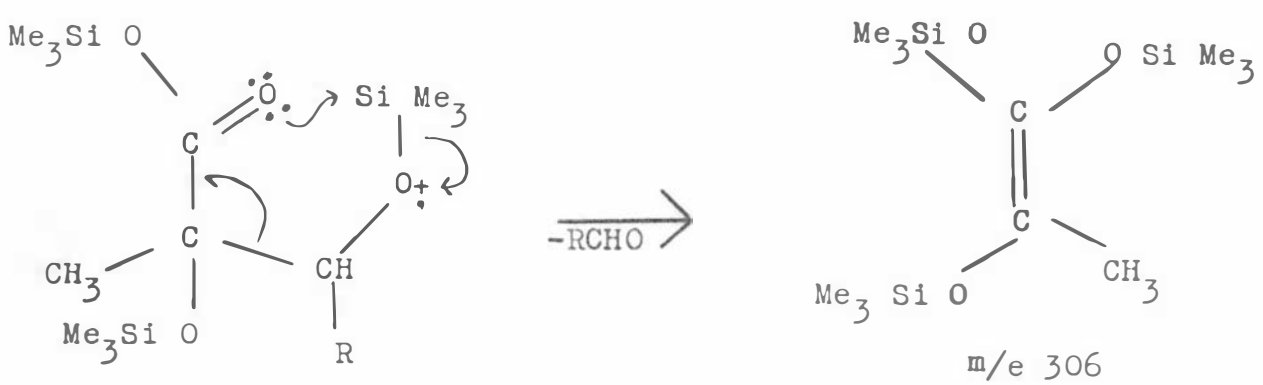
Figure 4·9·3 : Gas liquid chromatograph of silyl derivatives of nickel extract from P. metalli fera.
3% OV-101, 90°- 240°C ×8°min.$^{-1}$
see text for explanation of C and D.

a parent compound corresponding to $C_5 H_{10} O_5$.

The mass spectra of trimethylsilyl ethers of various pentoses have been reported by Chizhov et al., (1967), Petersson and Samuelson (1968) and Petersson (1970a). The most intense peaks in all the spectra are those at m/e 204 and 217 corresponding to the ions $(TMS\ O\text{-}CH{=}CH\text{-}OTMS)^+$ and $TMSO\text{-}CH{=}CH\text{-}CH{=}\overset{+}{O}TMS$ respectively. The peak at m/e 191 in the mass spectra of the TMS ethers of aldopentoses also has a high intensity (Chizhov et al., 1967; Petersson and Samuelson, 1968). In the mass spectrum of the TMS derivative from the nickel-containing extract of P. metallifera no ion appears at m/e 204 and the ions at m/e 191 and 217 are of low abundance (Figure 4.9.4).

Any reasonable interpretation for the decomposition of the molecular ion in the mass spectrum shown in Figure 4.9.4 must explain the loss of CH_2O from the M-15 ion, and the presence of the abundant ions at m/e 306, 234, 205 and 117. The very abundant ions at m/e 73 and 147 are characteristic of TMS ethers of carbohydrates and aliphatic hydroxy acids in general and represent the fragments $(CH_3)_3 Si^+$ and $(CH_3)_3 Si = \overset{+}{O} - Si(CH_3)_2$ respectively (Eglinton et al., 1968).

A metastable peak (m^* = 364.5 to 366; calc: 365.2) demonstrates the formation of m/e 393 from the m/e 423 molecular ion through the loss of formaldehyde. There are two possible explanations for the formation of the odd-electron ion at m/e 306. One possibility is its formation from the M-15 peak through the loss of -COO Si Me_3. The most probable explanation, however, is one derived from the work of Petersson (1970, 1972). This involves a McLafferty-type rearrangement similar to that given for the formation of the very abundant m/e 292 ion, prominent for acids with a 2,3 - dihydroxy acid structure. The m/e 306 ion results through the loss of - RCHO as follows:



m/e 306

With a molecular ion of mass 438 the above rearrangement would occur with the loss of $O{=}CH\text{-}CH_2OTMS$. It is, however not possible to

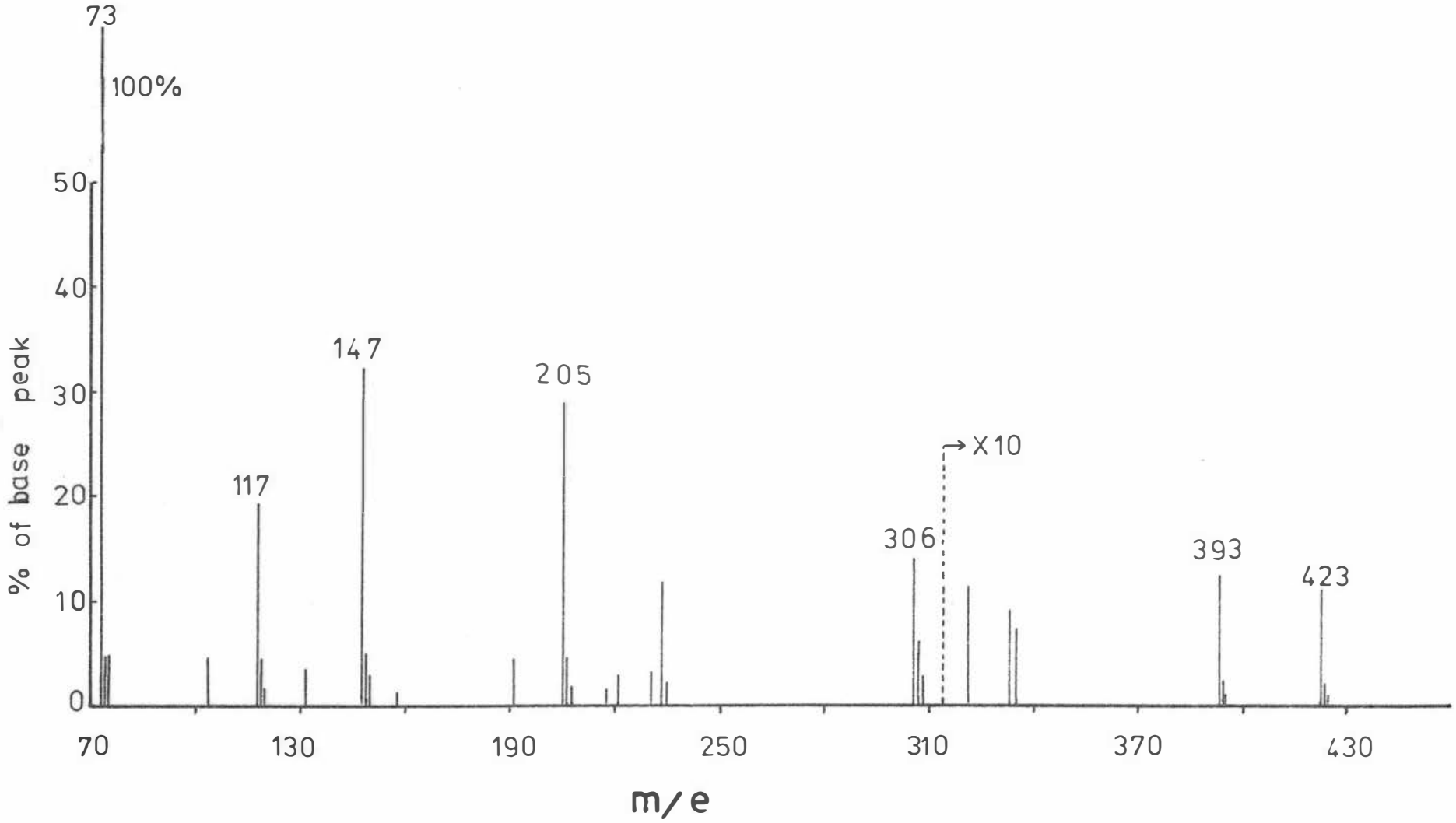


Figure 4·9·4: Mass spectrum of TMS-derivative from nickel-containing extract of P. metallifera. AEI MS 30, 20 eV, separator temp. 200°C. Peak heights relative to that of m/e 73.

distinguish by mass between the loss of this fragment and the loss of -COOTMS from M-15. For pentuloses $^{m}/e$ 306 is the most intense peak with $^{m}/e$ 307, $^{m}/e$ 234 and $^{m}/e$ 205 following in that order (Petersson, 1972). Petersson (1970) reports that 2- deoxypentonic acids also exhibit a relatively intense peak at $^{m}/e$ 306 and that this ion is probably formed by a rearrangement similar to that given above, but involving a seven-membered ring since the normally preferred six-membered ring gives a structurally less favourable ion for these acids. The low intensities of the M-117 - nx90 peaks and the complete absence of peaks at $^{m}/e$ 292 are also characteristic.

A number of TMS derivatives with a mass of 438 exist that would lead directly to $^{m}/e$ 205 ($TMSOCH_2$-CHOTMS-). Such structures would need to possess a terminal $HOCH_2$-CHOH- sequence. Petersson (1970) has reported the mass spectra of 2- deoxy-erythro - pentonic and 3-deoxy- erythro - pentonic acids, both of which contain substantial $^{m}/e$ 205 peaks. However, only a small peak at $^{m}/e$ 117 is observed. It appears that the fragment -COOTMS is not sufficiently stable to produce an abundant ion at $^{m}/e$ 117 and quickly loses CO_2 to leave the $(CH_3)_3$ Si^+ fragment of mass 73. A more stable fragment of mass 117 is CH_3 - CH-OTMS as in lactic acid. The mass spectrum of this acid contains a very intense peak at $^{m}/e$ 117. (Petersson, 1970). A very abundant $^{m}/e$ 117 ion is also characteristic of ω-deoxyaldonic acids such as 4 - deoxyerythronic acid. A comparison with the fragmentation of the other types of acids leads to the conclusion that the $^{m}/e$ 117 ions are formed almost exclusively from the hydroxyl-substituted portion of the molecule.

The dihydroxy - (hydroxy methyl) butanoic acids offer TMS - derivatives that might eliminate HCHO - from the molecular ion or the M-15 ion. Petersson (1970) reported the mass spectrum of one of the six possible isomers: 3 - deoxy - 2 - C - (hydroxymethyl) tetronic acid, and the appearance of peaks corresponding to M-30 and M-15-30 was observed.

4.9.4 Discussion

From the mass spectrum of the TMS derivative of the nickel-containing extract of P. metallifera, and consultation of the literature on this subject, certain peaks may be explained in part, but overall, on the basis of the information obtained to date, it is difficult to

assign a definite structure. The fact that a g.l.c. peak with the same mass spectrum as Figure 4.9.4 occurs in the gas chromatograph of the total organic acids extract of P. metallifera indicates that the unknown parent compound is an acidic compound rather than one of neutral charge,as the separation procedure involved cation and anion exchange. Further elucidation of the nickel-binding agent in P. metallifera must await further investigation on larger quantities of extract, when this can be obtained. Possible methods might involve, fragmentation of the ligand through periodate oxidation and identification of the subsequent acids produced through mass spectrometry of their TMS derivatives, and the use of proton N.M.R. in distinguishing different environments of the hydrogen atom. The culture of this Rhodesian species may be required if adequate amounts of material are to be obtained for subsequent work.

5.

General Discussion

The findings of this thesis have been outlined in three chapters. The results of each chapter have already been discussed and some conclusions drawn as to the nature of nickel accumulation. The present discussion will therefore serve to collate and summarize the individual conclusions that have been made, and to offer some explanations and hypotheses regarding the uptake of abnormal quantities of nickel.

The general aim was to investigate various aspects of nickel accumulation by New Caledonian serpentine flora. This was approached using both biogeochemical (Chapters 2 and 3) and phytochemical (Chapter 4) techniques.

Nearly 2000 herbarium specimens and 232 species of the genera Homalium and Hybanthus were analysed for nickel in order to identify plant accumulators of this element which were indicative of nickeliferous (usually ultrabasic) rocks. The survey resulted in the re-identification of all five previously known hyperaccumulator species (> 1000 µg/g on a dry weight basis), and in the discovery of five additional New Caledonian hyperaccumulators from these two genera. Fourteen previously unknown strong accumulators (100-1000 µg/g) of nickel were also discovered. Most of these were found growing over ultrabasic rocks. The survey was able to pinpoint many of the world's ultrabasic areas in temperate and tropical regions. The principle of the method could be applicable to other genera and other elements and may be used in mineral exploration to delineate areas of possible mineralization.

Elemental concentrations were studied in three New Caledonian hyperaccumulators of nickel and in the soils supporting these plants. The depletion of already low potassium, phosphorus and calcium levels in the soil by excessive laterization was noted. However the ability of Hybanthus austrocaledonicus, Homalium guillainii and Homalium kanaliense to obtain adequate amounts of these elements was notable. Both nickel and cobalt are available to the three investigated plants in substantial quantities. Only nickel, however, is actively absorbed and

accumulated, with cobalt uptake being restricted to slightly above normal levels. Chromium uptake is also minimal, although this may be due largely to the depressed availability in the soil. It therefore appears that accumulation is specific for nickel only and is restricted to a small group of unusual plant species. However with further sampling of herbarium specimens, this list is growing (Brooks, 1977). The majority of the New Caledonian flora growing over nickeliferous rocks is able to tolerate high levels in the soil through some exclusion process.

Because of the paucity of relationships between nickel and other elements, it was suggested that the high nickel levels in the accumulator plants may be controlled by organic constituents.

Phytochemical studies of nickel in a number of New Caledonian accumulators resulted in the isolation and identification of an anionic citrato-complex of nickel. The complex was identified using a combination of gel chromatography, spectrophotometry, electrophoresis, gas liquid chromatography and mass spectrometry. Analysis for total concentrations of citric acid and nickel in New Caledonian serpentine plants resulted in a highly significant correlation between the two. With increasing nickel concentrations in the leaves, a corresponding increase in citric acid content was observed. In those species which take up nickel ions in considerable amounts, nickel translocation appears to be metabolic dependent on some organic constituent. The bulk of the nickel in the exudate (latex) of S. acuminata and in the leaves of other species was complexed with citrate that was certainly of metabolic origin.

One possible consequence of excessive nickel levels in plant tissue as found in the New Caledonian accumulator plants, is the inhibition by nickel of the function of certain enzymes in the citric acid cycle, such as, aconitase and isocitric dehydrogenase. A lowering of the enzymatic activity may result in a subsequent build up of citric acid. Kratochvil et al. (1967) and O'Leary and Limburg (1977) have studied the inhibitory effect of nickel on isocitric dehydrogenase, a N.A.D.P. dependent enzyme requiring a metal ion for activity and responsible for the oxidation of isocitric acid to α-ketoglutarate with the release of carbon dioxide. The catalytic activity of the enzyme was ten-fold lower in the presence of nickel ions than of magnesium ions (O'Leary and Limburg, 1977). The nickel ion appeared to function less efficiently than magnesium in either the oxidation step or the decarboxylation

step. O'Leary and Limburg noted that a change of the metal in some way affects rates of substrate and product binding dissociation.

The production of complexing agents for the development of tolerance has been documented for several elements (copper : Reilly *et al.*, 1970; aluminium : Jones, 1961; zinc : Mathys, 1977; chromium : Lyon *et al.*, 1969). Citric and malic acids have been implicated in the mobility of calcium in xylem (Bradfield, 1976). Tiffin (1970) describes the translocation of iron citrate in xylem exudate of soybean. In these investigations the formation of the analysed complexes as artefacts of the extraction procedure cannot be excluded. Mathys (1977) noted that tolerance based on the action of complexing agents has to be documented by qualitative or large quantitative differences between resistant and non-resistant ecotypes of the same species. In this study the nickel-citrate complex was clearly evident in the latex of *S. acuminata*, and with the excellent correlation between nickel and citrate in the leaves of this and other accumulating and non-accumulating species, there is reason to suppose that complexing is a part of the normal metabolic function. A suggestion for future work might be the analysis of nickel and citrate in different populations of *Hybanthus caledonicus* which is a hyperaccumulator of nickel on ultrabasic substrates, but is also found growing in more normal areas.

Spectral studies of solutions with various citrate to nickel mole ratios indicated the formation of a 2 : 1 anionic species $(Ni(HC)_2)^{2-}$ in solutions in which citrate to nickel approached and exceeded a 2 : 1 mole ratio. *In vivo*, however, it is likely that, in the hyperaccumulators at least, where the citric acid/nickel ratio is generally less than or equal to one, and molar concentrations are low, the 1 : 1 species exists. Mere changes in concentration may induce large shifts in equilibria, and the actions of cation and anion exchange resins certainly cause shifts in equilibria. For these reasons the various nickel complexes obtained by Tiffin (1971), Tiffin and Thompson (1974) and Thompson and Tiffin (1974) by electrophoresis and anion exchange may in fact represent different nickel citrate species of the kind discussed in Section 4.7.

Because of the general chelating ability of citric acid (more stable chelates with copper and iron occur) and its widespread occurrence in most vegetation, it seems unlikely that nickel is absorbed at the roots as a citrate complex, although it forms such a complex once inside the plant cell. A more specific mechanism must be invoked

to account for the selectivity of the nickel absorption by the New Caledonian hyperaccumulating plants. At this stage it must be pointed out that a high citric acid concentration in plants does not necessarily imply nickel tolerance nor does it necessarily cause accumulation of this element. Vanselow (1951) noted that varying amounts of nickel added to the soil in an available form were extremely toxic to citrus plants.

A likely hypothesis based on the experimental work in this thesis, the carrier concept and the work of Mathys (1977) is schematically given in Figure 5.1. To alleviate the toxicity of ionic nickel, chelation takes place immediately after entry into the cytoplasm. In the hyperaccumulators from New Caledonia specificity of nickel absorption is envisaged to arise through the possession by these plants of a highly selective membrane (plasmalemma), possibly containing a specific nickel chelating agent. Certain oximes, for instance, may fulfil this role. This transport leads to an accumulation of nickel, firstly in the cytoplasm itself. In the plasma, however, the nickel concentration is kept relatively low through the operation of a second transport system which removes ionic nickel into the vacuole via the tonoplast. Once in the vacuole, a terminal acceptor which in this study has been identified as citric acid, forms a more stable complex with the nickel. The carrier is then able to move back through the tonoplast to be involved with further transport. Mathys (1977) has proposed and given evidence for malate as being such a transport vehicle for zinc, in zinc tolerant plants. Malate is mainly located in the plasma (Torri and Laties, 1966). The New Caledonian hyperaccumulators contained a high concentration of malic acid and a similar transport as envisaged by Mathys may be involved. As nickel-malate is less stable than a nickel citrate complex ($\log K^{Ni}_{NiHM} = 3.17$; $\log K^{Ni}_{NiHC} = 5.40$) transfer of nickel to the citrate in the vacuole is facilitated.

The complexing agent in the vacuole need only have one requirement; it must have a greater affinity for nickel than the transporting agent. In Pearsonia metallifera another complexing agent is involved.

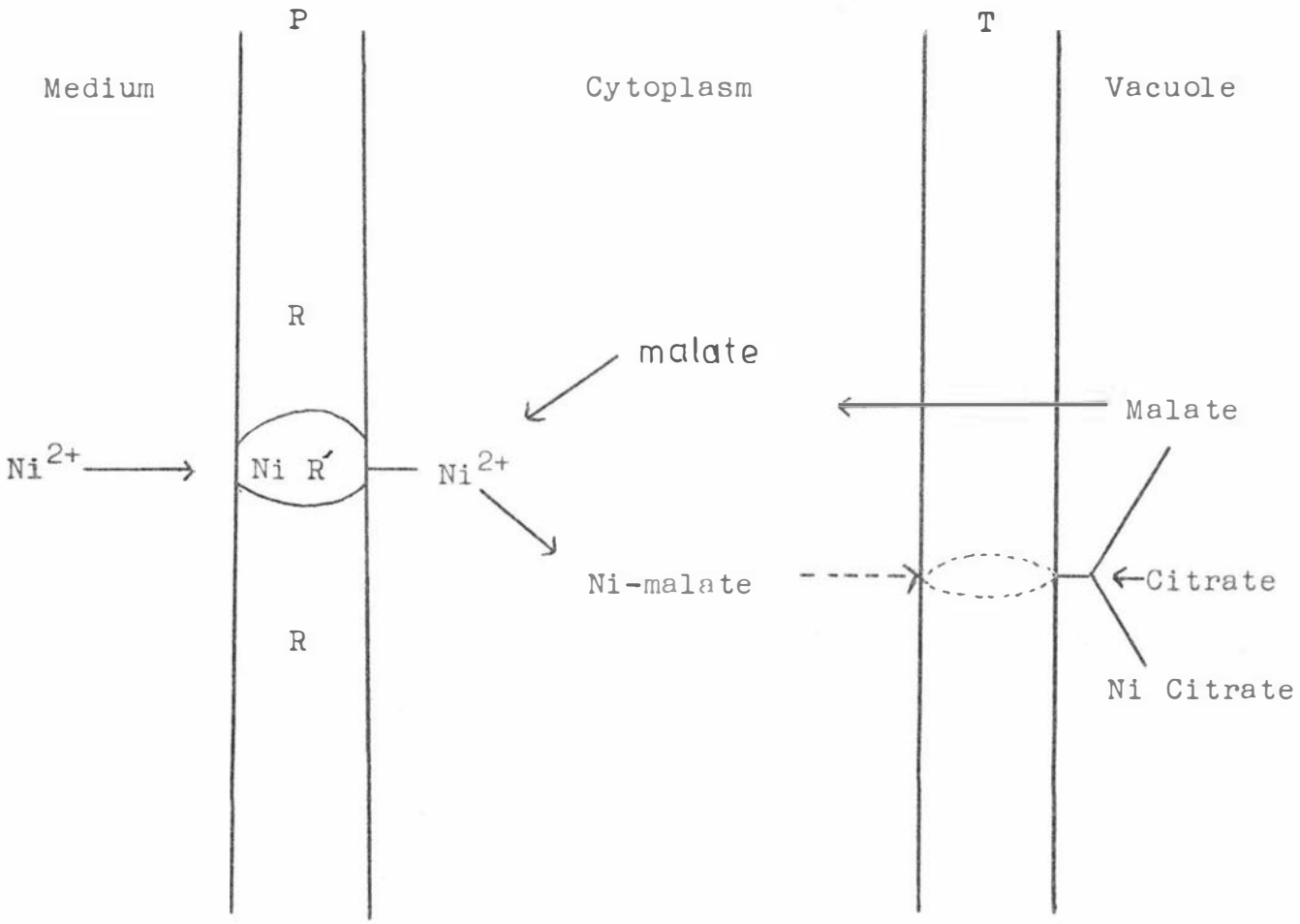


Figure 5.1 Schematic representation of the process of nickel absorption.

P = plasmalemma, T = tonoplast
R = amphoteric carrier.

Such a mechanism as this may operate in a number of species with the degree of accumulation dependent on the permeability of the root membrane towards nickel. The absence of negative correlation between nickel and other elements in the leaves of the New Caledonian hyper-accumulators is indicative of the specific nature of the plasmalemma in its absorption of nickel.

Future work should examine the rate of uptake of nickel supplied in both ionic and chelated forms using radioactive techniques. Detailed studies of the root exudates of both accumulator and non-accumulator species growing on ultrabasic substates could be carried out. Much of the future work may necessitate the establishment of laboratory-grown hyperaccumulating species under controlled conditions.

The leaf material used for phytochemical studies in this thesis contained more than 2,000 µg/g nickel on a dry weight which enabled extraction and purification of milligram quantities of complexed nickel. To identify metal complexes in plant species where concentrations are appreciably lower than this, an extension of the techniques used here to incorporate preparative thin-layer chromatography and continuous electrophoresis would be required. Hence small samples could be chromatographed and isolated subsequent to gas-liquid chromatography and mass spectrometry of volatile derivatives.

REFERENCES CITED.

Al-Rais, A.H., Myers, A., and L. Watson. 1971. The isolation and properties of oxalate crystals from plants. Ann. Bot. 35 : 1213 - 1218.

Baker, D. 1973. Analysis of organic acids in fruit products by anion exchange isolation and gas-liquid chromatography determination. J. Ass. Off. Anal. Chem. 56 (5), 1257 - 1263.

Barta, A.L., and C.A. Osmond. 1973. Improvements for gas chromatographic analysis of organic acids in plant tissue. J. Ag. Food Chem. 21 (2), 316 - 318.

Battzer, F., Canavet, J., Guillon, J.H., Launay, J.C., and J.J. Trescases. 1967. Weathering of the New Caledonian ultramafic rocks and consecutive sedimentation : A research programme. N.Z. Jl. Geol. Geophys. 10 : 1197 - 1199.

Bennett, E.M. 1969. The genus Hybanthus Jacquin in Australia including the cytology and anatomy of the Western Australian species. M. Sc. Thesis, Univ. Western Australia.

Bennett, E.M. 1972. A revision of the Australian species of Hybanthus Jacquin (Violaceae). Nuytsia 1 (3), 218 - 241.

Bennett - Clark, T.A. 1933. The role of organic acids in plant metabolism. New Phytologist, 32 : 37 - 76.

Birrell, K.S., and A.C.S. Wright. 1945. A serpentine soil in New Caledonia. N.Z. J. Sci. Tech. 27 : 72 - 76.

Bobtelsky, M., and C. Heitner. 1951. Étude spectrophotometrique des complèxes des citrates et tartrates de nickel, cobalt, cuivre et chrome. Bull. de la Société Chimique de France. 494 - 502.

Boland, R.L., and G.B. Garner. 1973. Determination of organic acids in tall fescue (Festuca avundinacea) by gas-liquid chromatography. J. Agr. Food. Chem. 21 (4), 661 - 665.

Bollard, E.G., and G.W. Butler. 1966. Mineral nutrition of plants. Ann. Rev. Plant Physiol. 17 : 77 - 112.

Bowen, H.J.M. 1966. Trace Elements in Biochemistry. Academic Press, Lond.

Bradfield, E.G. 1976. Calcium complexes in the xylem sap of apple shoots. Plant and Soil. 44 : 495 - 499.

Bremner, I., and A.H. Knight. 1970. The complexes of zinc, copper, and manganese present in ryegrass. Brit. J. Nut. 24 : 280 - 289.

Brenchley, W.E. 1938. Comparative effects of cobalt, nickel and copper on plant growth. Ann. Appl. Biol. 25 : 671 - 694.

Brobst, K.M., and C.E. Lott. 1966. Determination of some components of corn syrup by gas-liquid chromatography of the trimethylsilyl derivatives. Cereal Chem. 43 : 35.

Brooks, C.J., Betteley, I.G., and S.M. Loxston. 1966. Mathematics and Statistics for Chemists. Wiley, N.Y.

Brooks, R.R. 1972. Geobotany and Biogeochemistry in Mineral Exploration. Harper and Row, N.Y.

Brooks, R.R. 1977. Hyperaccumulation of nickel by plants: A review. submitted for publication.

Brooks, R.R., Lee, J., and T. Jaffré 1974. Some New Zealand and New Caledonian plant accumulators of nickel. J. Ecol. 62 : 523 - 529.

Brooks, R.R., Lee, J., Reeves, R.D., and T. Jaffré. 1977. Detection of nickeliferous rocks by analysis of herbarium specimens of indicator plants. J. Geochem. Explor. 7 : 49 - 57.

Brooks, R.R., and E.D. Wither. 1977. Nickel accumulation by Rinorea bengalensis (Wall) O.K. J. Geochem. Explor. 1 : 295-300.

Brown, J.C., and R.L. Chaney. 1971. Effect of iron on the transport of citrate in the xylem of soybeans and tomatoes. Plant Physiol. 47 : 836 - 840.

Brown, J.C., and L.O. Tiffin. 1965. Iron stress as related to the iron and citrate occurring in stem exudate. Plant Physiol. 40 : 395-400.

Camp, B.J., and L. Farmer. 1967. A rapid spectrophotometric method for the determination of citric acid in blood. Clin. Chem 13 : 501 - 505.

Campi, E., Ostacoli, G., Meirone, M., and G. Sainii., 1964. Stability of the complexes of tricarballylic and citric acids with bivalent metal ions in aqueous solution. J. Inorg. Nucl. Chem. 26 : 553 - 564.

Chaney, E.J. 1963. Plants containing substantially no assimilated nickel ions. U.S. 3 : 107, 163 (C1. 71 - 1) Oct. 15, 1963, Appl. May 28, 1959, pp.3. Cited Mishra and Kar, 1974.

Chang, A.T., and G.D. Sherman. 1953. The nickel content of some Hawaiian soils and plants and the relation of nickel to plant growth. Hawaii Agr. Expt. Sta. Tech. Bull. No 19, 3 - 25.

Charlanes, M. 1960. Differences de compartement de quelques variete's d' avoine (Avena sativa L.) en presence de manganese soluble et insoluble. C.R. Acad. Sci. (Paris) 250 : 2713 - 2715.

Chenery, E.M. 1948. Aluminium in the plant world. Part 1. General survey in dicotyledons. Kew Bull. 173.

Chenery, E.M., and K.R. Sporne. 1976. A note on the evolutionary status of aluminium accumulators among dicotyledons. New Phytol. 76 : 551 - 554.

Chizhov, O.S., Molodtstov, M.V., and N.K. Kochetkov. 1967. Mass spectrometry of TMS ethers of carbohydrates. Carbohyd. Res. 4 273 - 276.

Clark, R.B. 1969. Organic acids from leaves of several crop plants by gas chromatography. Crop Sci. 9 : 341.

Cole, M.M. 1971. The importance of environment in biogeographical / geobotanical and biogeochemical investigations. Can. Inst. Min. Metall. Spec. Vol. 11:414-425.

Cole, M.M. 1973. Geobotanical and biogeochemical investigations in the sclerophyllous woodland and shrub associations of the eastern goldfields area of Western Australia, with particular reference to the role of Hybanthus floribundus (Lindl.) F. Muell. as a nickel indicator and accumulator plant. J. Appl. Ecol. 10 (1), 269 - 320.

Committee on Medical and Biologic Effects of Environmental Pollutants. 1975. Medical and Biologic Effects of Environmental pollutants. Nickel. National academy of Sciences, Washington, D.C. pp 1 - 277.

Connor, J., and H.T. Shacklette. 1975. Background geochemistry of some rocks, soils, plants and vegetables in the conterminous United States. Geol. Surv. Prof. Paper 574-F : 1 - 167.

Cotton, M. 1930. Toxic effects of iodine and nickel on buckwheat grown in solution cultures. Bull. Torrey Bot. Club 57 : 127 - 140.

Craig, G.C. 1977. A method of measuring heavy metal tolerance in grasses. Trans. Rhodesian Scientific Assoc. 58 (2), 9 - 16.

Cronquist, A. 1968. The Evolution and Classification of Flowering plants. Nelson, London.

Crooke, W.M. 1954. Effect of nickel versenate on oat plants. Nature 173 : 403 - 404.

Crooke, W.M., 1956. Effect of soil reaction on uptake of nickel from a serpentine soil. Soil Sci. 81 : 269 - 276.

Crooke, W.M., Hunter, J.G., and O. Vergnano. 1954. The relationship between nickel toxicity and iron supply. Ann. Appl. Biol. 41 : 311 - 234.

Crooke, W.M., and A.H. Knight. 1955. The relation between nickel toxicity symptoms and the absorption of iron and nickel. Ann. Appl. Biol. 43 : 454 - 464.

David, D.J. 1959. Determination of calcium in plant material by atomic absorption spectrophometry. Analyst 84 : 536 - 545.

De Jongh, D.C., Radford, T., Hribar, J.D., Hanessian, S., Bieber, M., Dawson, G., and C.C. Sweeley. 1969. Analysis of trimethylsilyl derivatives of carbohydrates by gas-liquid chromatography mass spectrometry. J. Amer. Chem. Soc. 91 (7), 1728 - 1740.

De Kock, P.C. 1956. Heavy metal toxicity and iron chlorosis. Ann. Botany (London) 20 : 133 - 141.

De Kock, P.C., and R.L. Mitchell. 1957. Absorption of metal chelates by plants. Soil Sci. 84 : 55-62.

Diekman, J., Thomson, J.B., and C. Djerassi. 1968. Mass spectrometry in structural and sterochemical problems. CLV. Electron Impact Induced fragmentations and rearrangements of some trimethylsilyl ethers of aliphatic glycols and related compounds. J. Org. Chem. 33 (6), 2271 - 2284.

Dinnin, J.J. 1960. Releasing effects of flame photometry determination of calcium. Anal. Chem. 32 : 1475 - 1480.

Dixon, M.E., Gazzola, C., Blakeley, R.L., and Zerner, B. 1975. Jack Bean urease (EC 3.5.1.5). A metalloenzyme. A simple biological role of nickel? J.Amer. Chem. Soc. 97 (14) 4131 - 4133.

Draffan, G.H., Stillwell, R.N., and J.A. McCloskey. 1968. Electron impact induced rearrangement of trimethylsilyl groups in long chain compounds. Org. Mass. Spec. 1 : 669 - 685.

Dutton, G.S. 1974. Application of gas-liquid chromatography to carbohydrates Part II. Adv. Carbohyd. Chem. Biochem. 30: 9 - 110.

Efron, M. 1960. High voltage paper electrophoresis in chromatographic and electrophoretic techniques. Vol. 2 : 188 - 189. ed. I. Smith.

Eglinton, G., Hunnesman, D.H., and A. McCormick. 1968. Gas chromatographic - mass spectrometric studies of long chain hydroxy acids III. The mass spectra of the methyl esters, trimethysilyl ethers of aliphatic hydroxy acids. A facile method of double bond location. Org. Mass. Spec. 1 : 593 - 611.

Elwell, W.T., and J.A.F. Gidley, 1967. Atomic Absorption Spectrophotometry. Pergamon Press, Oxford.

Epstein, E. 1960. Spaces, barriers and ion carriers : Ion absorption by plants. Amer. J. Bot. 47 : 393 - 399.

Epstein, E. 1962. Mutual effects of ions in their absorption by plants. Agne Chimica 4 : 293 - 322.

Epstein, E., and R.L. Jefferies. 1964. The genetic basis of selective ion transport in plants. Ann. Rev. Plant Physiol. 15 : 169 - 184.

Ernst, W. 1972. Ecophysiological studies on heavy metal plants in South Central Africa. Kirkia. 8 : 125 - 145.

Ernst, W. 1974. Mechanismen der Schwermetallresistienz. Sonderdruck : Verhandlungen der Gesellschaft fur Ökologie, Erlangen 1974, 189 - 197.

Farago, M.E., Clark A.J., and M.J. Pitt. 1975. The chemistry of plants which accumulate metals. Co-ordination Chem. Rev 16 : 1-8.

Feigl, F. 1966. Spot tests in Organic Analysis. 7th Edition. Amsterdam.

Field, T.B., Coburn, J., McCourt, J.L., and W. McBryde. 1975 Composition and stability of some metal citrate and di glycolate complexes in aqueous solution. Anal. Chim. Acta, 75 (1), 101 - 6.

Garibaldi, J.A. and J.B. Neilands. 1955. Isolation and properties of Ferrichrome A. J. Am. Chem. Soc. 77 : 2429 - 2430.

Gauch, H.G. 1957. Mineral nutrition of plants. Ann. Rev. Pl. Physiol. 8 : 31 - 64.

Gauch, H.G. 1972. Inorganic Plant Nutrition. Dowden, Hutchinson and Ross Inc., Stroudsburg, Pa. 488p.

Gauch, H.G., and W.M. Dugger. 1953. The role of boron in the translocation of sucrose. Plant Physiol. 28 : 457 - 467.

Gerloff, G.C. 1963. Comparative mineral nutrition of plants. Ann. Rev. Plant Physiol. 14 : 107 - 124.

Giron, H.C. 1973. Comparison between dry ashing and wet ashing in the preparation of plant material for atomic absorption analysis. Atomic Absorption Newsletter 12: 28 - 29.

Gomah, A.M., and R.J. Davies. 1974. Identification of the active ligands chelating zinc in some plant water extracts. Pl. Soil : 1 - 19.

Goodman, G.T., and M.T. Roberts. 1971. Plants and soils as indicators of metals in the air. Nature 231 : 287 - 292.

Gregory, R.P.G., and A.D. Bradshaw. 1965. Heavy metal tolerance in populations of Agrostis tenuis Sibth and other grasses. New Phytologist. 64 : 131 - 143.

Grigg, J.L. 1953. Determination of available molybdenum of scils. N.Z. J. Sci. Tech. 34 (A) : 405 - 414.

Grigoreva, V.V., and S.M. Tsimbler. 1968. Interaction of cobalt (II) and nickel (II) compounds with certain hydroxy acids. Russ. J. Inorg. Chem. 13 (2), 259 - 263.

Grigoreva, V.V., and S.M. Tsimbler. 1970. Composition and relative stability of complex cobalt and nickel compounds with hydroxy acids at pH 6. Visn. Kiw. Univ. Ser. Khim. No 11, 17 - 23.

Halstead, R.L., Finn, B.J., and A.J. Maclean. 1969. Extractability of nickel added to soils and its concentration in plants. Can. J. Soil Sci. 49 (3), 335 - 342.

Harmon, M.A., and H.W. Doelle. 1969. Gas chromatographic separation and determination of micro-quantities of the esters of the tricarboxylic acid cycle acids and related compounds.

Haselhoff, E. 1893. Versuche über die schädliche Wirkung von nickel - haltigem wasser auf pflanzen. Landw. Jahrb. 22 : 1862 - 1868.

Heitner-Wirguin, C., Friedman, D., Goldschmidt, J.M.E., and J. Shamier. 1958. Contribution à l' etude des complexes des citrates et tartrates. 3. Le complex du nickel avec le citrate. Bull. Chim. France, 864 - 867.

Hewitt, E.J. 1951. The role of mineral elements in plant nutrition. Ann. Rev. Pl. Physiol. 2 : 25 - 52.

Hewitt, E.J. 1963. The essential nutrient elements : requirements and interactions in plants pp 137 - 360. In Plant Physiology 3 : 811 pp., Ed. F.C. Steward, Academic Press N-Y.

Hewitt, E.J., and E.W. Bolle-Jones. 1952. Possible micronutrient elements for higher plants. I. Experiments with cobalt, nickel and gallium in seed culture. Ann. Rept. Agr. and Hort. Res. Sta. Long Ashton, Bristol 1951 : 62 - 66.

Hill, J.R.C. 1973. The Rhodesian approach to the vegetating of slimes dams. Jl. S. Afr. Inst. Min. Metall. 74 : 197 - 208.

Hodgson, J.F. 1963. Chemistry of the micronutrient elements in soils. Advan. Agron. 15 : 119 - 159.

Holligan, P.M. 1971. Routine analysis by gas-liquid chromatography of soluble carbohydrates in extracts of plant tissues. New Phytol. 70 : 239 - 269.

Honert, T.H. van den. 1963. Vergadering Vereeninging Proefstations Personnel 16 : 85 - 93 (cited Gauch, 1972).

Horii, Z., Makita, M., and Tamura, Y. 1965. Gas-liquid chromatographic separation of acids of Krebs cycle as trimethylsilyl derivatives. Chemy Ind., 1494.

Hunter, J.G., and O. Vergnano. 1953. Trace element toxicities in oat plants. Ann. Appl. Biol. 39 : 279 - 284.

Hunter, S.H. 1948. Essentiality of constituents of sea water for growth of a marine diatom. Trans. N.Y. Acad. Sci. 10 : 136 - 141.

Hunter, S.H., Provasoli, L., Schatz, A., and C.P. Hachins. 1950. Proc. Ann. Phil. Soc. 94 : 152 - 170.

Irving, H., and R.P.J. Williams. 1953. The stability of transition metal complexes. J. Chem. Soc. 3192 - 3210.

Jacobs, M., and D.M. Moore. 1971. Violaceae. Flora Malesiana Ser. 1 7 (1), 179 - 212.

Jacobson, L., and L. Ordin. 1954. Organic acid metabolism and ion absorption in roots. Plant Physiol. 29, 70 - 75.

Jacobson, L., and R. Overstreet. 1947. A study of the mechanism of ion absorption by plant roots using radioactive elements. Amer. J. Bot. 34 : 415 - 420.

Jacobson, L., Overstreet, R., King, H.M., and R. Handley. 1950. A study of potassium absorption by barley roots. Plant Physiol. 25 : 639 - 647.

Jaffré , T. 1969. Recherches sur la végétation des roches ultrabasiques en Nouvelle - Calédonie. O.R.S.T.O.M./Nouméa, 30p.

Jaffré, T. 1973. Variations de la composition floristique et de la structure de la végétation du massif du Boulinda en fonction du substrat édaphique et de l'altitude. Note presentee au Colloque d' écologie de la Pacific Science Association. Guam, Mai 1972, 9p.

Jaffré , T. 1974. La végétation et la flore d'un massif de roches ultrabasiques de Nouvelle Calédonie. Candollea 29 : 427 - 456.

Jaffré , T. 1976. Composition chimique et conditions de l' alimentation minérale des plantes sur roches ultrabasiques (Nouvelle-Calédonie) Cah. O.R.S.T.O.M., Sér. Biol. XI, No.1, 53 - 63.

Jaffré , T., Latham, M., and P. Quantin. 1971. Les sols des Massifs Miniers de Nouvelle - Calédonie et leur relation avec la végétation. Sp. Rep. O.R.S.T.O.M., Nouméa, New Caledonia.

Jaffré, T., and M. Latham. 1974. Contribution a l'etude des relations sol - végétation sur un massif des roches ultrabasiques de la côte ouest de la Nouvelle - Calédonie : Le Boulinda. Adansonia 14 : 311 - 336.

Jaffré T., and M. Schmid. 1974. Accumulation du nickel par une Rubiacée de Nouvelle Calédonie; Psychotria douarrei (G.Beauvisage) Däniker. c.r. hebd. Seanc. Acad. Sci., Paris, 278 : D1727 - 1730.

Johnston, H.W. 1956. Chelation between copper and organic ions. N.Z. J. Sci. Technol. 37B : 522 - 537.

Jones, L.H. 1961. Aluminium uptake and toxicity in plants. Plant Soil, 13 : 297 - 310.

Jowett, D. 1958. Populations of Agrostis spp. tolerant of heavy metals Nature (Lond.) 182 : 816.

Jowett, D. 1964. Population studies on lead tolerant Agrostis tenuis. Evolution, Laucs. Pa., 18 : 70 - 80.

Kashin, V.K. 1968. Physiological role of nickel in living organisms. Mikrochem. Sib. 6 : 78 - 87. (Russ) C.A. 12093m.

Kelly, P.C., Brooks, R.R., Dilli,S., and T. Jaffré. 1975. Preliminary observations on the ecology and plant chemistry of some nickel accumulating plants from New Caledonia. Proc. Roy. Soc. Ser. B. 189 : 69 - 80.

Kirkby, E.A., and K. Mengel. 1967. Ionic balance in different tissues of the tomato plant in relation to nitrate, urea, or ammonium nutrition. Plant Physiol. 42 : 6 - 14.

Kratochvil, B., Boyer, S.L. and G.P. Hicks. 1967. Effects of metals on the activation and inhibition of isocitric dehydrogenase. Anal. Chem. 39 : 45 - 51.

Krause, W. 1958. Other special soils. pp. 755 - 806. In 'Encyclopedia of Plant Physiology' IV, W. Ruhland, ed. Springer-Verlag, Berlin (Ger).

Kruckeberg, A.R. 1951. Intra-specific variability in the response of certain native plant species to serpentine soil. Amer. J. Bot. 38 : 408 - 419.

Kruckeberg, A.R. 1954. The ecology of serpentine soils III. Plant species in relation to serpentine soils. Ecology, 35 : 267 - 274.

Latham, M. 1972. Etude préliminaire du potentiel agropédologique de la region de Koné - Voh-Témala O.R.S.T.O.M./Nouméa, 12p multigr.

Lee, J. 1974. Biogeochemical studies on some nickel accumulating plants from New Zealand and New Caledonian serpentine areas. M.Sc. Thesis, Massey University, Palmerston North.

Lee, J. Brooks, R.R., Reeves, R.D., and C.R. Boswell. 1973. Soil factors controlling a New Zealand serpentine flora. Plant Soil 42 (1), 153 - 60.

Lee, S.G., Aronoff, S. 1967. Boron in plants : A biochemical role. Science 158 : 798 - 799.

Levinson, A.A. 1974. Introduction to Exploration Geochemistry. Calgary, Applied Publishing, 612p.

Li, N.C., Lindenbaum, A., and J.M. White. 1959. Some metal complexes of citric and tricarballylic acids. J. Inorg. Nucl. Chem. 12 : 122 - 128.

Lillie, A.R., and R.N. Brothers. 1970. The geology of New Caledonia. N.Z. Jl. Geol. Geophys. 13 (1), 145 - 183.

Loew, O., and W. May. 1901. The relation of lime and magnesia to plant growth. I. Liming of soils from a physiological stand point. II. Experimental study of the relation of lime and magnesia to plant growth. Bull. Bur. Pl. Ind. U.S. Dept. Agric. 1 : 53.

Lounamaa, J. 1956. Trace elements in plants growing wild on different rocks in Finland. Ann. Bot. Soc. Zool -bot. Fenn. Vanamo 29 : 1 - 195.

Lyon, G.L. 1969. Trace elements in New Zealand Plants. Ph.D. Thesis, Massey University, New Zealand.

Lyon, G.L., Brooks, R.R., Peterson, P.J., and G.W. Butler. 1970. Some trace elements in plants from serpentine soils. N.Z. J. Sci. 13 : 133 - 139.

Lyon, G.L., Peterson, P.J., and R.R. Brooks. 1969. Chromium -51 transport in the xylem sap of Leptospermum scoparium N.Z. J. Sci. 12 : 541 - 545.

Malyuga, D.P. 1964. Biogeochemical methods of prospecting. Consultants Bureau, N.Y.

Mamer, D.A., Crawhall, J.C., and S.S. Tjoa. 1971. The identification of urinary acids by coupled gas chromatography- mass spectrometry. Clin. Chim. Acta. 32 : 171 - 184.

Marriage, P.B., and D.G. Wilson. 1971. Analysis of the organic acids of Bryophyllum calycinum. Can. J. Biochem. 49 : 282 - 296.

Martell, A.E., and M. Calvin. 1952. Chemistry of metal chelate compounds. Prentice Hall Inc., Englewood Cliffs, N.Y. 613p.

Martini, A. 1930. Der phytomikrochemische Nachweis des Nickels und sein Vorkommen im Pflanzenreich. Mikrochemie 8 : 41 - 45.

Mathys, W. 1977. The role of malate, oxalate, and mustard oil glucosides in the evolution of zinc-resistence in herbage plants. Physiol. Plant. 40 : 130 - 136.

Mavrodineanu, R. 1970. Analytical Flame Spectroscopy. Selected topics by C. Th. J. Alkemade (and others). Macmillan, Lond.

Merkel, D., and H. Jungh. 1973. Gas chromatographic estimation of non-volatile carboxylic acids in plant material. Pflanzenernachr. Bodenk. 134 (1) 1 - 9. (Ger).

Menezes de Sequeira, E. 1969. Toxicity and movement of heavy metals in serpentinic rocks (North-eastern Portugal). Agronomia Lusit. 30 : 115 - 154.

Mertz, W. 1970. Some aspects of nutritional trace element research. Fed. Proc. 29 : 1482 - 1488.

Minguzzi, C., and O. Vergnano. 1948. I1 contenuto di nickel nelle ceneri di Alyssum bertolonii Desv. Memorie Soc. Tosc. Sci. Nat. Ser. A 55 : 49 - 77.

Mishra, D., and M. Kar. 1974. Nickel in plant growth and metabolism Bot. Rev. 40 (4), 395 - 452.

Misra, S.G., and P. Pande. 1974. Evaluation of a suitable extractant for available nickel in soils. Plant Soil 41 (3), 697 - 700.

Moore, D.P. 1972. Mechanisms of micronutrient uptake by plants. pp. 171 - 198. In 'Micronutrients in Agriculture.' eds. Mortvedt, J.J., Giordano, P.M., and W.L. Lindsay. Madison. Soil Sci. Soc. Amer.

Mitchell, R.L. 1945. Cobalt and nickel in soils and plants. Soil Sci. 60 : 63 - 70.

Muir, J.W., Morrison, R.I., Brown, C.J., and J. Logan. 1964. The mobilization of iron by aqueous extracts of plants. I. composition of the amino-acid and organic acid fractions of an aqueous extract of pine needles. J. Soil Sci. 15 : 220 - 225.

Nason, A., and W.D. McElroy. 1963. Modes of action of the essential mineral elements in, Plant Physiology - A Treatise, Vol. III, Inorganic nutrition of plants, ed., F.C. Steward, Academic Press, N.Y. pp. 451 - 536.

Natelson, S., Pincus, J.B., and J.K. Lugovoy. 1948. Micro-estimation of citric acid; A new colorimetric reaction for pentabromo acetone. J. Biol. Chem. 175 : 745 - 750.

Niehaus, D., and H. Kinzel. 1971. Vergleichende Unter-suchungen über die organischen Säuren in Blättern höherer Pflanzen. 2.. Pflanzen Physiol. 64 : 107.

Nielson, F.H. 1971. Studies on the essentiality of nickel, pp. 215 - 253. In, New Trace Elements in Nutrition; eds., W. Mertz and W.E. Cornatzer, Marrel Dekker, I.A.C., N.Y.

Nielson, F.H., and D.A. Ollerich. 1974. Nickel: a new essential trace element. Fed. Proc. 1974.

Nomoto, S., Decsy, M.I., Murphy, J.R., and F.W. Sunderman. 1973. Isolation of ^{63}Ni-labelled nickeloplasmin from rabbit serum. Biochem. Med. 8 (2), 171-181.

Nomoto. S., McNeely, M.D., and F.W. Sunderman. 1971. Isolation of a nickel α_2-macroglobulin from rabbit serum. Biochem. 10 : 1647 - 1651.

Norvell, W.A. 1972. Equilibria of metal chelates in soil solution pp. 115 - 138. In 'Micronutrients in Agriculture.' eds. J.J. Mortvedt, P.M. Giordano, W.L. Lindsay. Soil. Sci. Soc. Amer. Madison, U.S.A.

O'Leary, M.H., and Limburg, J.A. 1977. Isotope effect studies of the role of metals ions in isocitrate dehydrogenase. Biochem. 16(6), 1129.

Overstreet, R., and L. Jac bson. 1952. Mechanisms of ion absorption by roots. Ann, Rev. Plant Physiol. 3 : 189 - 206.

Pande, P., and G.G. Misra. 1975. Availability of soil nickel as affected by phosphate, carbonate and complexants. An Inst. Nac. Invest. Agrar. (Spain), Ser; Gen. 3 : 71 -80.

Paribok, T.A., and N.V. Alekseeva-Popova. 1966. Control of some chemical elements in the wild plants of the Polar urals as related to the problem of the serpentine vegetation. Botan. Zh. 51 : 339 - 353.

Patnaik, R.K., and S. Pani. 1965. Studies on the citrate complex of nickel by solubility, pH -titration and conductometric titration methods. J. Ind. Chem. Soc. 42 (11), 793 - 798.

Pelosi, P., Fiorentini , R., and C. Galoppini. 1976. On the nature of nickel compounds in Alyssum bertolonii Desv. Agri. Biol. Chem. 40 (8), 1641 - 1642.

Pelosi, P., Galoppini, C., and O. Vergnano-Gambi. 1974. Sulla natura dei composti del nickel presenta in Alyssum bertolonii Desv. L' Agricoltura Ital. 29 : 1 - 5.

Persson, H. 1956. Studies of the so-called 'copper mosses' J. Hattori Bot. Lab. 17 : 1 - 18.

Pestemer, M., and A. Alslev-Kliuker. 1949. The photoabsorption of certain organic nickel salts. Z. Elektrochem. 53 : 387-389.

Petersson, G. 1970. Mass spectrometry of aldonic and deoxyaldonic acids as trimethylsilyl derivatives. Tetrahedron 26 : 3413 - 3428.

Petersson, G. 1970a. Mass spectra of trimethysilyl -O- (Trimethysilyl) monosaccharides. Arch. Mass Spectral Data, 1 (4) : 624 - 680.

Petersson, G. 1972. A McLafferty-type rearrangement of a trimethylsilyl group in silylated hydroxy carbonyl compounds. Org. Mass Spec. 6 : 577 - 592.

Petersson, G. 1974. Gas-chromatographic analysis of sugars and related hydroxy acids as acyclic oxime and ester trimethylsilyl derivatives. Carbohyd. Res. 33 (1), 47 - 61.

Petersson, G., and Samuelson, O. 1968. Determination of the number and position of methoxyl groups in methylated aldopentoses by mass spectrometry of their trimethylsilyl derivatives. Svensk Papperstidning 71 (3), 77 - 84.

Petersson, G., Samuelson, O., Anjou, K., and E. von Sydow. 1967. Mass spectrometric identification of aldonolactones of trimethylsilyl ethers. Acta. Chem. Scand. 21 (5), 1251 - 1256.

Peterson, P.J. 1969. The distribution of zinc -65 in Agrostis tenuis Sibth and A. stolonifera L. tissues. J. Expt. Bot. 20 (65), 863 - 875.

Pfeffer, W. 1900. The Physiology of Plants Vol. I. Transl. and ed. by A.J. Ewart, Oxford Univ. Press, oxford.

Phillips, R.D., and Jennings D.H. 1976. The estimation of plant organic acids by gas-liquid chromatography. New Phytol. 77 (2), 333-339.

Pickering, W.F. 1960. The absorption of bivalent metal ions by filter paper. J. Chrom. 4 : 481 - 484.

Pierce, E.C., and C.O. Appleman. 1943. Role of ether soluble organic acids in the cation-anion balance in plants. Plant Physiol. 18 224 - 238.

Pinelli, A., and A. Colombo. 1976. Gas chromatographic separation of Krebs cycle metabolites. J. Chrom. 118 : 236 - 239.

Poole, R.J., and L.W. Poel. 1965. Carbon dioxide and pH in relation to salt uptake by beetroot tissue. J. Exp. Bot. 16 : 453 - 461.

Price, C.A. 1970. Molecular Approaches to Plant Physiology. McGraw-Hill N.Y. 398 pp.

Proctor, J. 1971. Plant ecology of serpentine III. The influence of a high magnesium/calcium ratio and high nickel and chromium levels in some British and Swedish soils. J. Ecol. 59 (3), 837 - 842.

Pucher, G.W., Vickery, H.B., and A.J. Wakeman. 1938. Relation of the organic acids of tobacco to the inorganic basic constituents. Plant Physiol. 13 : 621 - 630.

Quantin, P. 1969. Note sur la fertilité de quelques sols de Nouvelle - Calédonie. O.R.S.T.O.M./Nouméa, 11 p, multigr.

Rasmussen, G.K., and P.F. Smith. 1961. Effects of Ca, K, Mg, on oxalic, malic and nitric acid content on Valencia orange leaf tissue. Plant Physiol. 36 : 99 - 101.

Rauthina, V.I. 1969. Citrate compounds of nickel. Tr. Dagestan. Sel' skokhoz. Inst. 20, (1), 130 - 136. (Russ).

Reid., P.E. Donaldson, B., Secret, D.W., and B. Bradford. 1970. A simple, rapid isothermal chromatographic procedure for the analysis of monosaccharide mixtures. J. Chromat. 47 : 199.

Reilly, C. 1969. The uptake and accumulation of copper by Becium homblei (de Wild). Duvign and Plancke. New Phytol. 68 : 1081-1087.

Reilly, C., Rowel, J., and J. Stone. 1970. The accumulation and binding of copper in leaf tissue of Becium homblei (de Wild). Duvign. and Plancke. New Phytol., 69 : 993 - 997.

Repp, G. 1963. Die Kupferresistenz des Protoplasmas höherer Pflanzen auf Kupfererzböden. Protoplasma, 57 : 643 - 569.

Rivière, J. 1960. Etude de la rhizosphere du blé. Ann. Agron. 11 : 397 - 440.

Roberts, R.B., Roberts, I., and D.B. Cowie. 1949. Potassium metabolism in Escherichia Coli. II. Metabolism in the presence of carbohydrates and their metabolic derivatives. J. Cellular Comp. Physiol. 34 : 259 - 292.

Robertson, R.N. 1958. The uptake of minerals. In., 'Encyclopedia of Plant Physiology,' Vol. IV, 243 - 279.

Robertson, R.N. 1968. Protons, electrons, phosphorylation and active transport. Cambridge Monogr. Exp. Biol. No. 15. Cambridge Univ. Press, Cambridge. 96 p.

Robinson, W.O., Edington, G., and W.G. Byers. 1935. Chemical studies of infertile soils derived from rocks generally high in magnesium, and generally high in chromium and nickel. U.S. Dept. Agr. Tech. Bull. 471.

Rosenberg, T. 1948. On accumulation and active transport in biological systems. Acta Chem. Scand. 2 : 14 - 33.

Rühling., A., and G. Tyler. 1969. Ecology of heavy metals - a regional and historical study. Bot. Notisier 122 : 248 - 259.

Rune, O. 1953. Plant life on serpentine and related rocks in the north of Sweden. Acta Phytogeogr. suec. 31 : 1 - 139.

Sarosiek, J. 1964. Ecological analysis of some plants growing on serpentine soils in Lower Silesia. Monographiae Bot. 18 : 1 - 105.

Schmid, M. 1974. Végétation et Flore de Nouvelle - Caledonie. Dossier du Pacifique (Societé des Oceanistes). Archives du Centre O.R.S.T.O.M. de Nouméa.

Schmid, W.E., and G.C. Gerloff. 1961. A naturally occurring chelate of iron in xylem exudate. Plant Physiol. 36, 226.

Schramm, R.W. 1973. Problems of organic acid analysis. Bull. Soc. Amis. Sci. Lett. Poznan, Ser. D 14 : 193 - 209. (Eng).

Schroeder, H.A. 1968. The biological trace elements or peripatetics through the periodic table. J. Chron. Dis. 18 : 217 - 228.

Schroeder, H.A., Balassa, J.J., and J.H. Tipton. 1962. Abnormal trace elements in man - nickel. J. Chron. Dis. 15 : 51 - 65.

Schroeder, H.A., and A.P. Nason. 1971. Trace element analysis in clinical chemistry. Clin. Chem. 17 : 461 - 474.

Schulze, G.K. 1938. Morphologisch - systematische Studien über die (Gattung Hybanthus. Mit besonderer Berücksichtigung der südamerikanischen Arten. Bot. Jb. 67 : 437 - 492. (1936).

Schütte, K. 1964. Biology of the Trace elements : their Role in Nutrition. Philadelphia, Pa. J.B. Lippincott. 228p.

Severne, B.C. 1972. Botantical methods for mineral exploration in Western Australia. Ph.D. Thesis, Massey University, Palmerston North, New Zealand.

Severne, B.C. and R.R. Brooks. 1962. A nickel-accumulating plant from Western Australia. Planta 103 : 91 - 94.

Shaw, W.H.R. 1960. Studies in biogeochemistry 1. A biogeochemical periodic table. The Data. Geochim. Cosmochim. Acta. 19 : 196-215.

Sillen, L.G. and A.E. Martell. 1964. Stability constants of metal ion complexes. 2nd ed. Special publication No. 17. Chemical Society, London. 754p.

Sleumer, H. 1954. Flacourtiaceae p. 1 - 106. In Flora Malesiana. Ser. I. 5, Ed C.G.G.J. van Steenis.

Sleumer, H. 1972. Addenda, corrigenda et emendanda : Flacourtiaceae. Flora Malesiana. Ser. I. 6 (6), 943 - 944 (Eng).

Sleumer, H. 1974. A concise revision of the Flacourtiaceae of New Caledonia and the Loyalty Islands. Blumea 22 : 123 - 147.

Smith, R.A.H., and A.D. Bradshaw. 1970. The reclamation of toxic metalliferous waste. Nature, Lond. 277 : 376 - 377.

Soane, B.D. and D.H. Saunder. 1959. Nickel and chromium toxicity of serpentine soils in Southern Rhodesia. Soil Sci. 88 : 322 - 330.

Soderstrom. T.R. 1962. The isocitric acid content of crassulacean plants and a few succulent species from other families. Am. J. Bot. 49 : 850.

Spence, D.H.N. 1957. Studies on the vegetation of Shetland. I. The serpentine debris in Unst. J. Ecol. 45 : 917 - 945.

Steveninck van R.F.M. 1966. Some metabolic implications of the tris effect in beetroot tissue. Aust. J. Biol. Sci. 19 : 271 - 281.

Stewart, I. 1963. Chelation in the absorption and translocation of mineral elements. Ann. Rev. Plant Physiol. 14 : 295 - 310.

Sunderman, F.W., Decsy, M.I., and M.D. McNeely. 1972. Nickel metabolism in health and disease. Ann. N.Y. Acad. Sci. 199 : 300 - 312.

Sunderman, F.W., Nomoto, S., Morang, R., Mechay, M.W., Burke, C.N., and S.W. Neilsen. 1972a. Nickel deprivation in chicks. J. Nutr. 102 : 259 - 267.

Sweeley, C.C., Bentley, R., Makita, M., and W.W. Wells. 1963. Gas-liquid chromatography of trimethylsilyl derivatives of sugars and related substances. J. Am. Chem. Soc. 83 : 2497 - 2507.

Takagi, S. 1976. Naturally-occurring iron-chelating compounds in oat and rice-root washings. Soil Sci. Plant Nutr. 22 , 423 - 433.

Thompson, J.F., and L.O. Tiffin. 1974. Fractionation of nickel complexing compounds of peanut seeds with ion-exchange resins. Plant. Physiol. Suppl. p. 23.

Tiffin, L.O. 1966. Iron translocation II. Citrate/Iron ratios in plant stem exudates. Plant Physiol. 41 : 515 - 518.

Tiffin, L.O. 1967. Translocation of manganese, iron, cobalt and zinc in tomato. Plant Physiol. 42 : 1427 - 1432.

Tiffin, L.O. 1970. Translocation of iron citrate and phosphorus in xylem exudate of soybean. Plant Physiol. 45 : 280 - 283.

Tiffin, L.O. 1971. Translocation of nickel in xylem exudate of plants. Plant Physiol. 48 : 273 - 277.

Tiffin, L.O. 1972. Translocation of micronutrients in plants. In Micronutrients in Agriculture, pp. 199 - 229, eds., Mortvedt, J. J., Giordano, P.M., Lindsay, W.L. Soil Sci. Soc. Amer. Madison.

Tiffin, L.O., and J.C. Brown. 1962. Iron chelates in soybean exudates. Science 135 : 311 - 313.

Tiffin, L.O. and J.F. Thompson. 1974. Electrophoretic study of nickel complexing by organic carriers in peanut. Plant Physiol. Suppl. p. 22.

Timberlake, C.F. 1964. Iron - malate and Iron - citrate complexes. J. Chem. Soc. 5078 - 5085.

Timperley, M.H. 1971. Biogeochemical studies of nickel and copper in New Zealand. Ph. D. Thesis, Massey University, Palmerston North New Zealand.

Torii, K., and G.G. Laties. 1966. Organic acid synthesis in response to excess cation absorption in vacuolate and non-vacuolate sections of corn and barley roots. Plant Cell Physiol. 7 : 395 - 403.

Tsitovich, I.K., and N.G. Nikitina. 1963. Complex formation of the transition elements of the fourth period in citric solutions. Isv. Vysshikh. Uchebn. Zavedenii, Khim. ikhim. Tekhnol. 6 (4) 507 - 571.

Turekian, K.K. 1963. The chromium and nickel distribution in basaltic rocks and eclogites. Geochim. et. Cosmochim. Acta, 27 : 835 - 846.

Turner, R.G. 1970. The sub cellular distribution of zinc and copper within the roots of metal-tolerant clones of Agrostis tenuis Sibth. New Phytol. 69 : 725 - 731.

Tyler, G. 1970. Moss analysis - a method for surveying heavy metal deposition. 2nd International Clean Air Conference Proceedings. Washington, D.C., 129 - 132.

Ulrich, A. 1941. Metabolism of non-volatile organic acids in excised barley roots as related to cation-anion balance during salt accumulation. Amer. J. Bot. 28 : 526 - 537.

Vančura, V. 1964. Root exudates of plants. I. Analysis of root exudates of barley and wheat in their initial phases of growth. Plant Soil, 21 : 231 - 248.

Vančura, V., and A. Hovadik. 1965. Root exudates of plants II. Composition of root exudates of some vegetables. Plant Soil 22 : 21 - 32.

Van Goor, B.J., and D. Wiersma. 1976. Chemical forms of manganese and zinc in phloem exudates. Physiol. Plant, 36 : 213 - 216.

Vanselow, A.P. 1951. Micro-element research with citrus. Citrograph 37 (2), 77 - 80.

Vanselow, A.P. 1966. Nickel, pp. 302 - 309. In H.D. Chapman, ed. Diagnostic Criteria for Plants and Soils. Riverside University of California, Division of Agricultural Science.

Vergnano, O. 1953. Action of nickel on plants in serpentine soils. Nuovo giorn botan. ital. 60 : 109 - 183.

Vergnano-Gambi, O. 1967. Primi dati sulla localizzazione istologica del nickel in Alyssum bertolonii Desv., Giornale Botanico Italiano, 101 (1) : 59-60.

Walker, R.B. 1954. The ecology of serpentine soils. III. Factors affecting plant growth in serpentine soils. Ecology, 35 : 359 - 366.

Wallace, A. 1963. Solute uptake by intact plants. Arthur Wallace, Los Angeles, p 142 - 146.

Walser, M. 1961. Ion-association V. Dissociation constants for complexes of citrate with sodium, potassium, calcium and magnesium ions. J. Phys. Chem. 65 : 159 - 161.

Whittaker, G.H. 1954. The ecology of serpentine soils IV. The vegetational response to serpentine soils. Ecology 35 (2), 275 - 288.

Wilkins, D.A. 1957. A Technique for the measurement of lead tolerance in plants. Nature, Lond. 180 : 37 - 38.

Wild, H. 1965. The flora of the Great Dyke of S. Rhodesia with special reference to serpentine soils. Kirkia 5 (1) : 49 - 86.

Wild, H. 1971. The taxonomy, ecology and possible method of evolution of a new metalliferous species of Dicoma Cass. (Compositae) Mitt. Bot. St. Samml., Münch. 10 : 266 - 274.

Wild, H. 1974. Indigenous plants and chromium in Rhodesia. Kirkia, 9 : 233 - 241.

Williams, R.J.P. 1959. Co-ordination, chelation, and catalysis p 391 - 441. In P.D. Boyer, H. Hardy, and K. Myrback (eds) The Enzymes (2nd ed.) Academic Press, N.Y.

Willis, J.C. 1973. A Dictionary of the Flowering Plants and Ferns. 8th Edition. Cambridge.

Wilson, S.B., and D.J.D. Nicholas. 1967. A cobalt requirement for non-nodulated legumes and for wheat. Phytochemistry 6 : 1057 - 1067.

Wither, E.D., and R.R. Brooks. 1977. Hyperaccumulation of nickel by some plants of S.E. Asia. J. Geochem. Explor. (in press).

Wu, L., and J. Antonovics. 1976. Experimental ecological genetics in Plantago. II. Lead tolerance in Plantago lanceolata and Cynodon dactylon from a roadside. Ecology, 57 : 205 - 208.

Wyn-Jones, R.S., Sutcliffe, M., and C. Marshall. 1971. Physiological and biochemical basis for heavy metal tolerance in clones of Agrostis tenuis pp575 - 591. In 'Recent Advances in Plant Nutrition' Vol. 2.

Appendix I. List of co-operating herbaria.

	Country.	Institution.	Address.
1.	Argentina	Fundacion Miguel Lillo	San Miguel de Tucuman
2.	Argentina	Museo de Ciencias Naturales	Buenos Aires
3.	Australia	Herbarium Australiense, C.S.I.R.O.	Canberra
4.	Brazil	Inst. de Pesquisa Agropecuaria	Belem
5.	Brazil	Museo Nacional, Universidade Federal	Rio de Janeiro
6.	France	Museum National d' Histoire Naturelle	Paris F75005
7.	Germany	Bot. Garten und Museum	Berlin-Dahlem
8.	Germany	Herbarium Haussknecht	Jena (DDR)
9.	India	Banaras Hindu Univ.	Varanasi 5
10.	India	Blattner Herbarium, St. Xaviers College	Bombay
11.	India	Central National Herbarium	Howrah 3
12.	India	Central Circle, Bot. Survey India	Allahabad
13.	India	Eastern Circle, Bot. Survey India	Shillong 3
14.	India	Southern Circle, Bot. Survey India	Coimbatore
15.	Indonesia	Herbarium Bogoriense	Bogor
16	Netherlands	Rijksherbarium	Leiden
17.	New Caledonia	O.R.S.T.O.M.	Noumea
18.	Philippines	Pambansang Museo	Manila
19.	Portugal	Centro Botanico	Lisbon 3
20.	Portugal	Instituto Botanico, Univ. of Coimbra	Coimbra
21.	Portugal	Instituto Botanico, Univ. of Lisboa	Lisbon 2
22.	South Africa	Bolus Herbarium, Univ. of Cape Town	Cape Town

continued...

Appendix I. continued....

23.	South Africa	Botanical Res. Inst. D.S.I.R.	Durban
24.	South Africa	Compton Herbarium, Kirstenbosch	Cape Town
25.	South Africa	National Herbarium	Pretoria
26.	Sweden	Inst. Systematic Bot. Univ. of Uppsala	Uppsala
27	Switzerland	Bot. Garten, Univ. of Zurich	Zurich
28.	United Kingdom	Royal Botanical Gardens	Kew
29.	United Kingdom	Royal Botanical Garden	Edinburgh
30.	United States	Dept. of Botany, Univ. of California	Berkeley, Calif.
31.	United States	Field Museum of Natural History	Chicago, Ill
32.	United States	Missouri Botanic Gardens	St. Louis, Mo.
33.	United States	Academy of Natural Sciences	Philadelphia, Pa.
34.	United States	New York Botanical Garden	Bronx, N.Y.
35.	United States	Univ. of Missouri Herbarium	Columbia, Mo.

Appendix II. Data for nickel and cobalt in Homalium plants containing more than 15 µg/g nickel (Dry weight).

Species	No.	Ni (<15µg) Mean	Ni (<15µg) Range	Co Mean	Co Range	Anomalous values. Ni	Ni/Co	Location
abdessammadi	13	3.1	0.7-6.7	10	0.2-44	19	1.6	Sudan
africanum	18	4.3	0.2-13	12	1.8-46	16	-	Cameroons
						21	1.2	"
angustifolium	5	9.2	6.6-12			21	3.7	Sierre Leone
						37	1.0	" "
						155	1.6	" "
austrocaledonicum	6	anomalous values only				432	10	New Caledonia
						561	8.9	" "
						1049	124	" "
						1075	7.9	" "
						1680	-	" "
						1805	-	" "
buchholzii	6	5.6	2.9-11	12	2.9-38	16	0.3	Cameroons.
circumpinnatum	3	4.3	3.6-5.0	1.4	-	25	0.9	Queensland
decurrens	5	anomalous values only				42	1.8	New Caledonia
						42	6.7	" "
						71	12	" "
						87	-	" "
						176	6.8	" "
dentatum	18	3.9	0.8-11	7.4	1.0-31	44	-	Rhodesia

continued...

Appendix II. continued...

Species	No.	Ni (<15µg) Mean	Ni (<15µg) Range	Co Mean	Co Range	Anomalous values. Ni	Ni/Co	Location
deplanchei	10	11	-	10	-	28	-	New Caledonia
						88	10	" "
						140	15	" "
						170	11	" "
						408	-	" "
						538	48	" "
						764	-	" "
						1226	40	" "
						1850	-	" "
ealaense	2	14	-	-	-	18	1.5	Zaire
foetidum	19	4.3	0.7-13	4.3	0.7-11	20	3.8	New Guinea
francii	7	anomalous values only				1502	100	New Caledonia
						4316	-	" "
						4469	-	" "
						4900	-	" "
						10420	-	" "
						11172	5.7	" "
						14500	381	" "
gitingense	1					144	144	Philippines
guillainii	2					2260	251	New Caledonia
						6926	161	" "

continued...

Appendix II. continued...

Species	No.	Ni (<15µg) Mean	Range	Mean	Range	Anomalous values. Ni	Ni/Co	Location
intermedium	2	0.5	-	2.3	-	17	3.1	New Caledonia
juxtapositum	1	anomalous value only				32	2.0	" "
kanaliense	6	anomalous value only				845	37	" "
						3760	-	" "
						4380	151	" "
						7079	90	" "
						7854	314	" "
						9420	-	" "
le-ratiorum	6	anomalous value only				23	1.2	" "
						26	0.5	" "
						40	3.2	" "
						149	40	" "
						153	14	" "
						643	17	" "
longifolium	4	5.0	0.7-11	9.5	2-17	61	-	Malaya
longistylum	10	5.0	0.8-99	8.9	1.6-22	18	0.1	Zaire
mathieuanum	2	anomalous value only				22	1.5	New Caledonia
						1694	33	" "
mollissimum	5	4.3	1.6-77	17	3.0-31	19	2.1	Hainan
multiflorum	3	2.1	1.6-2.5	0.6	0.3-1.0	41	6.0	Philippines

continued...

Appendix II. continued....

Species	No.	Ni (< 15µg) Mean	Range	Mean	Range	Anomalous values. Ni	Ni/Co	Location
nepalense	4	2.3	1.2-3.3	8.3	-	20	16	India
panayanum	10	4.4	1.2-8.3	4.8	1.2-12	39	1.7	Borneo
pleiandrum	3	1.0	-	1.0		112	22	Puerto Rico
						343	70	" "
racemosum	59	3.4	0.3-14	3.2	0.1-11	19	2.3	" "
						23	18	" "
rivulare	2	4.4	-	33	-	52	2.0	New Caledonia
rubiginosum	1	anomalous value only				397	100	" "
rubrocostatum	2	anomalous value only				476	77	" "
						1157	214	" "
samarense	4	2.5	0.7-43	5.6	2.5-8.6	23	23	Philippines
serratum	6	10	10-11	1.1	10-12	19	38	New Caledonia
						35	-	" "
						88	4.0	" "
						116	12	" "
travancoricum	2	7.5	-	30	-	49	2.0	India
villarianum	4	4.4	30-5.4	8	4-10	17	2.1	Philippines
viridiflorum	1	anomalous value only				17	.8	Congo
vitiense	9	2.6	0.5-5.4	.75	0.5-1.0	17	0.5	Fiji
						20	0.8	"
						33	10	"

continued...

Appendix III. Data for nickel and cobalt in Hybanthus plants containing more than 15 µg/g nickel (Dry weight).

		Ni (< 15µg)		Co		Anomalous values.		
Species	No.	Mean	Range	Mean	Range	Ni	Ni/Co	Location
austrocaledonicus	4	anomalous values only				8891	171	New Caledonia
						10138	274	" "
						13600	680	" "
						13750	371	" "
brevilabris	4	17	13-21	-	-	57	0.9	W. Australia
						229	0.6	" "
caledonicus	11	43	2.0-8.1	3.8	0.7-5.4	40	40	New Caledonia
						68	2.7	" "
						405	101	" "
						4635	22	" "
						8338	124	" "
						5917	204	" "
communis	32	3.3	0.5-8.2	1.1	0.5-2.2	18	18	Paraguay
concolor	33	3.9	0.5-9.1	0.6	0.1-1.5	17	-	U.S.A.
						29	-	"
						38	126	"
enneaspermus	97	5.7	0.2-14	4.8	0.4-28	16	1.6	Zaire
						17	2.2	N.Terr. Australia
						24	13	Cameroons

continued...

Appendix III Continued....

Species	No.	Ni (< 15µg) Mean	Ni (< 15µg) Range	Co Mean	Co Range	Anomalous values Ni	Anomalous values Ni/Co	Anomalous values Location
enneaspermus continued..						24	10	Cameroons
						25	33	Rhodesia
						61	-	Philippines
						99	33	Ruanda Urundi
floribundus	13	8.8	7.5-10	1.3	1.2-1.5	34	9.1	S. Australia
						42	1.0	" "
						43	0.4	" "
						76	1.5	W. Australia
						89	2.5	" "
						240	2.5	" "
						290	0.3	S. Australia
						485	1.1	W. Australia
						1058	11	" "
						6680	74	" "
glabor	2	1	-	0.1	-	16	26	Guatemala
glutinosus	8.	3.9	1-7.2	1.6	1-4	40	50	Paraguay
havanensis	11	4.8	0.1-8.5	0.9	0.1-1.6	34	5.1	Cuba
ipepecuanha	24	4.2	0.5-13	1.3	0.2-2.5	21	-	Guyana
latifolius	1	anomalous value only				48	2.1	New Caledonia
linearifolius	11	4.7	0.7-8.1	1.5	0.5-2.5	22	27	Cuba

continued...

Appendix III Continued...

		Ni (< 15µg)				Anomalous values		
Species	No	Mean	Range	Mean	Range	Ni	Ni/Co	Location
longipes	1	anomalus value only				17	-	Mexico
oppositifolius	22	4.6	0.7-12	3.7	0.7-8.0	16	-	Brazil
						19		"
						20		"
parietariifolius	3	4.1	0.6-7.6	2.6	10-30	21	26	Mexico
parviflorus	33	3.9	0.3-12	1.6	0.4-6.0	62	-	Columbia
prunifolius	13	4.0	0.9-11	1.7	0.6-3.0	25	16	Venezuela
serrulatus	3	5.4	3.5-7.2	-	-	18	30	Mexico
setigerus	3	4.3	2.9-5.7	1.0	-	130	100	Brazil
		anomalous value only				16	16	Brazil
thiemei	7	1.8	1.0-3.3	-	-	21	3.0	Honduras
wrightii	2	anomalous value only				35	-	Cuba
						350	7.0	"
yucatanensis	12	3.6	0.2-7.7	2.0	1.0-2.6	134	268	Mexico

Appendix IV.

Important and characteristic $^{m}/e$ values in the mass spectra of the trimethysilyl ether of some organic acids

$^{m}/e$	relative intensity	$^{m}/e$	relative intensity
Quinic acid - 4TMS (552)		Unknown (B)	(450)
537	1.00	435	0.24
462	0.44	393	0.07
447	0.27	345	0.48
435	2.11	333	0.16
423	0.56	319	0.43
419	0.94	305	0.72
372	0.88	291	0.47
347	4.44	255	0.29
346	10.00	243	0.55
345	31.11	231	0.86
335	1.01	217	7.92
334	3.33	206	3.96
305	0.89	205	9.00
292	2.55	204	48.60
256	2.66	192	3.36
255	11.11	191	19.80
243	1.11	147	16.56
231	0.88	129	5.40
217	2.00	117	5.40
205	1.55	103	5.04
204	5.11	73	100.00
191	5.00		
147	22.22		
133	3.00		
131	2.00		
129	2.00		
117	0.77		
73	100.00		

Appendix IV. continued...

m/e	relative intensity	m/e	intensity
Citric acid - 4TMS (480)		Malic acid - 3TMS (350)	
465	1.86	335	2.52
375	2.17	319	0.31
363	2.79	307	0.95
347	1.55	306	0.70
333	0.31	305	0.36
319	0.53	265	1.12
307	0.21	263	0.62
305	0.71	245	5.04
291	0.18	233	10.08
285	0.18	221	0.63
273	8.68	217	1.68
257	0.39	191	2.52
245	0.14	190	4.20
231	1.24	189	4.62
221	0.93	175	3.36
217	0.93	171	1.26
215	0.43	147	35.70
211	0.43	133	5.04
183	0.51	117	2.52
147	13.33	101	4.83
133	2.48	73	100.00
131	1.55		
117	1.24		
75	13.64		
73	100.00		

Appendix IV. continued....

m/e	relative intensity	m/e	relative intensity
Unknown (A) (364)		Malonic acid - 2TMS (248)	
364	0.19	248	1.26
349	1.71	233	8.19
336	0.95	217	0.38
335	2.09	204	0.46
334	7.41	189	0.75
319	0.95	173	0.50
292	0.19	147	151.20
259	0.95	143	2.52
245	3.80	133	5.04
244	1.14	131	4.41
231	1.90	117	1.89
221	0.95	116	0.95
217	2.47	115	1.58
205	0.76	109	1.58
204	0.76	101	2.52
203	0.95	99	6.30
191	1.33	75	50.40
189	2.47	73	100.00
179	0.57		
157	1.33		
147	19.00		
133	3.61		
129	2.85		
117	2.66		
103	15.20		
73	100.00		

PUBLICATIONS ARISING FROM THIS THESIS.

Brooks, R.R., Lee, J., Reeves, R.D. and Jaffré, T.
Detection of nickeliferous rocks by analysis of herbarium specimens of indicator plants.
J. Geochem. Explor. 7, 49-57 (1977).

Lee, J., Brooks, R.R., Reeves, R.D., Boswell, C.R. and Jaffré, T.
Plant-soil relationships in a New Caledonian serpentine flora.
Plant and Soil 46 675-680 (1977).

Lee, J., Brooks, R.R., Reeves, R.D. and Jaffré, T.
A chromium accumulating bryophyte from New Caledonia.
The Bryologist. 80, 203 (1977)

Lee, J., Reeves, R.D., Brooks, R.R. and Jaffré, T.
Isolation and identification of a citrato-complex of nickel from nickel accumulating plants.
Phytochemistry (in press). 16(10), 1503-1505 (1977)

Jaffré, T., Brooks, R.R., Lee, J. and Reeves, R.D.
Sebertia acuminata: A hyperaccumulator of nickel from New Caledonia.
Science 193, 579-580 (1976).