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Determination of phytosiderophores by anion-exchange chromatography with pulsed amperometric detection

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Abstract

Phytosiderophores of the mugineic acid family are separated by anion-exchange HPLC using NaOH gradient elution. Separation of mugineic acid (MA), 2′-deoxymugineic acid (DMA), 3-hydroxymugineic acid (HMA) and 3-epi-hydroxymugineic acid (epi-HMA) is obtained within 15 min. Detection of the underivatised phytosiderophores is performed using pulsed amperometric detection (PAD) at pH 13. The sensitivity of the detection increases in the order DMA<MA<HMA< epi-HMA and respective detection limits of 5 μ*M* (DMA), 1 μ*M* (MA) and <0.5 μ*M* (HMA, epi-HMA) are achieved. PAD is discussed in comparison with the well-established fluorimetric detection method after post-column derivatisation with *ortho*-phthaldialdehyde. The main advantage of PAD is the simplicity of the method (no derivatisation) and the high sensitivity for hydroxylated mugineic acids. The method is used for the determination of phytosiderophores in root washings of iron-deficient and non-deficient wheat and barley plants.

Keywords: Pulsed amperometric detection; Phytosiderophores; Mugineic acids

1. Introduction

Phytosiderophores are highly effective iron chelators, released from roots of graminaceous plant species in response to iron deficiency, which occurs particularly in alkaline soils with low iron availability [1]. The respective compounds are mugineic acid (MA), 2′-deoxymugineic acid (DMA), 3-hydroxymugineic acid (HMA) and 3-epi-hydroxymugineic acid (epi-HMA) (Fig. 1), and related compounds such as avenic acid and distichonic acid. Methods for highly sensitive and simultaneous determinations of the different mugineic acids are a prerequisite for investigation of the mechanisms of iron uptake by graminaceous plants [2].

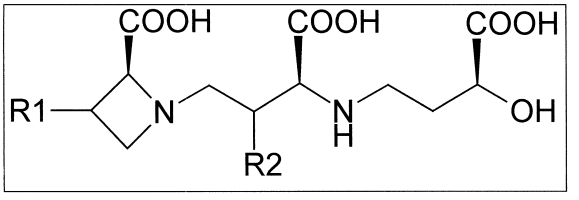

Fig. 1. Chemical structure of mugineic acids. R1=H, R2=β-OH, mugineic acid (MA); R1=H, R2=H, 2′-deoxymugineic acid (DMA); R1=R2=β-OH, 3-hydroxymugineic acid (HMA); R1=α-OH, R2=β-OH, 3-epi-hydroxymugineic acid (e-HMA).

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Chromatographic methods for the quantitation of different mugineic acids include paper chromatography [3] or thin-layer chromatography [4], but, more recently, HPLC methods have been proposed, using cation-exchange or ion-pair chromatography with fluorescence detection after post-column derivatisation with *ortho*-phthaldialdehyde (OPA) [3,5–7]. While these methods are very sensitive, there are still some problems with time-consuming sample preparation or separation [8] or incomplete separation of some mugineic acids (especially MA/DMA or HMA/epi-HMA) [3,7]. Recently, it has been shown that some of these problems can be overcome by using anion-exchange chromatography and fluorescence detection after OPA derivatisation [9]. However, for this procedure, two post-column derivatisation steps are necessary, making the whole system relatively complicated. Moreover, the sensitivity is decreased due to sample dilution associated with post-column derivatisation.

In this paper, pulsed amperometric detection (PAD) is presented as an alternative detection method for mugineic acids after anion-exchange chromatography. This detection method is already well-established for carbohydrates [10–16] and has also been used for amino acids [17,18]. One advantage of PAD is that no derivatisation is necessary for the detection of mugineic acids.

2. Experimental

2.1. Plant culture and collection of samples

Hydroponic cultures of wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) were used for the experiments. Details of the germination, composition of the nutrient solution, and collection of the samples for phytosiderophore analysis were similar to those described previously [9]. All phytosiderophore standards and plant samples were isolated at the ''Institut für Pflanzenernährung'' in Stuttgart and were first analysed using HPLC with fluorescence detection after OPA derivatisation [9]. Frozen aliquots of the samples (−18°C) were then transported to Dortmund, where they were re-analysed using HPLC–PAD.

2.2. HPLC system

HPLC was performed using a Knauer gradient pump K1001 with solvent organizer K1500, solvent degasser, titanium mixing chamber and Rheodyne 9010 injection valve with a 50 μL sample loop. The separation column was a Dionex AS11 (250×4 mm I.D.) with a Dionex AG11 guard column (50×4 mm I.D.). All capillaries and connections were made of PEEK.

2.3. Gradient elution

The following sodium hydroxide gradient was mixed from ultrapure (Seradest) water (solvent A) and 125 m*M* NaOH (solvent B): 0–8 min, 90% A+10% B; 8–18 min, linear gradient to 80% A+20% B. Then, 18–20 min, a column clean-up was performed using a linear gradient to 60% A+40% B and holding this concentration from 20 to 21 min. Then the column was re-equilibrated (21–24 min) using a linear gradient down to 10% B in A and holding this concentration until 30 min. The flow-rate was 1 mL/min.

Precautions: in order to obtain reproducible retention times, the carbonate content of eluents should be kept to a minimum by using only freshly prepared, helium-degassed solvents. Also, iron contamination of the column affects the retention times and resolution of phytosiderophores. Therefore, the column should be regenerated periodically with 100 m*M* oxalic acid at a flow-rate of 1 mL/min for 1 to 2 h.

2.4. Electrochemical detection

For pulsed amperometric detection, an ESA Coulochem II detector was used with an ESA 5040 analytical cell, equipped with a gold working electrode. An ISMATEC peristaltic pump was used for post-column addition of 0.5 *M* NaOH at a flow-rate of 0.3 mL/min to adjust the pH to 13. A triple-step waveform was used for PAD: detection at +0.1 V ($t = 300$ ms, 200 ms acquisition delay), followed by oxidative cleaning at +0.6 V ($t = 120$ ms) and regeneration at −0.8 V ($t = 300$ ms).

3. Results and discussion

3.1. Separation and pulsed amperometric detection of phytosiderophores

Phytosiderophores of the mugineic acid family are tricarboxylic amino acids, which differ in their hydroxylation pattern (see Fig. 1). The ability of anion-exchange chromatography to separate these phytosiderophores results from the increase of negative charges in the order DMA<MA<HMA<epi-HMA at pH 12–13. A typical chromatogram of phytosiderophore standards is shown in Fig. 2 using pulsed amperometric detection (PAD). To our knowledge, this is the first time that PAD has been used for the detection of mugineic acids. The detection parameters are similar to those commonly used for the detection of sugars or oligosaccharides [10–16]. This means that the detection mechanism can be described by ''Mode I'' according to Johnson and LaCourse [10], i.e. direct oxidation of aliphatic hydroxy groups at oxide-free gold surfaces in alkaline media. As a consequence of the increasing number of electroactive hydroxy groups in the order DMA<MA<HMA (=epi-HMA), the sensitivity of PAD increases in the same order. The different sensitivities for detection of the various phytosiderophores are illustrated in Fig. 3, which also demonstrates the linearity of PAD detection in the range

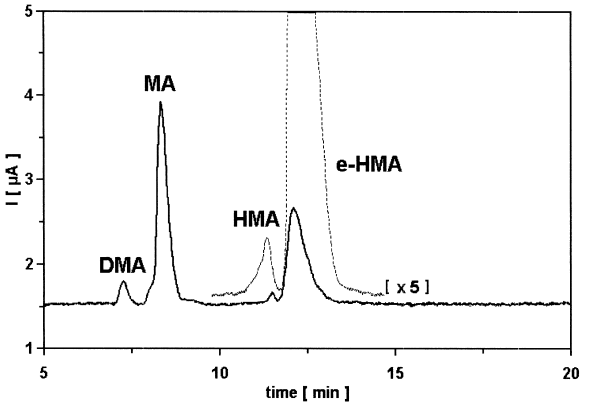

Fig. 2. Separation of mugineic acid standards. MA=mugineic acid (50 μ*M*), DMA=2′-deoxymugineic acid (25 μ*M*), HMA=3-hydroxymugineic acid (1 μ*M*), e-HMA=3-epi-hydroxymugineic acid (10 μ*M*); for HPLC and detection parameters, see Experimental.

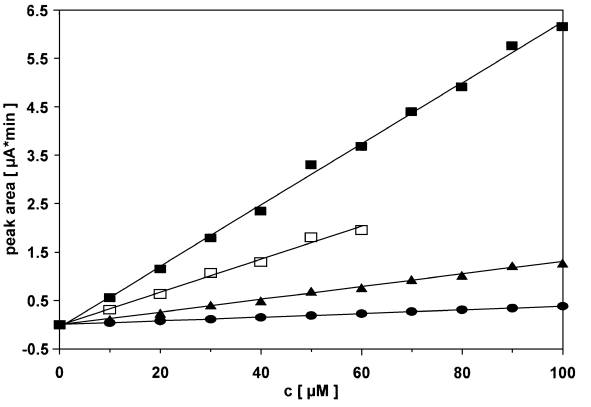

Fig. 3. Calibration of phytosiderophores by HPLC–PAD. (●) DMA, (▲) MA, (□) HMA, (■) epi-HMA.

1–100 μ*M*. The correlation coefficients of the linear regression analyses are better than 0.996 for all four compounds. Interestingly, the sensitivity of PAD (slope of the linear regression line) is not directly proportional to the number of hydroxy groups present: if the sensitivity for MA is set to 1.0, the respective sensitivity is 0.3 for DMA, 2.6 for HMA, and 4.8 for epi-HMA. The reason for the great difference in sensitivity for HMA and epi-HMA (both containing three hydroxy groups) is not yet clear. The most probable explanation would be a more favourable orientation of the hydroxy groups of epi-HMA towards the electrode surface, but the mechanism remains to be established.

The detection limits of PAD are determined by the fluctuations of the background current (baseline ''noise''). These fluctuations depend on the detection parameters (residual charging currents), on the presence of electroactive impurities (e.g. traces of iron(II)), and on the pH of the eluent. Thus, the NaOH gradient used for separation has an effect on sensitivity and detection limits. We compensated for this effect by post-column addition of NaOH to adjust to pH 13, because otherwise the differences in sensitivity for the four phytosiderophores would become even more pronounced. It should be noted, however, that detection of the phytosiderophores is still possible even without post-column pH adjustment; only the detection limits are higher. The detection limits at pH 13, as calculated from the fluctuations of the background current (3σ), are

approximately 5 μ*M* for DMA, 1 μ*M* for MA, and <0.5 μ*M* for HMA and epi-HMA, corresponding to 0.25 nmol DMA, 0.05 nmol MA, and <0.025 nmol HMA injected onto the column, which is comparable to the detection limits reported for the system with OPA post-column derivatisation [9].

3.2. Application to wheat and barley plants and comparison with fluorimetric detection

The method was applied to the analysis of phytosiderophores in root washings obtained from wheat and barley plants. Samples of both iron-deficient and non-deficient plants were analysed. The respective chromatograms are shown in Fig. 4 (DMA in wheat), Fig. 5 (MA in barley), and Fig. 6 (epi-HMA in barley). Quantitative results are given in Table 1 (please note the different dilution factors). Also shown in Table 1 are the results for the same samples using HPLC with fluorescence detection, which were obtained at Hohenheim University (Stuttgart). The results of both detection methods are in good agreement, perhaps with the exception of MA in iron-deficient barley, which is slightly higher for HPLC–PAD and also exhibits a larger standard deviation. The most important contribution to the measurement error for both methods is the limited stability of phytosiderophore solutions at room temperature (due to microbial or chemical degradation). Therefore, samples and standards were stored at −18°C, but small concentration changes cannot be

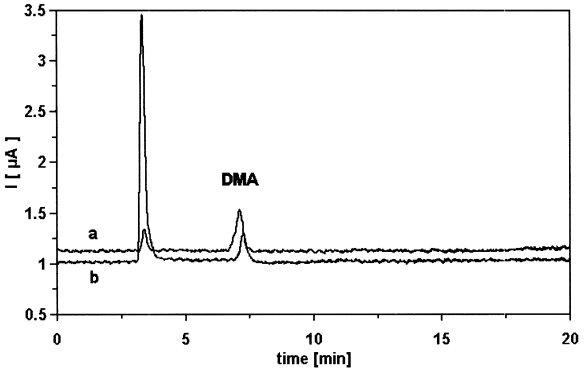

Fig. 4. HPLC–PAD of wheat (*Triticum aestivum* L.). (a) Iron-deficient wheat (1:20); (b) non-deficient wheat (undiluted). DMA, 2′-deoxymugineic acid.

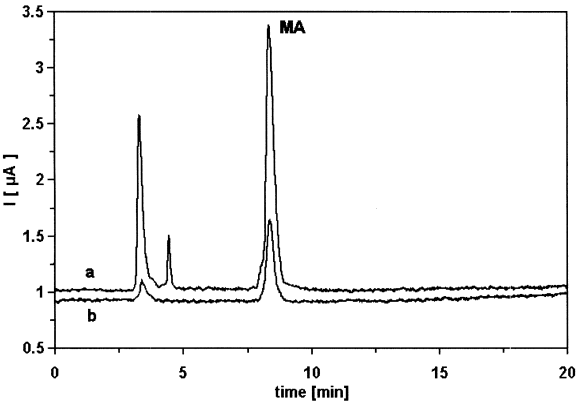

Fig. 5. HPLC–PAD of barley (*Hordeum vulgare* L., cv. Minori). (a) Non-deficient barley (undiluted); (b) iron-deficient barley (1:20). MA, mugineic acid.

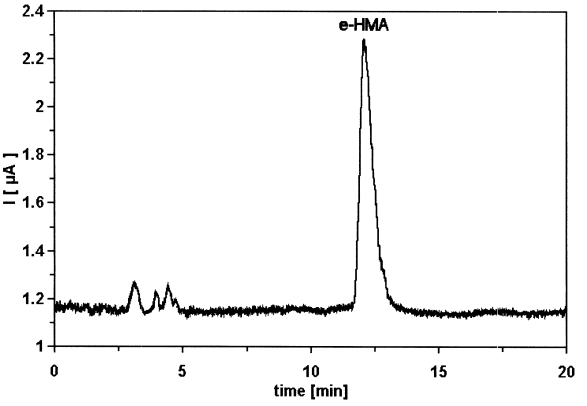

Fig. 6. HPLC–PAD of barley (*Hordeum vulgare* L., cv. Europa). e-HMA, 3-epi-hydroxymugineic acid (1:500).

Table 1
Phytosiderophore concentrations in wheat and barley plants

	c (μ*M*)±SD HPLC–PAD	*c* (μ*M*)±SD HPLC–fluorescence
DMA in wheat (−Fe), 1:20	40±2.6	42±3.8
DMA in wheat (+Fe)	21±2.1	24±2.2
MA in barley (−Fe), 1:20	14±2.4	9.4±0.5
MA in barley (+Fe)	48±1.8	45±2.1
Epi-HMA in barley, 1:500	9.9±0.6	–
Epi-HMA in barley, 1:50	–	93±4.6

(−Fe), samples from iron-deficient plants; (+Fe), samples from non-deficient plants; $n \geq 5$.

fully excluded when the samples are brought to room temperature for measurement.

The peaks found in all chromatograms in the retention range below 5 min result from the detection of electroactive amino acids. Especially amino acids containing hydroxy groups (serine, threonine, tyrosine) and also cysteine can contribute to these signals. We confirmed this by injecting standards of the respective compounds at micromolar concentrations and indeed obtained PAD-detectable signals in the retention range 1–5 min. Because of the coelution of some peaks, quantitation of the amino acids is not possible under the selected conditions, but simultaneous detection of amino acids and phytosiderophores may be achieved by modification of the gradient.

With the exception of amino acids (1–5 min) and phytosiderophores (7–15 min), no other electroactive compounds could be detected in chromatograms of root washings without further purification. From this it can be concluded that the proposed HPLC–PAD method is not prone to interferences. The method can also be used for (semi-) preparative purification of underivatised mugineic acids, which is an advantage compared to fluorimetric detection methods using OPA derivatisation.

4. Conclusion

Pulsed amperometric detection has been demonstrated as an alternative detection technique for phytosiderophores of the mugineic acid family after anion-exchange HPLC of root washings. No post-column derivatisation is needed and, with the exception of DMA, the detection limit is equal to (MA) or even better than (HMA, epi-HMA) fluorimetric detection.

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