Speciation of zinc in microliter volumes of plant sap by capillary HPLC-ICP-MS

Application note

Environmental

Author

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Introduction

Many metals are very important for normal plant growth and development. One of these metals is zinc – typically the second most abundant transition metal in organisms and a substantial micronutrient that plays different roles in plant physiology. Zinc is an essential component of over 300 enzymes. It is responsible for gene regulation and stabilization of protein structure including Zn fingers, Zn clusters and RING finger domains. It is also involved in essential processes such as photosynthesis and CO_2 fixation. Excess or deficiency of zinc in plants leads to high plant mortality, reduced and stunted growth, chlorosis, necrosis, small leaves and delay in flowering. All of these symptoms may cause serious implications for food security because of the significant reduction of crop yields that is correlated to zinc availability [1-3].

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Better understanding of plant physiology through the identification of low molecular weight metal-containing compounds present in plant saps, and the determination of their exact function will further inform nutritional, agricultural and environmental studies. However, the study of complex biological matrices such as plant samples may cause several issues during sample preparation or chromatographic separation. The main challenges relate to the low concentrations of metal complexes and their huge diversity. In addition, metal complexes are often unstable and can be degraded during extraction, off-line preconcentration steps or even during chromatographic separation. Of the chromatographic techniques that have been investigated thus far, size exclusion chromatography (SEC), hydrophilic interaction chromatography (HILIC) and reverse phase (RP) chromatography have proved to be the most suitable techniques to avoid degradation of the metal complexes during analysis [4]. A chromatographic system consisting of a preconcentration column and HILIC or RP separation column seems to be ideal for this kind of application.

This work proposes an ICP-MS-assisted metallomic approach to the separation of zinc species present in post-phloem of *Pisum sativum*. The embryo sac liquid was analyzed by capillary HPLC-ICP-MS with on-line preconcentration. The use of capillary chromatography was essential due to the low amount of plant sap available.

Experimental

Sample

Embryonic sac liquid (post-phloem) obtained from the developing pods of *Pisum sativum* (green pea) was studied.

Sample preparation

The pods were perforated with a glass capillary and the liquid endosperm was extracted using a peristaltic pump and put into an Eppendorf tube kept on ice. After collection, the samples were immediately frozen in liquid nitrogen and stored at -20 °C until further analysis. Just before analysis, the samples were thawed, diluted with acetonitrile to obtain a 1:2 ratio (sample:acetonitrile) and then centrifuged for 2 minutes at 10000 rpm. The supernatant was collected and analyzed immediately.

HPLC-ICP-MS system

An Agilent 1100 LC fitted with a capillary pump and manual valve (loop size: 100 µL) was used. 30 µL of supernatant was loaded on the SeQuant zwitterionic (ZIC-)HILIC guard column (Merck KGaA, Darmstadt, Germany, 5 mm x 1 mm i.d., 5 µm) using an isocratic flow of 20 µL/min of 90% acetonitrile and 10 mM ammonium formate buffer (pH 5.5). The sample was washed with the mobile phase for 4 min and then back-flushed onto the SeQuant ZIC-HILIC capillary column (Merck KGaA, Darmstadt, Germany, 150 mm x 0.3 mm i.d., 3.5 µm) that was used for compound-separation. Gradient elution, at a flow rate of 4 µL/min, was carried out using eluent A, 10 mM ammonium formate buffer (pH 5.5), and eluent B, acetonitrile. The gradient program is given in Table 1.

Table 1. HPLC elution program

Step	Eluent [%B]	Time [min]
1	90	0-5
2	90-65	5-17
3	65-52	17-47
4	52-35	47-53
5	35	53-65
6	35-90	65-70
7	90	70-75

HPLC-ICP-MS and ESI-MS/MS

The outlet of the separating column was connected to the Agilent 7700x ICP-MS (via the Agilent capLC interface, G3680A, Figure 1) or ESI-MS/MS.

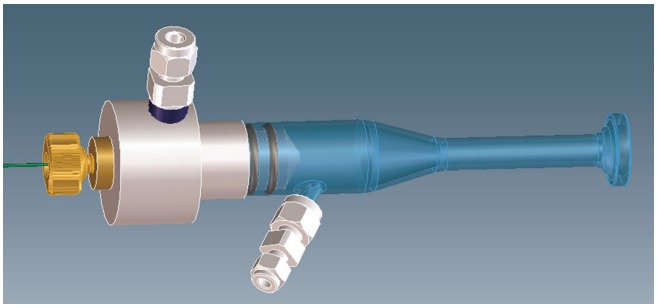

Figure 1. Agilent capillary LC interface kit (G3680A) which consists of a total consumption nebulizer inside a small quartz spray chamber

ICP-MS conditions were auto-optimized at the start of each day, using a tune solution containing 20 ppb of Y, Li, Tl, Ce in 2% nitric acid. The ORS^3 collision/reaction cell of the 7700x was operated in high energy helium mode to exclude polyatomic interferences that may occur on Zn isotopes. Signals for ^{64}Zn and ^{66}Zn were acquired using a dwell time of 60 ms. The ICP-MS operating conditions are given in Table 2.

Table 2. Agilent 7700x ICP-MS operating conditions

Parameter	Value
Nebulizer/spray chamber	Capillary LC Interface G3680A
Torch i.d.	1 mm
Cones	Platinum
RF power	1560 W
Sampling depth	7.5 mm
Carrier gas flow rate	0.78 L/ min
Optional gas (O_2) flow rate	0.04 L/ min
Lenses	
Extract 1	2.7 V
Extract 2	-180 V
Cell	
Octopole bias	-100 V
He flow	10 mL/ min
Kinetic energy discrimination	7 V

The ESI LTQ Orbitrap Velos mass spectrometer was operated in the positive ion mode at 3.0 kV. The vaporizer temperature of the source was set to 120 °C and the capillary temperature to 280 °C. The resolution in full MS mode was set at 100,000 (FWHM at *m/z* 400).

Results and discussion

The chromatogram obtained for ^{64}Zn for green pea post-phloem sample is shown in Figure 2. The use of capillary ZIC-HILIC ICP-MS with on-line preconcentration allowed us to obtain sharp and intense peaks and enabled the separation and detection of two zinc species (Figure 2a and 2b). Both metal complexes were identified using ESI Orbitrap MS/MS. ICP-MS detection was essential to determine the retention times of the different zinc species and to estimate the mass balance. This simplified the search for the zinc complexes in the ESI-MS mass spectra. The expanded parts of the mass spectra (Figure 2d and 2e) clearly show two ions containing the isotopic pattern for zinc. The retention times of these extracted ion chromatograms (EIC) are in agreement with the two zinc peaks observed via capillary ZIC-HILIC ICP-MS (Figure 2c). The data obtained allowed us to identify two zinc complexes: zinc-nicotianamine (NA) and zinc-(histidine)$_2$ (Table 3).

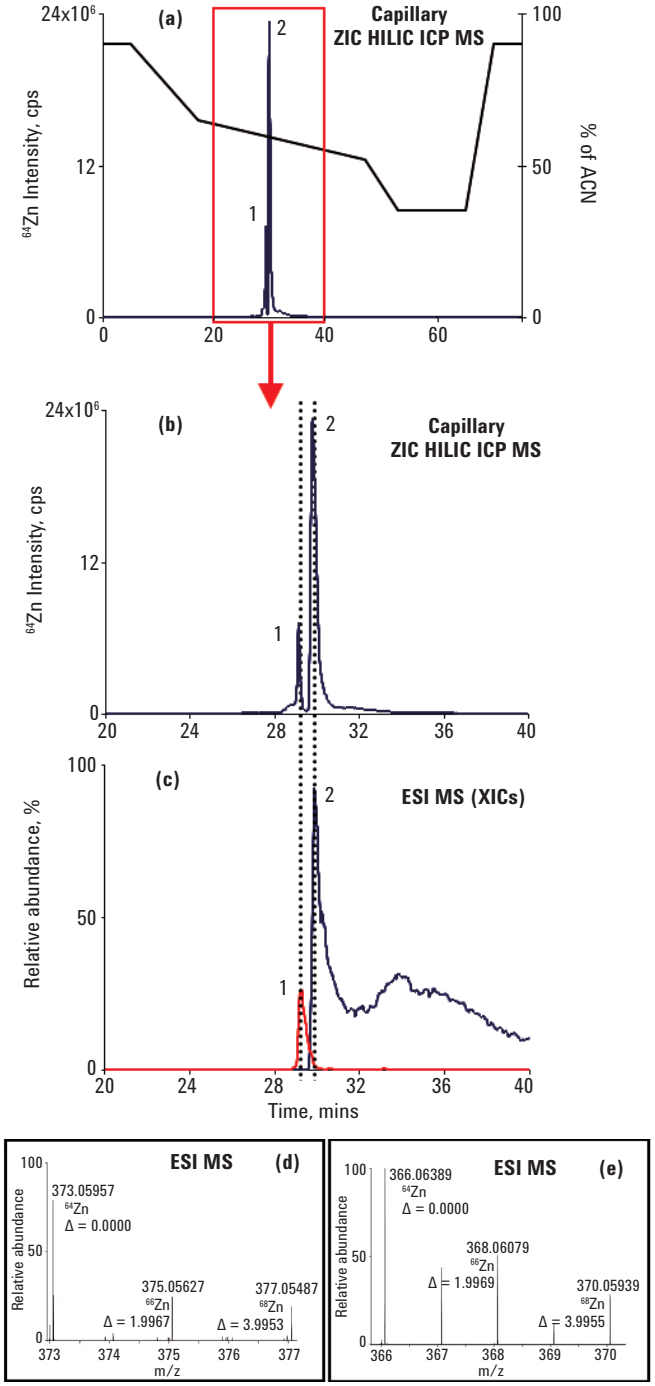

Figure 2. Chromatograms obtained by a) capillary ZIC-HILIC-ICP-MS; b) capillary ZIC-HILIC-ICP-MS – zoomed in section of chromatogram; c) capillary ZIC-HILIC-ESI-MS (selected ion chromatograms). d) and e) Zoomed in section of the ESI-MS spectrum containing zinc isotopic pattern.

Table 3. List of zinc containing complexes indentified in post phloem of *Pisum sativum* (green pea)

	Peak 1	Peak 2
Ligand	Histidine	Nicotianamine
Complex	$(His)_2$-Zn	NA-Zn
Formula (neutral form)	$C_{12}H_{16}O_4N_6Zn$	$C_{12}H_{19}O_6N_3Zn$
Theoretical mass	373.05973	366.06381
Experimental mass	373.05957	366.06389
Delta ppm	-0.437	0.216

The chromatographic system consisting of capillary HPLC and ICP-MS allowed us to achieve a detection limit of 75 ng/L for ^{64}Zn (~ 6 fmol of Zn-NA complex), calculated as 3x the standard deviation of 20 points of the base line. This value was compared with the signal of Zn-NA complex in respect to column recovery obtained for ^{64}Zn which was 70-80%.

Conclusions

The study shows an effective ICP-MS assisted metallomic approach for the separation and identification of zinc complexes present in post-phloem of *Pisum sativum*. The zinc species were preconcentrated using a ZIC-HILIC pre-column and then separated via a ZIC-HILIC capillary column. The combination of data obtained by coupling capillary HPLC to ICP-MS and ESI MS/MS instruments allowed the identification of different zinc complexes. The use of capillary chromatography was essential due to the low amount of plant sap available. Additionally the chromatographic system with on-line preconcentration is ideal to work with biological samples containing low concentrations of metal species that may also sometimes be unstable. This approach has been successfully used to identify two zinc species: zinc-nicotianamine (NA) and zinc-$(histidine)_2$ complexes proving that NA and histidine are two major ligands that complex zinc in post-phloem of green pea.

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