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Column chromatography and verification of phytosiderophores by phenylisothiocyanate derivatization and UV detection

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Abstract

A high-performance liquid chromatographic method was developed for the separation of phytosiderophores and for verification of the Fe^{3+}-complexing ability of chromatographic separates. The chromatographic procedure involves separation on a C_{18} reversed-phase column by linear gradient elution with sodium acetate (pH 6.4) and methanol with UV detection, following pre-column derivatization of the phytosiderophores with phenylisothiocyanate (PITC). A modification of the procedure involving pretreatment of the sample with Fe^{3+}, which blocks the derivatization by PITC, was used to confirm whether a given chromatographic peak was attributable to an Fe^{3+}-complexing compound. The practical detection limit was approximately 100 pmol of phytosiderophore.

Keywords: Derivatization, LC; Phytosiderophores; Phenylisothiocyanate; 2-Deoxymugineic acid; Mugineic acid; 3-epi-Hydroxymugineic acid

1. Introduction

Iron is an essential plant nutrient and is relatively abundant in most soils; however, it is often a limiting factor for plant growth, especially in neutral and alkaline soils. Within the pH range of calcareous soils, approximately 7.5 to 8.5, the concentration of dissolved iron is at an approximate minimum, and iron deficiencies are common [1]. Graminaceous plants exhibit a specific response to iron deficiency, which involves the release of phytosiderophore (i.e., mugineic acid) [2], a class of Fe^{3+}-chelating non-proteinaceous amino acid (Fig. 1), and specific absorption of the Fe^{3+}–phytosiderophore complex. The phytosiderophores react with soil Fe oxides [3], resulting in an increase in the concentration of dissolved iron in the immediate vicinity of the plant roots. These compounds may also influence the mobilization and uptake of other beneficial and non-beneficial metals by plants.

High-performance liquid chromatography (HPLC) procedures for the separation and detection of phytosiderophores were presented by Kawai et al. [4] and Mori et al. [5]. These procedures were modifications of the procedure developed by Ishida et al. [6] for the simultaneous fluorescent determination of primary and secondary amines. Each procedure involves three steps: (i) separation of the phytosiderophores on a cation-exchange column, (ii) post-column derivatization with *o*-phthalaldehyde (OPA) following pretreatment with NaClO, and (iii) detection by fluorescent spectroscopy. The separation is based on differences in pK_a and the influence of pH on the charge of the various phytosiderophores.

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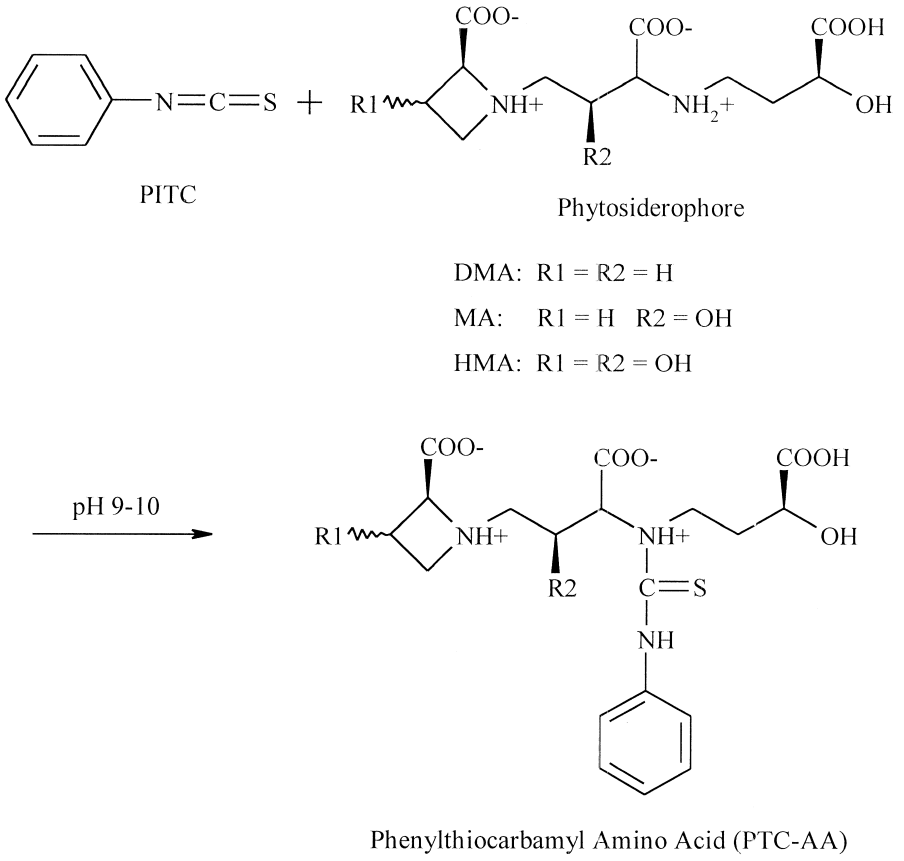

Fig. 1. Structure and derivatization reaction of phytosiderophores (2′-deoxymugineic acid, DMA; mugineic acid, MA and 3-epi-hydroxymugineic acid, HMA) with PITC, to produce the UV absorbing phenylthiocarbamyl amino acid.

Several difficulties are inherent in this procedure, e.g., poor stability of OPA derivatives, problems of Fe contamination with HPLC procedures involving the use of strong acids and low pH and the special equipment required for automated post-column derivatization. Also, the similarities in the pK_a values of the acid sites of the various phytosiderophores make it difficult to obtain adequate separation by cation-exchange procedures. Another recently introduced procedure involves anion-exchange at pH 10–13 rather than cation-exchange and post-column derivatization with OPA [7].

An alternative procedure for the assay of phytosiderophore involves pre-column derivatization followed by reversed-phase chromatography. The objectives of the current study were to develop a chromatographic procedure for (i) the routine assay of phytosiderophores from root exudates, and (ii) verification of whether unknown amino acids exhibit Fe-complexing characteristics.

2. Materials and methods

2.1. Phytosiderophore collection and concentration

Phytosiderophores for the methods development were collected from root exudates of three barley cultivars grown hydroponically in a modified Fe^{3+}-free Hoagland nutrient solution consisting of 0.25 mmol K_2HPO_4, 3 mmol KNO_3, 0.8 mmol $MgSO_4$, 6.7 μmol $MnCl_2$, 1.8 μmol $ZnSO_4$, 0.5 μmol $CuSO_4$, 0.3 μmol Na_2MoO_4 and 0.02 mmol H_3BO_3 l^{-1} adjusted to pH 6.0. Plants were aerated and

maintained in a controlled environment growth chamber with approximately 350 μmol photon m^{-2} s^{-1}, a 18 h/6 h light/dark cycle and temperature 22–24°C. The cultivars (*Hordeum vulgare* L. cv. Minorimugi; *H. vulgare* L. cv. Rikuzenmugi; *H. vulgare* L. var. *distichum* cv. Tochigi-Goldenmelon) were selected for their production of the target phytosiderophores (mugineic acid, MA; 2′-deoxymugineic acid, DMA; 3-epi-hydroxymugineic acid, HMA, respectively) (Fig. 1). The collection procedure, based on the procedure established by Takagi et al. [2], included (1) exudate collection in deionized water for 3 h in the morning beginning approximately 2 h after initiation of light for 10 consecutive days, (2) filtration of collected exudates through Whatman (Hillsboro, OR, USA) No. 2 filter paper, (3) concentration by adsorption of the cationic fraction on a H^+-saturated strong-acid cation-exchange resin, (4) elution with 1 *M* NH_4OH, and (5) rotary evaporation. Exudates for the 10-day collection period were combined, resuspended in 100 ml of deionized water following evaporation, frozen in 1-ml microcentrifuge tubes and stored at −20°C.

Phytosiderophore standards of MA, DMA and HMA were provided in crystal form by Dr. S. Kawai (Iwate University, Morioka, Japan). Standard and collected phytosiderophores were authenticated by positive fast atom bombardment (+FAB) mass spectrometry (MS). Spectra were acquired on a VG Analytical 70S (Manchester, UK) high-resolution, double focusing, magnetic sector mass spectrometer equipped with a BG 11/250J data system. Samples for analysis were prepared by mixing the sample with the thioglycerol matrix on the direct insertion probe tip. The probe was inserted into the instrument through a vacuum interlock and the sample bombarded with 8 eV xenon primary particles from an Ion Tech FAB gun operating at an emission current of 2 mA. Positive secondary ions were extracted and accelerated to 6 keV and then mass analyzed.

2.2. Derivatization procedure

The chromatographic procedure, which involves pre-column derivatization with phenylisothiocyanate (PITC), was adapted from the Waters Pico-Tag procedure [8,9]. In this pre-column derivatization procedure, PITC binds to the secondary amine site of the phytosiderophore (Fig. 1). Derivatization increases the UV absorptivity of the phytosiderophore and allows detection following chromatographic separation.

Standards were prepared by dissolution of approximately 1 mg phytosiderophore in 200 μl of deionized water and filtration through a 0.45-μm pore size membrane filter to remove particulate contaminants. Aliquots of the dissolved standards and collected phytosiderophores (5 and 20 μl, respectively; 10 and 40 μl of Fe-treated samples, see below) were dried in a Savant CVF-220H vacuum centrifuge (Savant Instruments, Hicksville, NY, USA) equipped with a Savant RVT 4104 refrigerated vapor trap, and derivatized with 10 μl of ethanol–deionized water–triethylamine (TEA)–PITC (7:1:1:1) (Sigma, St. Louis, MO, USA) for 20 min in the dark. The PITC reagent mixture was prepared 10 min prior to sample derivatization. Samples were redried and suspended in 1 ml of diluent (355 mg Na_2HPO_4 in 500 ml deionized water adjusted to pH 7.4 with 0.1% H_3PO_4). Samples were injected into the HPLC system within the next 6 h in 10 to 100 μl injections.

2.3. Modification for preparation of Fe^{3+}-treated samples

To prepare Fe^{3+}-treated samples, the dissolved standard or unknown sample was reacted with an equal volume of amorphous ferric hydroxide (ferrihydrite, two-line, [10]) suspension (10 g l^{-1}) in a microcentrifuge tube, by continuous shaking on a reciprocating shaker for 4 h. The ferrihydrite was prepared by dissolution of 40 g $Fe(NO_3)_3 \cdot 9H_2O$ into 500 ml of deionized water, and addition of approximately 330 ml of 1 mol KOH l^{-1} (CO_2 free) to obtain a pH of 7.5 (the last 20 ml was added dropwise). The precipitated product was washed three times with deionized water and diluted to 1 l. Selection of ferrihydrite as the iron source was based on the results of Inoue et al. [3], which indicated the relatively rapid reaction of phytosiderophore with ferrihydrite at pH 7.0 to form the Fe^{3+}–phytosiderophore complex. Following reaction with the phytosiderophore sample, the unreacted ferrihydrite was removed by centrifugal filtration on a 0.45-μm pore-size membrane filter followed by an Ultrafree-MC 5000 NMWL filter unit (Millipore Corporation,

Table 1
Recommended gradient for determination of phytosiderophore derivatives with Waters' Symmetry C_{18} column (Millipore Corporation)

Time range (min)	Eluent composition				Gradient
	Beginning		End		
	% A[a]	% B[a]	% A[a]	% B[a]	
0–12	100	0	50	50	Linear
12–13	50	50	0	100	Linear
13–15	0	100	100	0	Linear
15–25	100	0	100	0	Fixed

[a] A=$NaCH_3COO$ (pH 6.4); B=methanol.

Milford, MA, USA). Removal of all traces of colloidal iron is important, since iron contamination results in deterioration of the reversed-phase HPLC column.

2.4. Chromatography procedure

The Waters (Millipore Corporation) Nova-Pak or Symmetry silica-based reversed-phase C_{18} column (150×3.9 mm, 60 Å pore size and 4 μm particle size) was used for separation. The HPLC system consisted of a Waters 600 S controller and a 616 pump. A gradient elution system was utilized consisting of two solvents: (A) 19 g $NaCH_3COO{\cdot}3H_2O$ l^{-1} buffered with acetic acid to pH 6.4 with 500 μl triethylamine (TEA) l^{-1} and 0.2 mg ethylenediaminetetraacetic acid (EDTA) l^{-1}, and (B) 100% methanol. TEA was used to block exposed silanol sites on the C_{18} column while EDTA prevented dissolved Fe^{3+} species from binding to the column. Solvents were filtered, sonicated and degassed with helium and mixed according to the gradient in Table 1. Column temperature was set at 40°C and flow-rate at 1.0 ml min^{-1}. UV absorption spectra (from 200 to 400 nm) of the eluate components were obtained using a Waters 991 photodiode array detector. Data were collected and analyzed with the Waters Millennium software.

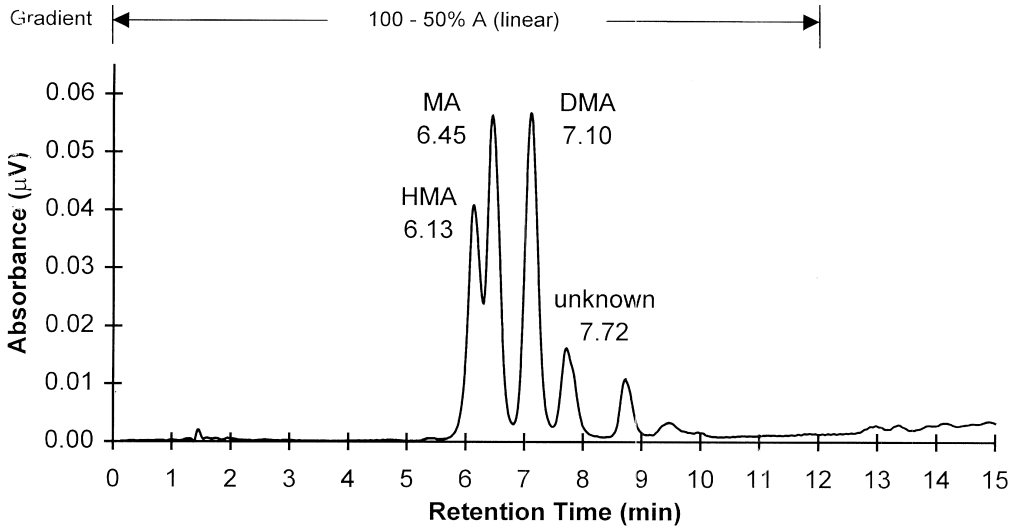

Fig. 2. Chromatogram of phytosiderophore standards HMA, MA and DMA using a Symmetry C_{18} column under the conditions summarized in Table 1.

3. Results and discussion

3.1. Chromatographic separation

Retention times on the C_{18} columns (Fig. 2) depended on the hydrophilicity of the phytosiderophore, with the most hydrophilic compound (HMA) eluting first, followed by MA and then DMA. The Symmetry column, which has a higher carbon loading and is more thoroughly endcapped than the Nova-Pak column, resulted in longer retention times for the derivatized phytosiderophores (data not shown). Endcapping also reduces the possibility of contamination from excess iron injected into the column during the separation of Fe^{3+}-treated samples.

Optimization of phytosiderophore separation was achieved by adjusting the pH, temperature, solvent and gradient. An eluent pH was selected within a range at which the net charge of the phytosiderophore was relatively unaffected by small changes in pH, approximately pH 6.4 (Fig. 3). Under these conditions, the elution of phytosiderophore was influenced by its hydrophilicity but not by its variable charge character. Higher temperatures resulted in reduced peak broadening and reduced retention times (data not shown). When solvent B was acetonitrile and the column was maintained at 60°C, the maximum operating temperature for this solvent, adequate separation of HMA and MA was obtained for peak identification, but baseline separation was not achieved. When solvent B was changed to the more polar methanol and column temperature was maintained at 40°C, separation was improved but baseline separation was still not obtained.

Isocratic, stepped, linear and curvilinear gradients were tested, and chromatograms were evaluated for peak symmetry, elution time and resolution. Slightly improved separations between HMA and MA were obtained when slow gradient changes from initial solvent A (aqueous) to B (organic) were used, but at the expense of significant peak broadening, reduced peak symmetry, and reduced separation between DMA and contaminants (Fig. 4A). Gradients with more initial solvent B produced chromatograms with sharper peaks, but the resolution between HMA and MA was decreased (Fig. 4B). The 12 min linear gradient from 100% to 50% A (Table 1, and chromatograms in Figs. 2 and 5) was finally selected as the best compromise between separation, peak shape and gradient simplicity.

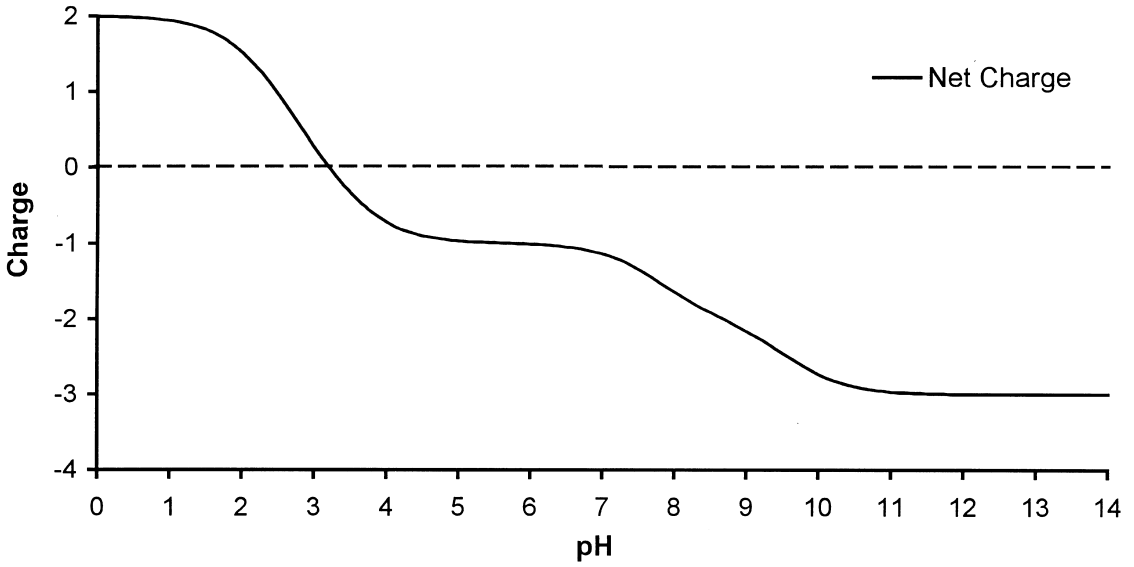

Fig. 3. Calculated net charge of mugineic acid with respect to pH. This curve was calculated using pK values reported by Sugiura et al. [11]: pK_1[COOH]=2.39; pK_2[COOH]=2.76; pK_3[COOH]=3.40; pK_4[NH^+]=7.78; pK_5[NH_2^+]=9.55.

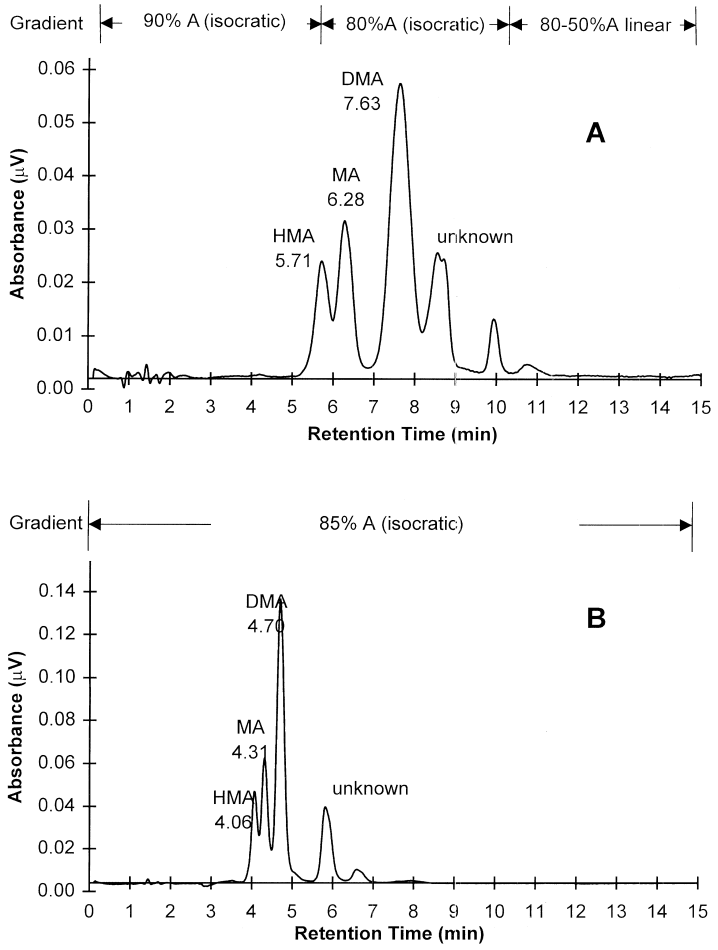

Fig. 4. Chromatograms and gradients of other separation conditions: (A) initially more hydrophilic gradient, and (B) initially more hydrophobic gradient.

3.2. Verification of phytosiderophore

During derivatization for HPLC analysis, the derivatizing agent attacks the secondary amine site of the phytosiderophore to form a compound with a relatively high molar absorptivity (Fig. 1), which then produces a signal from the photodiode array detector. If the phytosiderophore is complexed with Fe^{3+}, the secondary amine site is involved in Fe binding and is not available for attack by the derivatizing agent. As a result, no derivatization occurs, and the peak corresponding to the phytosiderophore is absent or significantly reduced (Fig. 5). The comparison of chromatographic patterns of derivatized samples of iron-treated and non iron-treated root exudates allowed the verification of whether a specific HPLC peak was attributable to a compound able to complex Fe^{3+}. For example, this procedure was used to verify that the elution peak at 6.45 min in Fig. 5 was attributable to a phytosiderophore, in this case MA. The reduction of the peak at 8.71 in the Fe-treated chromatogram suggests the presence of an unidentified Fe^{3+}-complexing compound.

3.3. Spectral verification of phytosiderophore

The authenticities of the standards were confirmed by FAB(+)-MS (Fig. 6A–C). The highest peaks in the spectra corresponded to the parent masses of

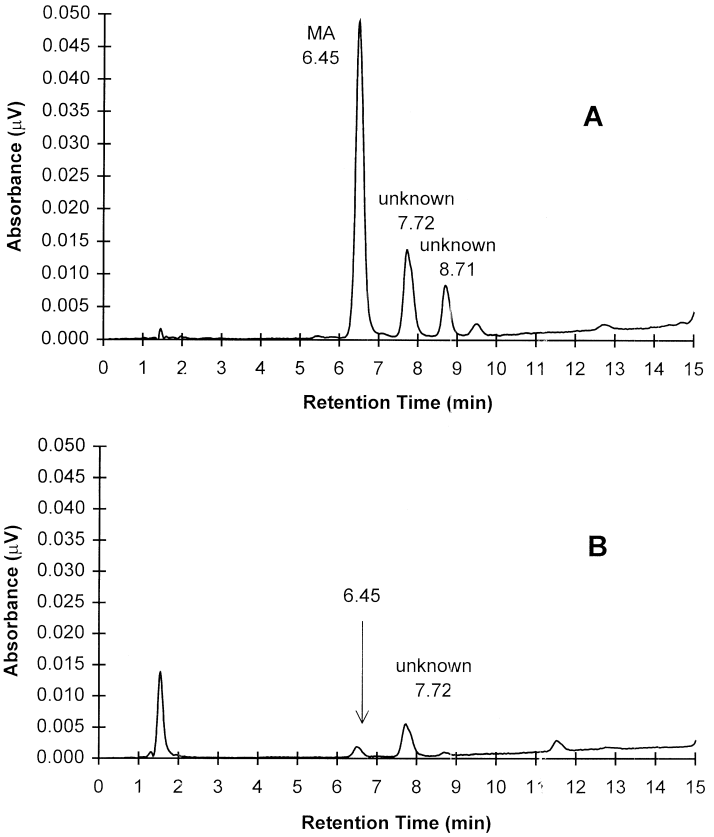

Fig. 5. Chromatograms of (A) MA standard, and (B) Fe-treated MA standard. The absence of the peak at 6.45 min in (B) indicates the presence of phytosiderophore. Conditions as in Fig. 2.

HMA, MA and DMA, of 337, 321 and 305 mass-to-charge (m/z) ratio, respectively. The collected phytosiderophores were also analyzed by FAB(+)-MS (Fig. 6D–F). As expected, the Rikuzenmugi, Minorimugi and Tochigi-Goldenmelon cultivars produced predominately DMA, MA and HMA, respectively. Minorimugi and Tochigi-Goldenmelon also produced smaller amounts of DMA. Peaks at 126, 149, 163, 181, 199 and 217 are attributable to the thioglycerol matrix.

Qualitative verification of phytosiderophore was also corroborated by comparisons of UV absorption spectra of unknown chromatographic peaks with those of the standards. The three derivatized phytosiderophores have similar UV absorption patterns (Fig. 7), with absorption maxima at 269.5 nm. Absorption spectra of contaminant peaks, attributable to degradation products of the PITC, did not have similar maxima or shapes as those of the phytosiderophores. For example, the absorption spectrum of the contaminant peak at 7.72 min. on the chromatogram in Fig. 2 had an absorption maximum at 249.6 nm (Fig. 7). By comparing the UV absorption spectra and the retention times of the compounds, it was possible to distinguish between peaks produced by phytosiderophores and contaminants.

3.4. Quantification of phytosiderophore

For quantitative analysis, standard curves were prepared by plotting peak areas of the individual chromatographic peaks versus amount of phytosiderophore standard injected. The linear range is approximately 0.1 to 10 nmol with r^2 values ranging from 0.9990 to 0.9998. In the current experiment the approximate production rates of the predominant phytosiderophores for the various cultivars were 1.46 (DMA; cv. Rikuzenmugi), 0.61 (MA; cv.

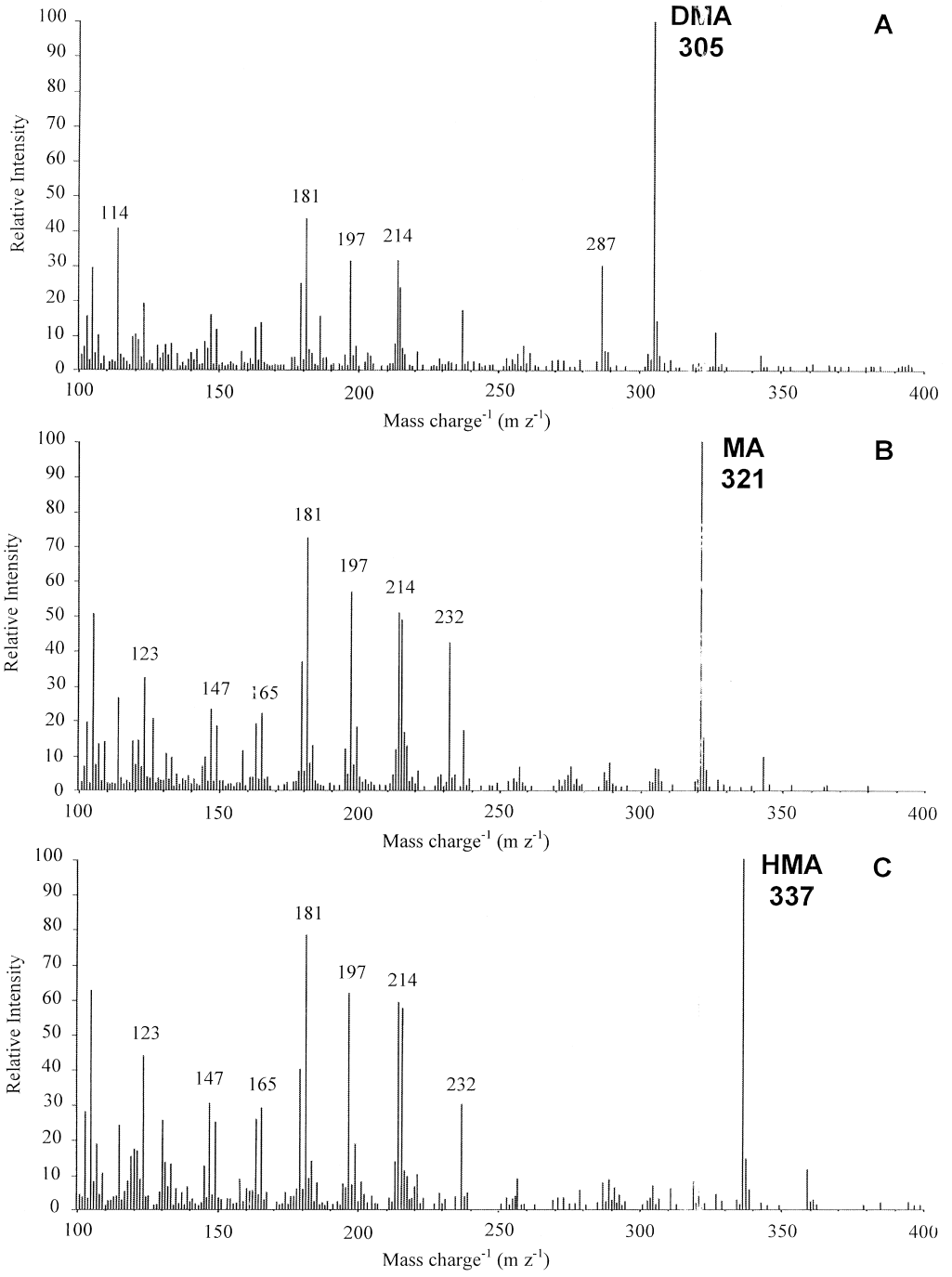

Fig. 6. Mass spectra of qualitative phytosiderophore standards (A–C) and collected phytosiderophores (D–F). Peaks at 126, 149, 163, 181, 199 and 217 originate from thioglycerol matrix.

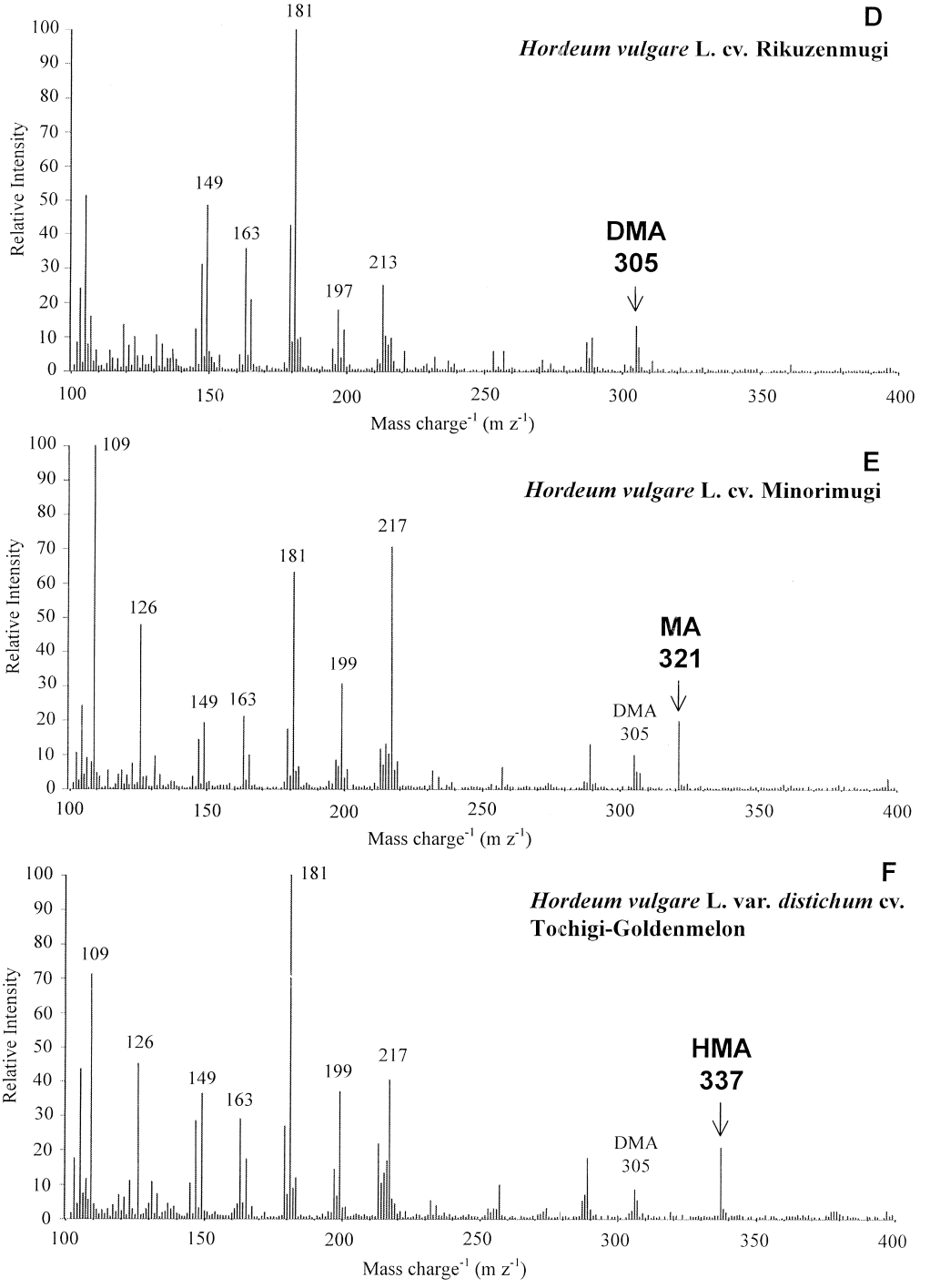

Fig. 6. (*continued*)

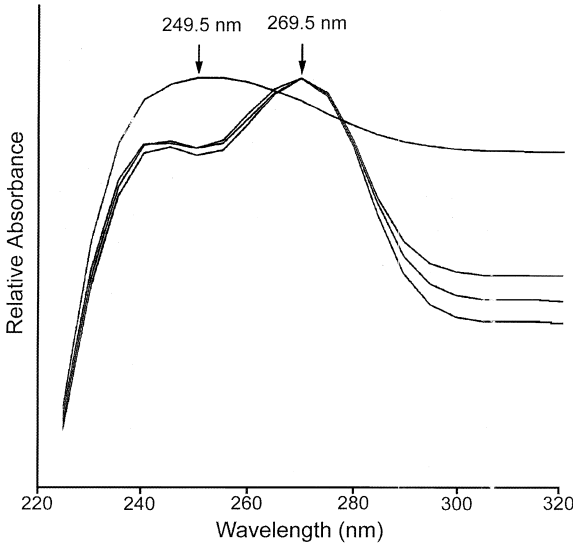

Fig. 7. UV absorption patterns of phytosiderophore standards (maxima at 269.5 nm) and a contaminant (maximum at 249.5 nm).

Minorimugi) and 0.75 (HMA; var. *distichum* cv. Tochigi-Goldenmelon) μmol per plant per 3 h daily collection period. These values are considered as conditional concentrations, since the standards contained some contamination (Fig. 6A–C) and the actual concentrations of phytosiderophores in the standard samples are unknown.

The detection limits of standard amino acids by the Waters Pico-Tag procedure were reported to be approximately 1 pmol [8]; however, in the current study we were unable to achieve this detection limit with the phytosiderophores. The practical detection limit was approximately 100 pmol.

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