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Monitoring of Phosphorus Oxide Ion for Analytical Speciation of Phosphite and Phosphate in Transgenic Plants by High-Performance Liquid Chromatography–Inductively Coupled Plasma Mass Spectrometry

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ABSTRACT: Large amounts of phosphate fertilizers utilized in agriculture and their relatively poor efficiency are of high ecological and economic concern. Therefore, transgenic plants capable of metabolizing phosphite are being engineered. In support of this biotechnological task, analytical speciation of phosphorus in biological tissues is required. In this study, plant extracts were analyzed by liquid chromatography–inductively coupled plasma mass spectrometry at m/z of elemental phosphorus and phosphorus oxide ions. Using polymeric-based anion exchange column and millimolar concentration of nitric acid in potassium phthalate mobile phase (pH 2.5), phosphite and phosphate ions were baseline resolved with retention times 6.95 ± 0.03 and 7.90 ± 0.03 min and with a total chromatographic run time 10 min. The detection limits were 1.58 and 1.74 μg P L^{-1} at m/z 47, as compared to 2.18 and 2.04 μg P L^{-1} at m/z 31, respectively. The results obtained in real world samples for the two detection modes were in good agreement, yet signal acquisition at m/z 47 enabled better precision without collision/reaction cell (RSD below 2%) as compared to RSD around 4% obtained at m/z 31 using He-pressurized cell (3.5 mL min^{-1}).

KEYWORDS: *phosphite, analytical speciation, HPLC, ICP-MS, transgenic plants*

INTRODUCTION

Phosphate-based fertilizers have long been used in agriculture even though these products present several drawbacks. In particular, phosphate [P(V)] tends to form insoluble compounds in soil and is also readily converted to organic forms by soil microorganisms; plants are able to take up and effectively use only a small part of phosphorus contained in the supplied fertilizers. The excessive utilization of P(V) seriously contributes to eutrophication processes and results in depletion of nonrenewable phosphate rock resource.[1] Another form of phosphorus in agricultural applications is phosphite [P(III)], commonly used as a crop fungicide and sometimes considered as a biostimulator or an alternative source of P for plant growth.[2,3] Owing to better mobility of P(III) and similar uptake rates as compared to P(V), a reduced amount of fertilizer might be applied; phosphite, however, cannot be metabolized by plants, and its adverse effects have been reported.[4] A biotechnological approach toward the efficient delivery and assimilation of phosphorus relies on the generation of transgenic plants that can use phosphite as a sole source of this element.[5] Because the specific target is to obtain plants capable of oxidation P(III) to P(V), quantitative data on these two phosphorus forms in biological tissues are required in support of plant engineering. It should be stressed at this point that in the majority of studies related to P(III)/P(V) in crop cultivation, total P was routinely evaluated, whereas speciation analysis of two phosphorus oxyanions in plants has seldom been undertaken.[3,6−10]

Owing to the variety of organic and inorganic phosphorus species present in the plant tissues and because of the complex chemical matrix, selective extraction and cleanup procedure as well as sensitive and interference-free quantification of phosphorus in the obtained fractions are the most challenging issues in any spectation scheme. In the reported studies, water, diluted formic or acetic acid, water/methanol mixtures at room or elevated temperature,and prolonged shaking time (up to 3 h) have been used; the obtained extracts were further purified by solid phase extraction for elimination of coextracted organic compounds and ionic species; however, evaluation of the extraction yield of individual phosphorus species has rarely been undertaken.[7,9−13]

Liquid chromatography with conductivity detector has been the predominant technique used in the analysis of phosphorus oxyanions in plant extracts, providing detection limits in the low parts per million concentration range.[8,9] It is worth mentioning that ion chromatography with gradient elution at elevated pH and with suppressed conductivity detection, as applied to chemically simple water samples, provided low- or even sub-parts per billon detection limits; however, in more demanding samples, poor selectivity was highlighted as an important limitation.[14,15] Alternatively, precolumn derivatization with diazomethane or *N*-methyl-*tert*-butyldimethylsilyltrifluoroacetamide was proposed for gas chromatography with flame ionization detector and, again, low parts per million detection limits were reported in plant analysis.[9,16] The application of ion trap mass spectrometry detection enabled an enhanced detection power (0.1 ng on column).[9] Selective phosphite fluorometric assay with a detection limit 5 μmol L^{-1} (about 0.4 mg L^{-1}) was also developed, on the basis of the

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enzymatic oxidation to phosphate in the presence of resazurin and subsequent fluorometric measurement of the reduced reagent form.[7] The hyphenation of atomic spectrometry techniques with liquid chromatography was used in speciation analysis of phosphorus in herbicides,[17] soil extracts,[18] and different natural water matrices,[15,19,20] yet the feasibility of such an approach in plant analysis has been only recently tested.[5]

Inductively coupled plasma mass spectrometry is generally considered a technique of choice in trace element speciation schemes; however, its application for monoisotopic phosphorus is compromised by the relatively high first ionization potential of this element and troublesome nitrogen-, oxygen-, and carbon-based polyatomic interferences.[21] Several strategies have been proposed to enhance the analytical performance of P determination by ICP-MS such as elimination of organic sample matrix, minimization of plasma sample load, and use of high-resolution instruments or collision/reaction cell technology. In particular, it was demonstrated that cells pressurized with helium stop polyatomic ions from being introduced to the quadrupole mass filter by discrimination of kinetic energy,[17,18,22] whereas oxygen introduced to dynamic reaction cells promotes formation of phosphorus oxide ion with m/z 47, which has less pronounced interferences than m/z 31.[23−25] On the other hand, the enhanced generation of phosphorus oxide ion was achieved by optimization of plasma operating conditions[26] and also when the analyzed solution contained nitric acid or methanol.[27] Even though as high enhancement of sensitivity as observed in the dynamic reaction cell cannot be expected, this latter option seems quite attractive due to its potential applicability even in the most austere ICP-MS instruments.

In this work, speciation analysis of phosphite and phosphate in transgenic plants had been undertaken by coupling anion exchange liquid chromatography with ICP-MS. Addition of nitric acid to the mobile phase was proposed with the aim to enhance the formation of phosphorus oxide for P detection at m/z 47, thus eliminating the need for oxygen introduction via collision/reaction cell. The sample cleanup procedure, chromatographic separation, and instrumental operating conditions were experimentally selected, and the analytical figures of merit were compared for the elemental and phosphorus oxide ion detection.

MATERIALS AND METHODS

Instrumentation. An Agilent series 1200 liquid chromatographic system equipped with a quaternary pump, a well plate autosampler, a diode array detector, and a ChemStation (Agilent Technologies, Palo Alto, CA, USA) was used. The chromatographic column was a Hamilton PRP-X100 (250 × 4.6 mm, 5 μm). For detection of phosphorus, the column effluent was online introduced to an inductively coupled plasma−mass spectrometry system via a short length (10 cm) Teflon tubing (0.3 mm i.d.).

A model 7500ce ICP-MS (Agilent Technologies, Tokyo, Japan) was used with a MiraMist Teflon nebulizer. A Peltier-cooled chamber was operated at 2 °C. Standard tuning procedure was performed using Li, Y, Tl, and Ce (1 μg L^{-1} each), and the obtained parameters were further refined for detection at m/z 47 using a P(V) standard solution in mobile phase (10 μg P L^{-1}). For phosphorus detection at m/z 31, an octopole collision reaction cell was used with a He flow rate of 3.5 mL min^{-1}.[22]

Reagents and Samples. All chemicals were of analytical reagent grade. Deionized water (18.2 MΩ cm, Labconco, Kansas City, MO, USA) and HPLC-grade methanol (Fisher Scientific, Pittsburgh, PA, USA) were used throughout.

Sodium phosphate dibasic [P(V)], sodium phosphite [P(III)], potassium hydrogen phthalate, nitric acid, EDTA, Supelclean LC-18 SPE tubes (3 mL), and Supelco and IC Acrodisc filters (0.2 μm) were obtained from Sigma (St. Louis, MO, USA).

Homogenized and freezed-dried transgenic tobacco plants grown in the presence of P(III) as a sole phosphorus source (samples 1, 2, and 3) and one pooled sample were kindly provided by Dr. Luis Herrera-Estrella from the National Laboratory of Genomics for Biodiversity, Mexico.

Procedures. For extraction of phosphate and phosphite ions, 1.0 mL of 10 mmol L^{-1} EDTA, pH 8.0, was added to 40−50 mg of freeze-dried plant material, and the mixture was placed in an ultrasonic bath for 30 min at room temperature. Afterward, the sample was centrifuged (10000g, 10 min), and the supernatant was collected and cleaned up with Supelclean LC-18 SPE tubes 3 mL (product 57012 Supelco). The tubes were conditioned with 2 mL of methanol and then washed with 2 mL of 10 mmol L^{-1} EDTA, pH 8 (solution used for extraction), and 1 mL of the crude extract was passed at a flow rate of 1 mL min^{-1}. Finally, the sample was filtered through an IC Acrodisc filter (0.2 μm) and, after appropriate dilution with the mobile phase (maximum 20-fold), 100 μL was introduced to the chromatographic system coupled with ICP-MS. Separation was achieved by isocratic elution with 5 mmol L^{-1} potassium hydrogen phthalate adjusted to pH 2.5 with nitric acid at total flow rate of 1.0 mL min^{-1} at room temperature. The ICP-MS instrument operating conditions established for detection at m/z 31 and 47 are presented in Table 1.

Table 1. ICP-MS Instrument Operating Conditions

ICP-MS detection	m/z 31	m/z 47
forward power	1500 W	
nebulizer gas flow	0.9 L min^{-1}	0.85 L min^{-1}
makeup gas	0.1 L min^{-1}	0.4 L min^{-1}
nebulizer	MiraMist Teflon	
spray chamber	Peltier cooled chamber, 2 °C	
sample and skimmer cones	platinum	
sample depth	9 mm	8.5 mm
ion lenses extract 1	2.5 V	−180 V
extract 2	−130 V	−60 V
cell entrance	−30 V	−36 V
QP focus	−15 V	−3 V
cell exit	−50 V	−60 V
OctP RF	100 V	
OctP bias	−18 V	−12 V
acquisition mode	time-resolved analysis	
dwell time	100 ms	
collision/reaction cell	3.5 mL min^{-1} He	no

Quantification was performed by external calibration using mixed P(III) + P(V) standard solutions at five concentration levels (0, 100, 250, 500, and 1000 μg L^{-1} of phosphorus as P(III) and as P(V), respectively) with five successive injections of each solution; peak area was always used as a signal mode.

Statistical Analysis. Descriptive statistics were performed to obtain means and standard deviations. For the tuning procedure, the surface responses were obtained using The Unscrambler 7.5 software package (CAMO, Oslo, Norway).

RESULTS AND DISCUSSION

The goal of this work was to establish a P(III)/P(V) speciation procedure in which nitric acid addition would enable reliable ICP-MS detection of phosphorus at m/z 47 without use of the collision/reaction cell. In the initial experiments, the separation was carried out on a SAX anion exchange column with 10 mmol L^{-1} potassium phthalate, pH 4.0; however, the chromatographic peaks were tailed, the background signal for

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ICP-MS detection at m/z 31 was elevated (>1600 cps), and sensitivity for m/z 47 was poor.[5] To enhance analytical performance, it was proposed to use a polymeric-based column and a more dilute mobile phase with the addition of nitric acid and to compare the results obtained for the detection of elemental and phosphorus oxide ions.

The effect of nitric acid on ICP-MS determination of phosphorus at m/z 31 and 47 had already been reported.[27] The main focus of the cited study was to evaluate the potential impact of nitric acid as introduced for the digestion of organic samples; therefore, relatively high acid concentrations (up to 2.5 mol L^{-1}) were used, and deteriorated detection limits were obtained for the two ions monitored; however, for lower acid concentrations (<0.1 mol L^{-1}), the undesirable background increase at m/z 47 and the signal decrease at m/z 31 were much less pronounced.[27] For this reason and also because low concentration would be required in the mobile phase, the effect of nitric acid on the ICP-MS m/z 47 signal was examined in the concentration range from 0 to 10 mmol L^{-1}. To this end, phosphate standard solution prepared in mobile phase and blank solution (5 mmol L^{-1} potassium phthalate or deionized water) containing different concentrations of nitric acid were directly aspirated to the plasma. The results are shown in Figure 1. It should be noted that the final tuning conditions

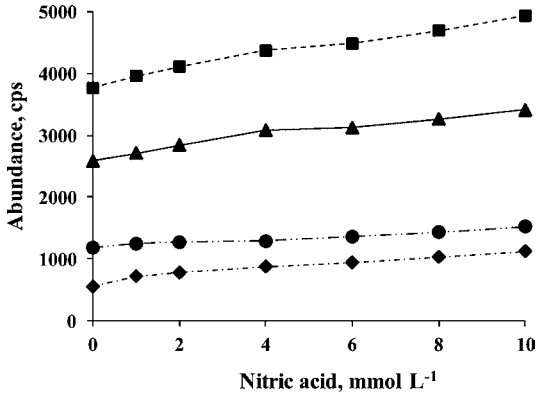

Figure 1. Effect of nitric acid on ICP-MS m/z 47 signal for (◆) deionized water; (●) potassium phthalate, 5 mmol L^{-1}; (■) 25 μg P L^{-1} as P(V) in potassium phthalate, 10 mmol L^{-1}; and (▲) phosphorus−phtalate signal.

were applied in this experiment for better clarity of presentation (Table 1). As can be observed, the increasing concentration of nitric acid caused a gradual increase of signal for all three solutions tested. The net signal evaluated for phosphate standard (25 μg P L^{-1}) rose from 2517 cps in the absence of acid to 3260 cps for its concentration of 10 mmol L^{-1} (30% increase) and, most importantly, this signal remained relatively stable in the concentration range of 4−8 mmol L^{-1} nitric acid (3092 ± 73 cps), indicating that this range can be safely used in chromatographic separation for phosphorus detection at m/z 47.

The next task was to set appropriate instrumental conditions for the detection of elemental and oxide ions in the presence of mobile phase. For m/z 31, the tuning settings and collision/reaction cell conditions were adopted from the previous work[22] (Table 1). To check for possible polyatomic interferences at m/z 47 ($^{47}Ti^{+}$, $^{94}Zr^{2+}$, $^{7}Li^{40}Ar^{+}$, $^{12}C^{35}Cl^{+}$, $^{14}N^{16}O^{16}O^{1}H^{+}$), the effect of He flow rate in the range from 0 to 4 mL min^{-1} was examined for phosphate standard prepared in the mobile phase and for blank (5 mmol L^{-1} potassium hydrogen phthalate containing 8 mmol L^{-1} nitric acid). As shown in Figure 2, the

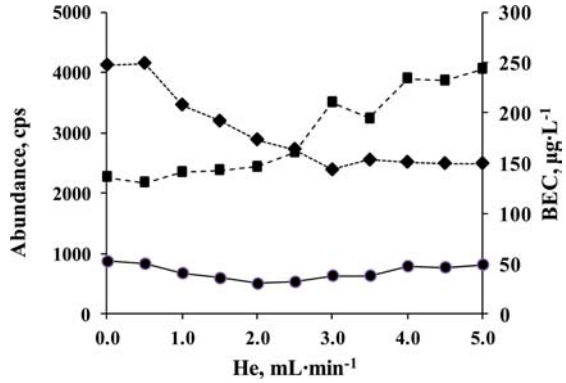

Figure 2. Effect of He flow rate in the octopole cell on ICP-MS signal at m/z 47: (■) BEC, μg P L^{-1}; (◆) phosphate, 25 μg P L^{-1} in the mobile phase; (●) potassium phthalate in 8 mmol L^{-1} nitric acid.

blank signal was always below 830 cps, the signal of phosphate standard solution (25 μgP L^{-1}) decreased significantly at a gas flow rate of >0.5 mL min^{-1}, and background equivalent concentration (BEC) gradually increased, indicating no need for reaction/collision cell at m/z 47. Plasma operating conditions and QP focus, cell exit, and OcP bias voltages were refined by varying one parameter at a time under a criterion of the highest sensitivity (net signal for 10 μg P L^{-1} as phosphate, direct aspiration to ICP-MS). In this regard, forward plasma power was kept at 1500 W and octopole RF voltage at 100 V for both m/z values. A short residence time in plasma favors oxide formation, for which the makeup gas flow and the sampling depth were examined in the ranges of 0.1−0.8 mL min^{-1} and 4−9 mm, respectively; the values finally chosen for oxide detection were 0.4 mL min^{-1} and 8.5 mm (Table 1). Because the octopole cell was not pressurized with gas for m/z 47, the selected values for QP focus, cell exit, and OcP bias were different with respect to m/z 31 (Table 1). Finally, for setting voltages of the ion lenses, net signal for 10 μg P L^{-1} as P(V) was registered using three different values at cell entrance (−44, −36, and −30 V) while the two other parameters were varied according to the four-level factorial design. Specifically, the voltages tested were −180, −150, −100, and −50 V for extract 1 and −130, −120, −90, and −60 V for extract 2. The optimized response surfaces were evaluated using The Unscrambler software, and the obtained results revealed relatively higher P signal intensities for the cell entrance voltage −36 V as compared to −44 and −30 V. In Figure 3, the response surface for cell entrance −36 V is presented, and it can be observed that as low as possible voltage at extract 1 (−180 V) and as high as possible voltage at extract 2 (−60 V) would provide the best sensitivity of phosphorus detection at m/z 47. The tuning parameters finally selected are summarized in Table 1.

For chromatographic separation, a Hamilton PRP-X100 column with chemically inert polymeric support was selected with the expectation of better peak symmetry and lower ICP-MS baseline as compared to a silica-based column, in which silica and residual silanol groups present affinity to ionic species and, thus, may potentially affect the retention and column recovery of phosphorus oxyanions. In ion chromatography with conductivity detection, alkaline mobile phases have been

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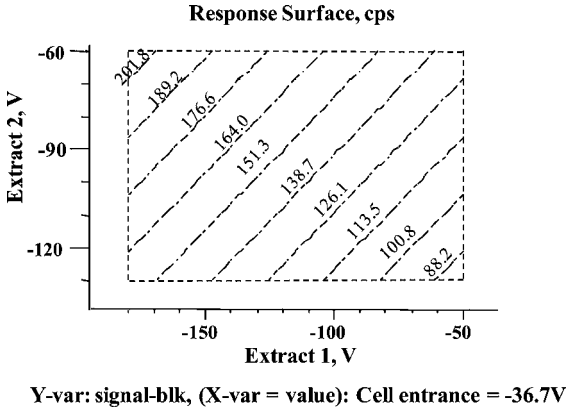

Figure 3. Response surface for net signal of 10 μg P L^{-1} as P(V) obtained at cell entrance voltage −36 , and variable voltages at extract 1 (−180 to −50 V) and extract 2 (−130 to −60 V).

typically used for separation of completely deprotonated phosphorus oxyanions;[14,15] however, elevated pH values were avoided in this work to protect the components of the HPLC-ICP-MS system and, most importantly, for PO^+ detection/quantification, nitric acid had to be present in the mobile phase at the concentration of 4−8 mmol L^{-1} (Figure 1). Several authors reported separation of P oxyanions in the pH range from 2.5 to 4.0 with the mobile phases containing organic ions such as phthalate, succinate, malonate, and citrate.[9,19,28,29] The single-charged $H_2PO_3^-$ and $H_2PO_4^-$ anions, as the primary species in such conditions,[30] would present lower retention with respect to double- or triple-charged anions and also shorter total chromatographic runs, especially advantageous when ICP-MS is to be used as the detection system. Because this technique does not tolerate high pH values, the intent was to apply low millimolar concentrations of potassium hydrogen phthalate/nitric acid mixture. To set the conditions enabling baseline separation of the two species in a short time and with good peak symmetry, several chromatograms for P(III) + P(V) standard mix were obtained, by varying the concentration of potassium hydrogen phthalate (2−10 mmol L^{-1}) with pH adjusted by the addition of 1 mol L^{-1} nitric acid (pH 2.5−3.5) and testing different column flow rates (0.6−1.5 mL min^{-1}) and temperatures (from room temperature up to 40 °C). The finally selected conditions are listed under Materials and Methods; it should be noted that the concentration of nitric acid in the mobile phase was about 6 mmol L^{-1} (5 mmol L^{-1} hydrogen phthalate, pH 2.5), within the previously selected concentration range (Figure 1). In Figure 4, typical chromatograms of two mixed standard solutions are presented; the calibration results and typical figures of merit are summarized in Table 2. Even though baseline at m/z 47 was more elevated with respect to m/z 31, monitoring phosphorus oxide in the established chromatographic and ICP-MS conditions provided higher sensitivity, lower detection limits, and better short-term precision with respect to m/z 31 (Table 2). The detection limits evaluated as P concentration injected on column were comparable with those reported for LC-ICP-MS speciation of different phosphorus species and sample types[18,19,22,31,32] yet significantly lower with respect to other procedures employed in the analysis of P oxyanions in plants.[7−9,16] As described under Materials and Methods, a 50 mg aliquot of biomass was treated with 1 mL of extracting solution, and the obtained extract was diluted up to 20 times before its introduction to the

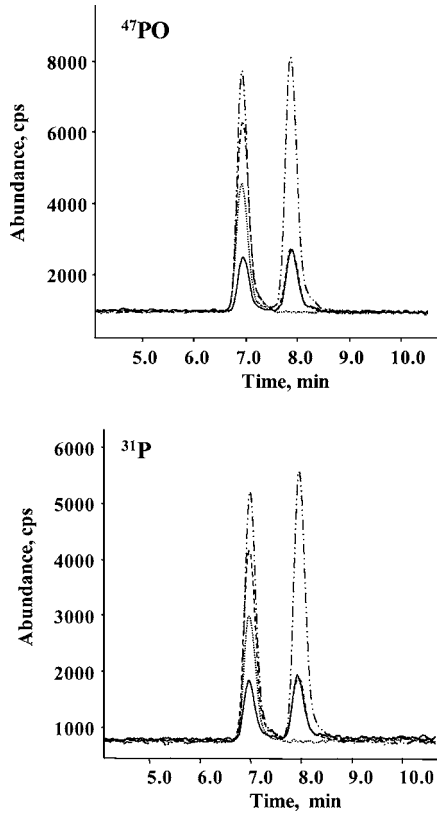

Figure 4. HPLC-ICP-MS chromatograms obtained for (—) phosphate + phosphite standard mix, 250 μg P L^{-1} each; (-·-·-) phosphate + phosphite standard mix, 1000 μg P L^{-1} each; (···) plant extract; (---) plant extract with P(III) + P(V) standard addition, 250 μg P L^{-1} each: (a) m/z 47; (b) m/z 31.

HPLC-ICP-MS system, for which the detection limits evaluated in plant biomass at m/z 31 were 0.82 μg P g^{-1} P(III) and 0.87 μg P g^{-1} P(V) and at m/z 47 were 0.70 μg P g^{-1} P(III) and 0.63 μg P g^{-1} P(V) (Table 2). These low values are important features of the procedure proposed here, because the quantitative evaluation of P(III) to P(V) conversion in transgenic plant becomes possible.

The real world samples analyzed were transgenic tobacco plants engineered for phosphite metabolization. In the first attempt, deionized water was used for extraction as reported elsewhere.[4,11,12] To do so, 1 mL of water was added to 50 mg of the pooled biomass, the mixture was placed in an ultrasonic bath for 30 min at room temperature, and the obtained extract was further cleaned up on a C18 cartridge. It should be noted that aqueous extract presented green color and contained organic matter; therefore, it could not be introduced directly to the anion exchange column or to ICP-MS. The reversed phase SPE is a common approach for the elimination of organic sample components, and it has also been reported as a cleanup step in the determination of P oxyanions in plant materials.[7,12] Afterward, the extract was filtered through a 0.2 μm Supor membrane, certified for ion chromatography, for elimination of residual fine particles. Finally, the sample extract was 20 times diluted prior to the HPLC-ICP-MS procedure. In triplicate analysis using m/z 31 for phosphorus detection, phosphite was not detected in this sample (<0.8 μg P g^{-1}), and the concentration found for phosphate was 0.204 ± 0.032 mg P g^{-1}. To examine the recovery of the procedure, 200 μL of phosphate and phosphite mixed standards (50 mg P L^{-1} each)

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Table 2. Analytical Figures of Merit Evaluated for the Anion Exchange Separation of Phosphate and Phosphate with ICP-MS Detection at ^{31}P and ^{47}PO (Mean Value, Standard Deviation, and CV Obtained for Five Replicates)

	m/z 31		*m/z* 47	
parameter	P(V)	P(III)	P(V)	P(III)
baseline[a] ± SD, cps	521 ± 17	576 ± 20	832 ± 29	858 ± 24
t_{ret} ± SD, min	6.95 ± 0.03	7.90 ± 0.04	6.95 ± 0.03	7.90 ± 0.04
calibration[b]	71.2*c* + 23	84.1*c* + 43	110.3*c* + 86.4	115.1*c* + 101
R^2	0.9994	0.9997	0.9997	0.9998
DL,[c] μg P L^{-1}	2.18	2.04	1.58	1.74
DL,[d] μg P g^{-1}	0.87	0.82	0.63	0.70
DL,[e] nmol P g^{-1}	28	26	20	23
QL,[f] μg P L^{-1}	3.64	3.42	2.65	2.89
CV,[g] %	1.0	1.1	0.7	0.7
CV,[h] %	2.7	2.5	1.5	1.4
CV,[i] %	3.9	3.7	2.1	2.2

[a]Acquired in the elution region of each species. [b]*c* = concentration, μg P L^{-1}. [c]Detection limit evaluated on the basis of six standard deviations of baseline measured in the elution region of the analyte. [d]Detection limit as referred to phosphorus mass per gram of biomass. [e]DL as nmol of each species (phosphite or phosphate) per gram of biomass. [f]Quantification limit evaluated on the basis of 10 standard deviations of baseline measured in the elution region of the analyte. [g]Coefficient of variation representing instrumental precision for 250 μg P L^{-1}. [h]Coefficient of variation representing within-run precision for 250 μg P L^{-1}. [i]Coefficient of variation representing between-run precision for 250 μg P L^{-1}.

Table 3. Quantification Results Obtained in the Analysis of Plant Samples by the Proposed Procedures with ICP-MS Detection at *m/z* 31 and 47[s]

	external calibration		standard addition	
sample	P(III)	P(V)	P(III)	P(V)
		***m/z* 47, mg P g^{-1} (dw) ± SD**		
1	0.035 ± 0.009	0.296 ± 0.015	0.032 ± 0.016	0.304 ± 0.019
2	nd	0.266 ± 0.012		0.270 ± 0.023
3	nd	0.203 ± 0.016		0.215 ± 0.027
		***m/z* 31, mg P g^{-1} (dw) ± SD**		
1	0.025 ± 0.010	0.274 ± 0.024	0.028 ± 0.015	0.289 ± 0.031
2	nd	0.255 ± 0.021		0.283 ± 0.019
3	nd	0.179 ± 0.017		0.202 ± 0.024

[s]Mean concentration values with respective SD based on triplicate analysis of each biomass are presented.

and 800 μL of deionized water were added to another portion of this same pooled sample, and the results obtained were 0.193 ± 0.020 mg P g^{-1} for P(III) and 0.368 ± 0.037 mg P g^{-1} for P(V), indicating 96.5 and 82.0% recoveries for the two species, respectively. As already mentioned before, many metals form poorly soluble phosphates; therefore, in further development extraction was carried out with 10 mmol L^{-1} EDTA at pH 8 for complexation of metal ions (principally Ca and Mg) and so to improve the extraction yield of P(V). An alkaline solution of EDTA (0.1 mol L^{-1} EDTA in 0.25 mol L^{-1} NaOH) is typically used for efficient extraction of phosphorus from soil (16 h in agitation);[33] however, lower concentrations of EDTA (<50 mmol L^{-1}) and less alkaline conditions (pH 8−8.5) were used for the extraction of soil phosphate available to plants,[34,35] and these milder conditions seemed suitable for the extraction of P oxyanions from tobacco biomass. The results obtained for this same pooled sample in the second experiment were 0.231 ± 0.037 mg P g^{-1} P(V) and P(III) not detected, whereas in the spiked sample 0.419 ± 0.035 mg P g^{-1} as P(V) and 0.197 ± 0.028 mg P g^{-1} as P(III) were found, yielding recovery values of 94.2 and 98.6% for the two species, respectively. On the basis of these results, EDTA extraction and a two-step purification procedure were applied in further analyses.

In Figure 4, the chromatograms acquired at *m/z* 31 and 47 for plant extract (sample 3) and for the spiked sample are presented. Specifically, 100 μL of the mixed standard solution (50 mg P L^{-1} P(III) and 50 mg P L^{-}P(V)) was added to the 50 mg sample aliquot, which means that after extraction, cleanup, and dilution, the standard addition resulted in the concentration 250 μg P L^{-1} of each species in the final solution. In Figure 4, chromatograms of two calibration solutions are also included, which enables confirmation of species identity. It can also be observed that the baseline for *m/z* 31 was lower as compared to *m/z* 47; however, it presented higher noise, in agreement with Figures 1 and 2 and Table 2. Three samples from different plants were analyzed, and the results obtained for the detection at two *m/z* values were in good agreement; however, monitoring phosphorus oxide ion resulted in better precision as compared to elemental ion (Table 3). Indeed, the relative standard deviation for P(V) determination was around 5% for *m/z* 47 and 9% for *m/z* 31 (Table 3). For accuracy checking, the determination was accomplished by a two-point standard addition (250 and 500 μg P L^{-1} of two species in the solution injected to the chromatographic system). The quantitative results were in good agreement with those obtained by external calibration (Table 3). In samples 2 and 3, only phosphate was found, whereas in sample 1 low phosphite concentration was also present. Biological interpretation of analytical data is out of scope; however, it should be noted that the results obtained in this work were consistent with those previously reported for these same transgenic plants.[5] In particular, samples 1−3 corresponded to plants

grown in phosphite as a sole P source, and the speciation results confirmed efficient oxidation of P(III) to P(V) by plant.

In conclusion, the feasibility of phosphorus monitoring at m/z 47 for the determination of phosphite and phosphate in plant material by anion exchange chromatography with ICP-MS detection has been demonstrated. In this procedure, potassium phthalate mobile phase containing a millimolar concentration of nitric acid enabled baseline resolution of the two element species within 10 min and favored the formation of phosphorus oxide ions in plasma. The results obtained in the analysis of transgenic plants using two detection modes (m/z 31 and 47) were in good agreement; however, phosphorus oxide monitoring yielded a slightly lower detection limit and better precision. Most importantly, the collision/reaction cell was not needed for phosphorus oxide ion monitoring, for which the procedure can be accomplished on any ICP-MS instrument. Many biotechnological studies focus today on phosphite as an alternative phosphorus source for plants; because phosphite utilization relies on its in vivo oxidation to phosphate, the implementation of the proposed procedure would provide useful, complementary analytical information in such studies.

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Notes

The authors declare no competing financial interest.

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