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Speciation of nickel by HPLC-UV/MS in pea nodules

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ABSTRACT

A new and sensitive methodology based on normal phase HPLC has been developed for the speciation of nickel in low-complexity plant extracts. The method combines a silica stationary phase column, a 9:1 (v/v) hexane:ethanol mixture as mobile phase, and the detection of nickel complexes by either UV or MS. The developed methodology was applied to the speciation of nickel complexes in the cytoplasm of pea root nodules. Results obtained indicate that nickel citrate and nickel malate accounts for 99% of nickel present in pea nodule cytoplasm fraction. The low detection limit of the method (<0.2 nM) enables nickel speciation in non-hyperaccumulator plants.

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1. Introduction

Different transition metals, such as nickel, cobalt, and zinc are essential micronutrients for plants as constituents of different enzymes. However, these elements also exhibit a high degree of toxicity even at moderate concentrations. Inside living cells, metal ions are not present in free form, but bound to organic ligands. Several compounds, such as organic acids, histidine, glutathione (GSH) and nicotianamine have been reported as potential ligands for transition metals in plants [1]. Speciation of these metal ions is a critical issue affecting their bioavailability and toxicity. Organic acids (i.e. citric acid, malic acid) have been associated with metal hyperaccumulation and tolerance in a range of plant species and have been proposed as important cellular ligands for Zn^{2+}, Cd^{2+} and Ni^{2+} [2,3]. By complexing free metal ions, these organic acids are involved in metal homeostasis, transport and sequestration [1].

In the case of plant–microbe endosymbiosis such as the *Rhizobium*-legume association, there is a higher level of complexity in the provision of metals for bacterial metalloenzyme synthesis. In this association the bacteria, modified into bacteroids to fix nitrogen inside the root nodules, are fully dependent on the plant cytoplasm content as source for macro and microelements. Synthesis of nitrogenase by bacteroids is one of the major sinks of iron for legume endosymbiotic bacteria, and depends on the transport of this metal complexed with citrate across the peribacteroid membrane [4]. In the case of *Rhizobium leguminosarum* UPM791/*Pisum sativum* symbiosis, a bacterial [NiFe] hydrogenase is co-induced with nitrogenase [5]. In this symbiosis, the concentration of nickel in the plant nutrient solution is a limiting factor for hydrogenase expression, and provision of high amounts of this element to the plant nutrient solution is required to ensure optimal levels of enzyme synthesis [6]. In that system, the potential diversity of nickel complexes in nodule cytosol from different legume species has been proposed to mediate the marked host effect on hydrogenase expression [7]. However, the information about the mechanisms mediating nickel provision for endosymbiotic bacterial cells is scarce, and requires the development of efficient and sensitive methodologies for the identification of nickel ligands in the cytoplasm of non-accumulator plants.

Different analytical procedures have been described in the literature for the quantification of nickel complexes in plant samples. The most common procedure used for metal speciation in plants requires its indirect determination after the hydrolysis of metallic complexes in a highly acidic media (pH < 1). This methodology, initially developed by Tolrà et al. [8] for zinc speciation, has been successfully applied to nickel speciation in hyperaccumulator plants [9,10]. For this purpose, total nickel is analysed by means of ICP-MS, while ligands are quantified by reversed phase chromatography with a H_3PO_4 mobile phase and UV detection. The main disadvantage of this methodology is its lack of selectivity, causing overestimation of nickel complexes due to hydrolysis of other metallic complexes.

Chromatographical methods based on LC–MS and GC–MS profiling have been applied to the speciation of nickel complexes in the hyperaccumulator plant *Sebertia acuminata* [11]. In this analy-

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sis nickel citrate was identified as the predominant Ni-containing species. Other approaches based on hyphenated techniques including size exclusion, HPLC and ICP-AES failed to separate citrate and malate nickel complexes [12].

Alternative approaches based on hydrophilic interaction chromatography (HILIC) have been applied to the direct separation of nickel complexes in plant samples [13,14]. Zwitterionic-type HILIC columns (i.e. sulfobetaine and phosphorylcholine) [13] either alone or in combination with size exclusion chromatography [14] were used for the separation of the different nickel complexes present in *Arabidopsis thaliana* xylem sap samples [13] or in *Thlaspi caerulescens* [14]. However, the detection limits obtained by these methodologies were not low enough to accurately quantify the amount of nickel complexes, except for nickel-nicotianamine.

The mechanisms involved in the separation of highly polar metallic complexes by different sorbents have been thoroughly evaluated by means of thin layer chromatography. Silica or polyacrylonitrile-based stationary phases have proven suitable for the separation of Co(III) or Ni(II) xanthates [15,16], Zn(II) dithiophosphates [17], or Co(III), Cr(III) and Ru(III) diketonates [18].

Considering that most plants are not nickel-accumulators, alternative direct speciation methodologies are required for the identification and quantification of nickel complexes when present in low amounts. Accordingly, the main aim of the present work is the development of a new analytical methodology for the direct identification and quantification of nickel complexes in low-complexity extracts of non-accumulator plants, as a tool to study the mechanism involved in the nickel provision for endosymbiotic bacterial cells. The normal phase liquid chromatography methodology set up performs fast and sensitive separation of nickel-organic complexes, and has been applied to the detection and quantification of such complexes in pea nodule cytosolic extracts.

2. Materials and methods

2.1. Chemicals

HPLC grade solvents (hexane, toluene, methanol, ethanol and n-propanol) were purchased from Scharlab. Citric and malic acids were purchased from Sigma–Aldrich. Nitric and phosphoric acids were purchased from Merck (Merck KGaA, Darmstadt, Germany). Nitric acid was purified by subboiling point distillation prior to use. Reagents KCl, KH_2PO_4, $MgSO_4{\cdot}7H_2O$, $MgSO_4{\cdot}7H_2O$, $MgSO_4{\cdot}7H_2O$, $ZnSO_4{\cdot}5H_2O$, $CuSO_4{\cdot}5H_2O$ and $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ and $NiCl_2{\cdot}6H_2O$ were of analytical grade and were supplied by Merck KGaA (Darmstadt, Germany), whereas Fe(III) citrate and H_3BO_3 were provided by Sigma–Aldrich (St Louis, MO). Nickel citrate and nickel malate complexes were prepared in the laboratory using a 10-fold excess of the corresponding ligands.

All other reagents used for bacterial or plant growth were of analytical grade, and were used without any further purification step.

2.2. Instrumentation

HPLC-UV measurements were performed in an HPLC system from Thermo Fischer Scientific consisting of a Constametric 4100 Series high pressure pump, a Spectromonitor 5000 diode array detector, and a Rheodyne 7100 injection valve, equipped with a 20 μL loop.

HPLC-MS measurements were performed in an Agilent 1100 HPLC system, consisting of an autosampler, a high pressure gradient HPLC pump, coupled to an Agilent 6120B electrospray ionization interface operating in the negative mode and a quadrupole MS detector.

Total nickel was analysed by means of a ICP-MS Thermo X-Series (Thermo Electron, Windsford, Cheshire, UK). Radiofrequency power was set at 1250 W. Plasma, auxiliary and carrier gas flows were set at 15.0, 0.73 and 0.7 L/min, respectively. ^{58}Ni, ^{60}Ni, ^{61}Ni and ^{62}Ni were monitored for total nickel analysis experiments. A Meinhar nebulisation was used with a double pass spray chamber. Sampler and skimmer cones used were made of platinum. Data have been acquired in the single ion monitoring (SIM) mode, being the dwell time of 100 ms.

2.3. Plant and bacterial growth conditions

Pea (*P. sativum* L. cv. Frisson) seeds were surface-sterilized and germinated on 1% water-agar plates at 28 °C in darkness. After 3 days, pea seedlings were planted in Leonard jar-type assemblies under bacteriologically controlled conditions [19]. Each seed was inoculated with 1 ml of a 2×10^8 c.f.u./ml *R. leguminosarum* bv. *viciae* SPF25 culture [20]. Strain SPF25 was grown to stationary phase at 28 °C in yeast-mannitol medium [21]. Plants were grown in a controlled environmental chamber with a 16/8 h light–dark cycle, 25/21 °C day–night temperature and a light intensity of 25 klx supplied by fluorescent and incandescent lamps. Plants were maintained in vermiculite as substrate with a nitrogen-free plant nutrient solution containing (per liter) 1 mmol KCl; 1.29 mmol KH_2PO_4; 1 mmol $MgSO_4{\cdot}7H_2O$; 2.53 mmol $CaSO_4$; 6.8 μmol Fe(III) citrate; 3.6 μmol $MnSO_4{\cdot}7H_2O$; 11.3 μmol H_3BO_3; 0.44 μmol $ZnSO_4{\cdot}5H_2O$; 0.16 μmol $CuSO_4{\cdot}5H_2O$ and 4.04 nmol $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$. When indicated, the standard nutrient solution was supplemented with different amounts of $NiCl_2$ (42.5, 85, and 170 μM) from day 10 after seedling inoculation [19].

2.4. Nodule cytosol fractions and bacteroid cells preparation

Nodule cytosol fractions and bacteroid cells were obtained from root nodules of 21 day-old plants. After excision, a fraction of 0.5 g of pea nodules collected from three plants was crushed in 7 ml of Dixon buffer (250 mM K_2HPO_4, 25 mM KH_2PO_4, 2.5 mM $MgCl_2$ at pH 7.0) with 0.17 g of polyvinylpolypyrrolidone (PVPP) to remove plant polyphenolic compounds. Extracts were sequentially centrifuged three times at $120 \times g$ for 1 min to discard plant material. Bacteroid cells were removed from nodule cytosol by centrifugation at $25{,}000 \times g$ for 10 min. The soluble fraction containing nodule cytosol was further clarified by passing through a 0.22 μm filter (Millipore, Bedford, USA).

2.5. HPLC analysis of β-hydroxyacids

Chromatographic separation of β-hydroxyacids was achieved by means of reversed phase liquid chromatography, using a Kromasil C_{18} column (250 mm × 4.6 mm, 5 μm, Scharlab) and a H_3PO_4 40 mM (pH 2.2) solution as mobile phase at a flow rate of 1 mL/min. Citric and malic acid contents in the samples were quantified by external calibration at 210 nm.

2.6. Nickel speciation

Nickel speciation was carried out by means of normal phase chromatography, using a silica HPLC column (Kromasil 100 Silica, 250 mm × 4.6 mm, 5 μm, Sigma–Aldrich) as stationary phase. Nickel compounds were best resolved in the chromatographic system with an hexane:ethanol (9:1, v/v) mobile phase at a flow rate of 1 mL/min, and monitored at both 240 and 302 nm (UV experiments) or at m/z 325 and 441 (MS experiments). Under these chromatographic conditions, nickel citrate and nickel malate eluted at 6.2 and 7.4 min, respectively. Such complexes were identified in the root nodule extracts by their retention times and their UV and MS

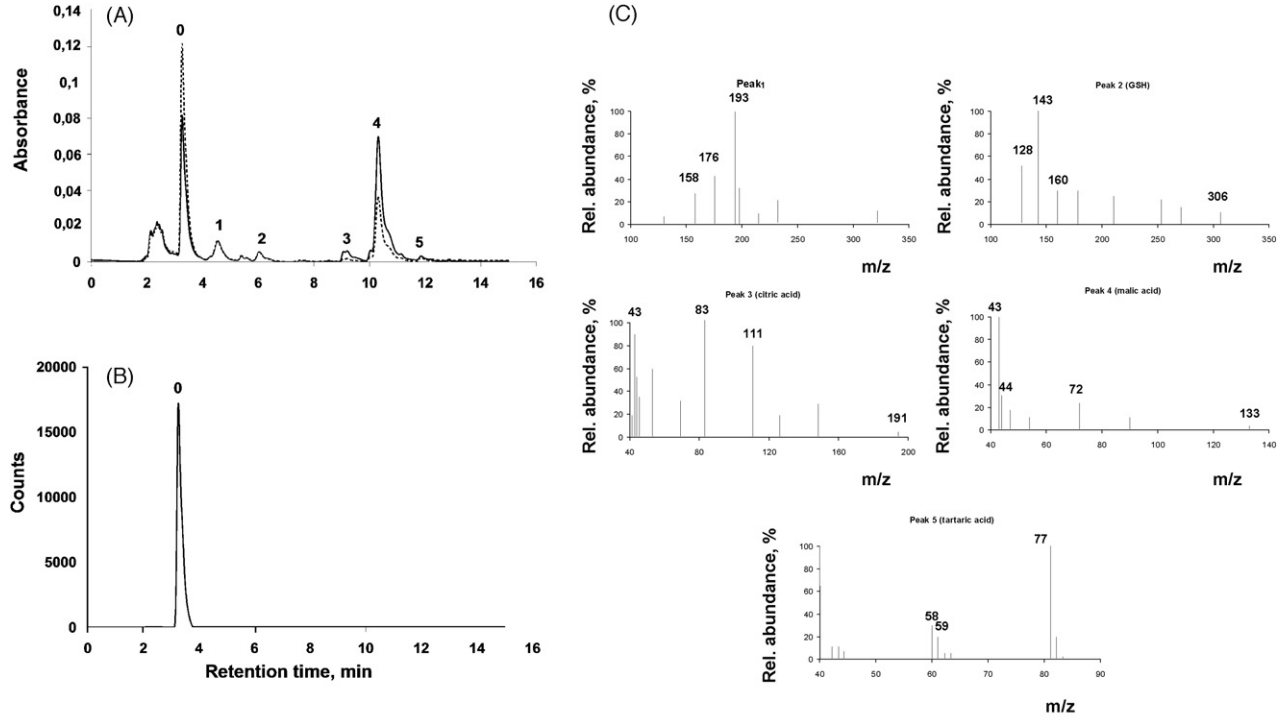

Fig. 1. RPLC analysis of nodule cytosolic fractions. (A) Chromatograms obtained for the UV monitorization (210 nm) of ligands before (straight lines) and after (dotted lines) incubation of the sample with 0.5 mM Ni^{2+}. (B) Chromatogram of the same sample as monitored by ICP-MS (*m*/*z* 58). (C) Electrospray mass spectra of fractions corresponding to peaks 1–5 in panel A. Peaks (identified by MS spectra) corresponded to GSH (2), citric acid (3), malic acid (4), and tartaric acid (5). Accurate identification of peak 1 was not possible. *m*/*z* values are shown for relevant peaks. Samples were obtained from pea plants grown on 42 μM $NiCl_2$. Chromatographic conditions: reversed phase chromatography on C18 (250 mm × 4.6 mm, 5 μm). Mobile phase: phosphoric acid 40 mM (pH 2.2). Mass spectra were recorded in the negative mode.

spectra. Their content in the samples were quantified by external calibration, as no matrix effects were detected.

3. Results and discussion

3.1. Optimization of the chromatographic separation of nickel complexes

Preliminary analysis of the different components present in the nodule cytosol extracts from plants grown with Ni supplementation (42.5 μM) was carried out using reversed phase chromatography (RPLC) in conditions described in Section 2.5. The resulting chromatograms, measured by UV (210 nm), contained five peaks (Fig. 1A). Searching of NIST MS spectra library allowed the identification of compounds corresponding to 4 out of the 5 peaks (Fig. 1C). Peaks appearing at 6.1, 9.2, 10.3 and 11.9 min were identified as GSH, citric acid, malic acid and tartaric acid, respectively, as major compounds, whereas peak 1 spectrum partially corresponded to homo-GSH, with some additional bands that suggest the presence of additional, co-eluting compounds. The identity of the compounds associated to peaks 2–5 was confirmed by spiking the extract with purified compounds (data not shown). ICP-MS data (*m*/*z* 58) indicated that, under these conditions, all Ni-containing compounds co-eluted within the column void volume (Fig. 1B).

In order to evaluate which of the identified species was able of chelating nickel, the nodule cytosol fraction of plants grown in the presence of nickel (42.5 μM) was incubated with 0.5 mM nickel chloride for 2 h, and the elution profile was compared to that of the original sample (Fig. 1A). In order to avoid interconversion of the Krebs' cycle acids, incubation with nickel was carried out in the dark at −18 °C. Incubation with Ni resulted in a significant increase of the signal in the dead volume (peak 0), along with a decrease of peaks corresponding to citric and malic acids (Fig. 1A). The rest of the peaks did not suffer significant alterations. Similar results were obtained with cytosol fractions from plants not supplemented with nickel (data not shown). These results suggest that citrate and malate are the preferred nickel ligands in the cytosol of pea nodules. Incubation experiments with pure compounds under the same chromatographic conditions used (40 mM phosphoric acid, pH 2.2) showed that nickel citrate and nickel malate were stable for at least 30 min in these conditions (data not shown), so these complexes could be present in the fraction corresponding to void volume in Fig. 1.

Since all nickel species present in the samples co-eluted in the void volume, a different chromatographic procedure should be employed in order to separate the different nickel species. An alternative approach based on normal phase liquid chromatography using a silica-based column as stationary phase was tested. As malate and citrate nickel complexes were expected to be the main ligands in nodule cytosol extracts, different mobile phases were tested in order to allow baseline separation of nickel citrate and nickel malate complexes in the lowest analysis time possible. To this aim, mobile phases consisting of mixtures of apolar solvents (hexane, toluene) with different ratios of alcohols (n-propanol, ethanol and methanol) have been evaluated. Fig. 2A shows the chromatographic resolution between nickel citrate and nickel malate for each mobile phase used. As it can be observed, methanol-based mobile phases were not able of baseline separating both complexes and were thus discarded. In contrast, mobile phases containing up to 10% ethanol or 35% n-propanol showed resolution values above 1.5, thus ensuring baseline separation.

Once the separation of both complexes was ensured, the retention times obtained under the different mobile phases were evaluated in order to minimize the duration of the chromatographic run (Fig. 2B). As it can be observed, retention times considerably diminished with increasing alcohol contents. Although no significant differences were obtained for the chromatographic resolution

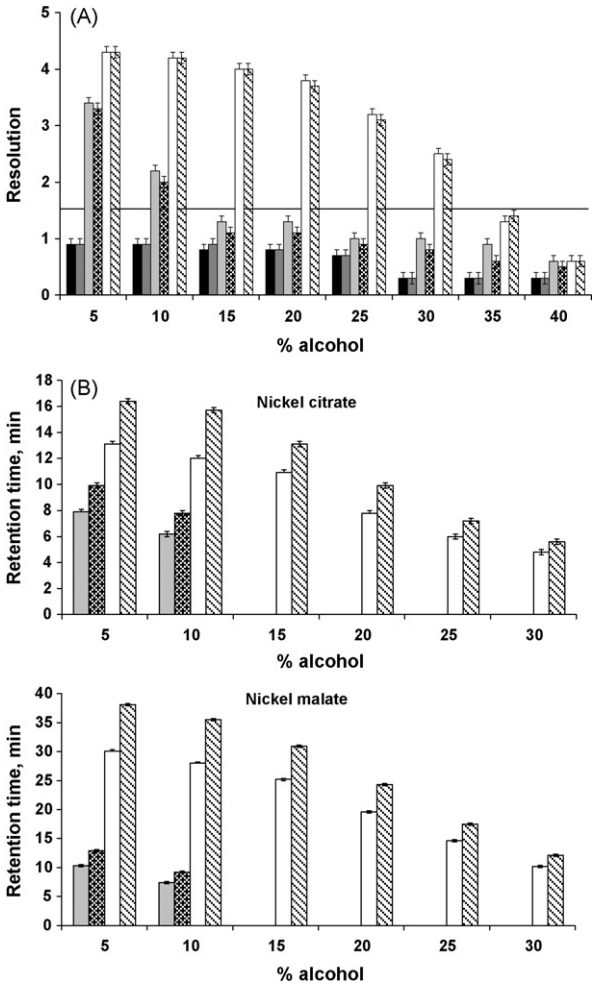

Fig. 2. Optimization of mobile phase for resolution of malate and citrate nickel complexes. (A) Effect of the mobile phase composition on the chromatographic resolution of nickel citrate and nickel malate. In each case, enough amount of an apolar solvent (hexane or toluene) was used to fill up the indicated percentage of alcohol (methanol, ethanol or propanol). The horizontal line indicates the threshold of chromatographic resolution. (B) Effect of the mobile phase composition on the retention times of nickel citrate and nickel malate. Solvent mixtures: hexane/methanol (black bars), toluene/methanol (dark grey), hexane/ethanol (light grey), toluene/ethanol (spotted bars), hexane/propanol (white bars), and toluene/propanol (hatched bars).

with hexane- or toluene-based mobile phases, retention times in the case of using toluene were considerably longer. According to these results, a mobile phase consisting of an hexane:ethanol (9:1) mixture was selected as optimum for the chromatographic separation. Fig. 3 shows the chromatogram obtained for the separation of nickel citrate and nickel malate (10 μM each) on the silica column under these conditions, where nickel citrate and nickel malate showed retention times of 6.2 and 7.4 min, respectively. Under the optimal conditions the determination of nickel complexes was not affected by either excess of nickel or ligands that could be present in the samples, as these eluted at 2.1 and 14–15.5 min, respectively (data not shown).

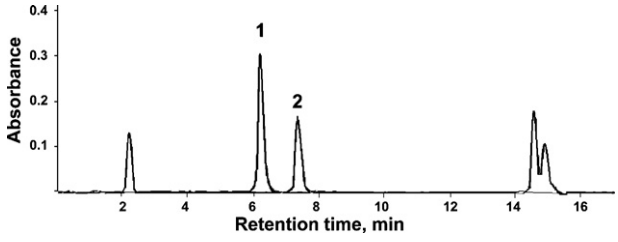

Fig. 3. Separation of nickel citrate and nickel malate by normal phase chromatography. The figure shows the chromatogram obtained from the normal phase LC separation of nickel citrate (1) and nickel malate (2) under the optimized chromatographic conditions. Chromatographic conditions: normal phase chromatography on Silica (250 mm × 4.6 mm, 5 μm). Mobile phase: hexane:ethanol (9:1, v/v). UV detector set at 240 nm.

3.2. Analytical characteristics

The accuracy of the developed HPLC method was estimated by using standard solutions of nickel citrate and nickel malate in concentrations up to 25 μM, and calculating the mass balance resulting from the comparison of the total Ni content determined by ICP-MS after nitric digestion vs. the total amount the Ni present in the different complexes. The developed method was linear in the range of concentrations studied, with regression correlation coefficients above 0.996 in all cases. The limits of detection were estimated as three times the signal of the background noise. Similarly, the limits of quantification were calculated as ten times the signal of the background noise. Limits of detection of about 0.17 and 0.19 nM were obtained for nickel citrate and nickel malate respectively, whereas the limits of quantification were 0.40 and 0.47 nM for the same complexes. The method set up in this paper allows direct identification of both citrate and malate complexes in the sub-nanomolar range.

As it has been stated in the introduction section, citric acid and malic acid are able of complexing other metallic ions, such as Fe^{2+}, Co^{2+}, Cu^{2+} or Zn^{2+}. Accordingly, the chromatographic performance of their corresponding citrate and malate complexes under the optimized conditions has been evaluated. First, a semi-quantitative ICP-MS analysis of the nodule cytosol samples from plants with no nickel added to the nutrient solution was carried out to seek for possible interferents. Results from these experiments demonstrated that the most abundant metallic compounds were iron (100 μM), nickel (0.5 μM), zinc (0.2 μM) and copper (0.05 μM). Accordingly, their citrate and malate chelates were evaluated as possible interferents in the quantification of nickel complexes under the optimized separation procedure. Results from these experiments demonstrated that iron complexes eluted at retention times below 5 min while zinc complexes appeared after 9 min. Copper citrate, on the other hand, eluted at 7.5 min and could thus interfere in the determination of nickel malate (eluting at 7.4 min). However, and due to the strong differences in the MS spectra of copper citrate and nickel malate, its potential interference in the case of samples with high copper content could easily be obviated by monitoring the chromatogram by mass spectrometry.

3.3. Nickel speciation in pea root nodules

The developed methodology has been applied for the direct speciation of nickel complexes in cytosolic fractions of pea nodules. For this purpose, different sets of pea plants were grown for 21 days with varying nickel concentrations in the nutrient solution. After 21 days, plants were collected and nodule cytosolic fractions were prepared as stated in Section 2. After dilution with the mobile phase (dilution factor 10), extracts were directly injected in the HPLC. Fig. 4 shows the chromatogram obtained from the analysis of nickel complexes in nodule cytosolic fractions from plants grown in the presence of 42.5 μM nickel. Two main peaks eluting at 6.2 and 7.4 min were evident in this chromatogram. These retention times corresponded to those from nickel citrate and nickel malate, respectively (Fig. 3). Furthermore, UV and MS spectra from the corresponding fractions indicated that these peaks indeed corresponded to citrate and malate nickel complexes. *m*/*z* values indicate that both complexes contain a 1:2 metal:ligand stoichiometry. The

Table 1-
Speciation of nickel in the cytosol fraction of pea nodules from plants grown in the presence of different nickel concentrations.

Ni added to plant growth media (μM)	Nickel citrate[a] (μM)	Nickel malate[a] (μM)	Total nickel[b] (μM)	% Ni in complexes	Free citrate[c] (mM)	Free malate[c] (mM)
0	0.09	0.32	0.42	97.6	2.55	17.17
42	1.17	1.50	2.65	100.7	2.04	16.32
85	2.42	3.05	5.46	100.2	1.85	15.01
170	3.49	4.71	8.07	101.6	1.42	15.09

Values are the average of five repetitions. In all cases RSD values were below 2.5%.
[a] Analysed by normal phase LC–MS.
[b] Analysed by ICP-MS.
[c] Determined by RPLC.

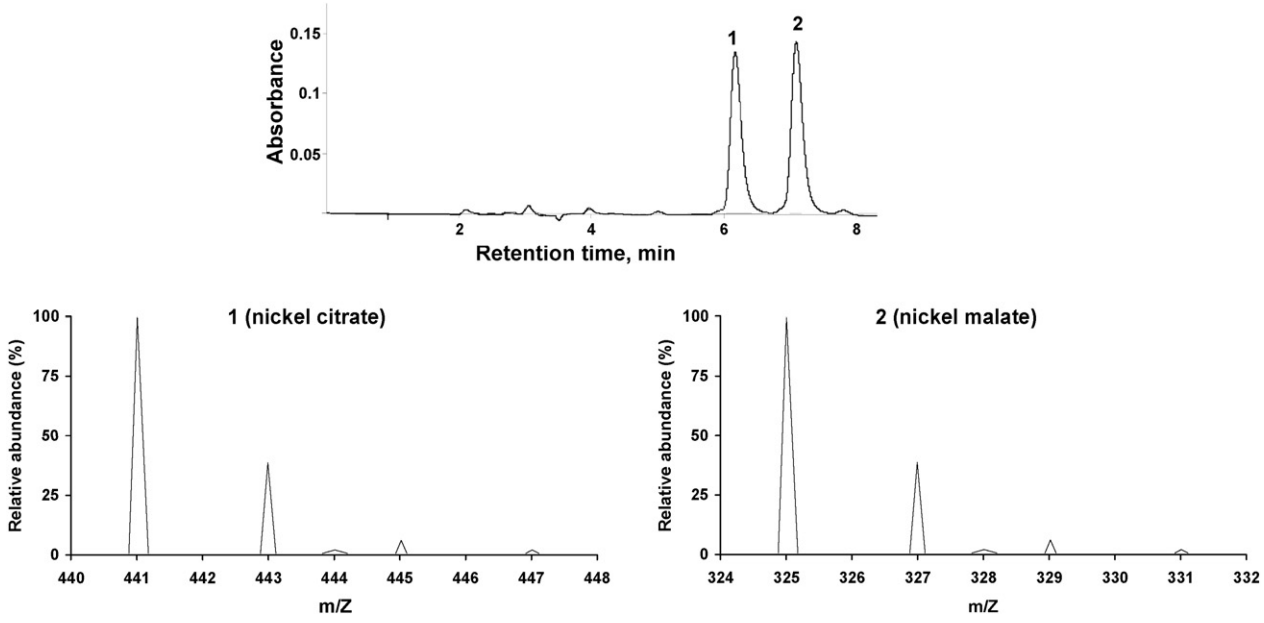

Fig. 4. Identification of nickel complexes in pea nodule samples. (A) Chromatogram (UV detector, 240 nm) obtained from the analysis of cytosolic fractions of pea nodules from plants grown in the presence of 42.5 μM $NiCl_2$. (B) Nickel isotope distribution of electrospray mass spectra of peaks 1 and 2. Chromatographic conditions: normal phase chromatography on silica (250 mm × 4.6 mm, 5 μm). Mobile phase: hexane:ethanol (9:1, v/v). Mass spectra were recorded in the negative mode.

isotope pattern deduced from MS data further confirmed the presence nickel in the complexes (Fig. 4B).

In order to quantify the complexes, both external and standard addition calibration have been evaluated. Results from this experiment demonstrated that both calibration protocols were not significantly different ($\alpha = 0.05$), thus proving the absence of matrix effect in the extracts, and allowing external calibration to be used in this method. Using this calibration method, the concentrations of both nickel complexes were determined in extracts from plants grown in the presence of increasing nickel concentrations (Table 1). These experiments demonstrated that the amount of both nickel citrate and nickel malate increased with increasing nickel contents present in the growth solution.

In order to check for other potential nickel species not determined by the developed methodology, the total amount of nickel present in the samples was determined by ICP-MS after hydrolysis of the complexes with HNO_3 (Table 1). The comparison of these results with the values deduced from the quantification of the complexes by LC–MS showed that nickel malate and nickel citrate account for virtually all (over 97%) of total nickel species present in pea root nodule cytoplasm. When plants were exposed to higher nickel concentrations the levels of citrate and malate nickel complexes significantly increased, leaving virtually no free nickel in the extract.

Concentration of both complexes in pea nodule cytosol extracts were on the same range and so, considering that citrate has a higher affinity for nickel than malate [22], the results obtained indicate that the level of free malate should be much higher than that of citrate. In order to confirm this point, contents of free malate and citrate were monitored by reversed phase chromatography, following the experimental procedure shown in Section 2.5. According to these experiments, concentrations of *ca.* 2 and 17 mM were found for free citrate and malate, respectively (Table 1), which is consistent with physiological evidences pointing towards malate as the main carbon source for pea bacteroids [23]. Both malate and citrate have been identified as predominant ligands for nickel in different hyperaccumulating plants [24]. Our results suggest that these organic acids contribute to protect the nodule environment against high nickel levels.

4. Concluding remarks

A new methodology has been developed for the fast speciation of nickel in plant samples, based on normal phase chromatography. This method is very simple compared with those based on the determination of ligands before and after hydrolysis. The developed methodology allows, for the first time, the direct determination of nickel complexes with very low detection limits (0.17–0.19 nM), thus demonstrating its suitability for the analysis of nickel complexes in non-hyperaccumulating plants.

The developed methodology has been applied to the speciation of nickel complexes in root nodules of pea plants grown in the presence of up to 170 μM $NiCl_2$. The results obtained demonstrate that nickel malate and nickel citrate are the two major species found in

these samples, accounting for over 97% of total nickel. An increase in the concentrations of both nickel species can be observed with increasing nickel contents in the plant nutrient solution. This technique is a promising tool for the analysis of nickel speciation in low-complexity extracts from different plants, and might pave the way to a better understanding of the behaviour of other metals in plant tissues.

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