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Mass spectrometry of natural organic phosphorus

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Abstract

This article provides a review of the use of modern mass spectrometry (MS) for quantitative and qualitative measurements of organic phosphorus compounds in nature. Included is a brief discussion of recent developments in large molecule mass spectrometry, focusing on time-of-flight (TOF) and ion cyclotron resonance (ICR) mass analysis techniques, as well as electrospray (ESI) and inductively coupled plasma (ICP) ionization. The use of ICP with high-resolution mass spectrometry for quantitative measurements of total phosphorus and as a detector coupled to HPLC and CE for defining organic phosphorus speciation is demonstrated using results from a study of phosphorus cycling in a treatment wetland. Qualitative identifications of individual phosphorus compounds by ultrahigh resolution Fourier-transform ion cyclotron resonance mass spectrometry (FT-ICR MS) is demonstrated using dissolved organic phosphorus isolated from this same wetland.

Keywords: Organic phosphorus; Mass spectrometry; ICP-mass spectrometry; Electrospray ionization; FT-ICR mass spectrometry

1. Introduction

There are few studies in the scientific literature in which mass spectrometry (MS) has been used to qualitatively identify or quantitatively measure natural organic phosphorus compounds in environmental matrices (e.g. soils, sediments, water). The reluctance to use this technique, which has played such an important role in defining the biogeochemical cycling and environmental impacts of so many other elements is unfortunate, given the growing awareness of the importance of organic phosphorus (org-P) in agriculture and environmental protection [1]. At present, little is known about the "speciation" (i.e. molecular forms) of naturally-derived organic phosphorus in the environment.

The molecular properties of organic phosphorus are largely responsible for this situation. Organic phosphorus is dominated by the PO_4 group(s) that imparts polarity and mass. It is thus difficult to ionize org-P molecules without substantial fragmentation, making qualitative identification of individual compounds quite difficult. Relatively recent advances in "soft" ionization techniques have changed this situation, however. Mass spectrometry of molecules such as nucleotides, proteins, and peptides is now a common part of many biochemical studies.

The inductively coupled plasma (ICP) is the ultimate "hard" ionization technique, reducing even ionic molecules to their elemental components. When used with a mass spectrometer that is monitoring an appropriate mass, highly sensitive and selective elemental analyses can be obtained. Low ionization efficiencies and the need for high-resolution mass analysis lower the absolute sensitivity of ICP-MS for phosphorus relative to what can be achieved with other elements. However, ICP-MS can compete with traditional methods for phosphorus quantitation in terms of detection limits and linear dynamic ranges, and is by far the preferred technique for P-specific detection with separation techniques such as liquid chromatography and capillary electrophoresis (CE).

This review will focus on organic phosphorus produced by "natural" sources, and will not include "anthropogenic"

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organic phosphorus compounds (e.g. pesticides, surfactants) for which numerous, highly specific techniques have been developed. We will begin with a brief discussion of mass spectrometry, emphasizing those techniques that have been developed recently for large molecules, particularly biomolecules. Many of these methods are directly applicable to studies of natural organic phosphorus. There are number very good reviews that include more detailed discussions of these techniques (see, for example, [2]). We will then describe the important ionization techniques useful for org-P mass spectrometry; "hard" ionization in an ICP to reduce org-P compounds to elemental phosphorus for quantitative analyses, and the "soft" electrospray ionization technique that makes identification of large org-P molecules possible by mass spectrometry. We will conclude with a description of our work on phosphorus speciation in the Florida Everglades. Those studies utilized high-resolution P-specific mass spectrometry with ICP ionization for quantitative analyses, and ultra-high resolution MS experiments with electrospray ionization for qualitative identification of individual compounds. These latter experiments included both single- and multi-stage Fourier-transform ion cyclotron resonance mass spectrometry (FT-ICR MS). Multi-stage FT-ICR MS is a form of MS/MS in which ions are selected by the first MS stage for fragmentation and product ion analysis in the second stage. MS/MS is one of the most powerful techniques available for identifying the formulas and structures of unknown molecules.

2. Large molecule mass spectrometry

Mass spectrometers produce information about the masses and abundances of gas-phase ions. Since molecular mass is the single most important piece of information necessary for characterizing an unknown chemical structure, MS has become an invaluable analytical technique for environmental studies. Mass spectrometry is an important quantitative tool as well because mass spectrometers are very sensitive and exhibit large linear dynamic ranges. Mass spectrometry can also provide element-specific detection by monitoring a single mass that corresponds to the element of interest (e.g. phosphorus at 30.974 Da). This is an application particularly useful for quantifying organic phosphorus compounds that normally exist in complex matrices dominated by other non-phosphorus organic compounds.

In this brief review of mass spectrometry, we will focus on relatively recent developments in time-of-flight (TOF) and ion cyclotron resonance (ICR) mass analyzers. These two mass analysis techniques, along with parallel advances in molecular ionization, are largely responsible for the current explosion in large-molecule mass spectrometry that has occurred over the past decade. However, a brief discussion of mass resolving power and mass accuracy is first necessary to better understand the power of these analyzers.

2.1. Mass resolution and mass accuracy

Mass resolution (R) is the ability of the analyzer to distinguish ions of similar mass-to-charge ratio. It is defined by Eq. (1):

$$R = \frac{m}{\Delta m} \tag{1}$$

In Eq. (1), m is the mass at which two adjacent peaks are resolved by one mass unit ($\Delta m = 1$). It should be noted that an analyzer with a resolution of 200 can provide unit mass resolution at mass 200 Da, but can only distinguish molecules that differ by five mass units at 1000 Da. Thus, high-resolution analyzers are necessary to characterize large molecules such as organic phosphates. Furthermore, unit mass resolution is not always sufficient when complex natural mixtures such as natural dissolved organic matter are being analyzed. Consider two fulvic acid molecules we have identified in the Suwannee River in northern Florida [3].

$C_{36}H_{20}O_{16}$; $m/z = 577.0801$;

$C_{37}H_{24}O_{15}$; $m/z = 577.1175$

For these two molecules, $M = 577$, $\Delta m = 0.0364$, and thus the resolution required to distinguish them is ~15,850. Mass accuracy is also a very important characteristic of mass spectrometers used for qualitative identification of unknown compounds. The greater the mass accuracy, the greater the confidence in the assignment of exact mass and chemical formula. Again, using these same two fulvic acid molecules as an example, we need a mass accuracy of $\Delta m/M$, or $0.0364/577 = 63 \times 10^{-6}$ (63 ppm). Obviously, just like resolution, greater mass accuracy is needed for larger molecules because of the increasing chemical formula possibilities as mass increases [4].

2.2. Time-of-flight mass analyzers

Time-of-flight mass analyzers are in essence velocity spectrometers. Ions are separated by m/z based upon different velocities acquired in an electrostatic field. Since all ions are accelerated through the same electrostatic field, they all have the same nominal kinetic energy, and their velocities will thus vary inversely with mass-to-charge ratio. A mass spectrum is obtained by monitoring ions arriving at the detector as a function of time after "drifting" through a field-free region. The defining relationships that equate drift time to mass-to-charge ratio are given in Eq. (2):

$$t = \frac{L}{v} = L\left(\frac{1}{2V}\right)\left(\frac{m}{z}\right)^{1/2} \tag{2}$$

where t is the time for the ion to reach the detector, L the length of the tube, v the ion velocity, and V is the accelerating voltage.

The most significant effect that limits resolution in time-of-flight analysers is the dispersion in kinetic energies pro-

duced during injection into the field-free region. Ions of a particular mass traveling faster than ions of the same mass arrive at the detector sooner, producing a spread in the peak for that molecule. While a number of approaches have been taken to minimize this effect, the most successful by far has been the development of the reflectron [5], a series of electrical lenses that compensate for variations in ion velocity. Ions traverse the first field-free region and enter the reflectron, where their velocity is reduced and then reversed. Ions are then injected back into a second field-free region and finally detected. Faster-moving ions penetrate farther into the lens system and thus spend less time in the field-free region than slower moving ions of the same mass. This extra time spent in the reflectron compensates for their faster velocity, and all ions of the same mass arrive at the detector in a narrow time window.

Time-delay extraction is an additional technique for enhancing the resolution of TOF analyzers. Ions are stored in an external field for a fixed time period, and then introduced into the reflectron TOF in a very narrow burst. Resolutions of up to 15,000 can be routinely achieved over very broad mass ranges, extending up to about 300,000 Da. Time-of-flight reflectron mass spectrometry with time-delay extraction is thus a popular technique for characterizing large molecules. They are also very fast, with mass spectra often obtainable in about 25 μs.

2.3. Fourier-transform ion cyclotron resonance mass spectrometry

Fourier-transform ion cyclotron resonance (FT-ICR) mass spectrometry uses the circular motion of ions in a magnetic field to measure mass–charge ratios. The velocity and thus angular frequency of this cyclotron motion is given by Eq. (3), where ω is frequency, B the magnitude of the magnetic field, and m and z have their usual meaning.

$$\omega = B\frac{z}{m} \tag{3}$$

In ICR mass spectrometry, frequency is measured, not arrival of ions at a detector. Frequency is inherently the most precise physical parameter that can be measured and largely accounts for the power of ICR mass spectrometry.

Fig. 1 is a schematic representation of an ICR trap. The first step in the FT-ICR experiment is to excite ions coherently to a larger and detectable radius. This is accomplished by applying a broadband radio frequency voltage pulse at the transmitter plates. When the radio frequency matches the cyclotron frequency of a particular ion, the ion absorbs energy and increases in velocity and radius. Ions of a particular m/z ratio will form a coherent ion packet, and the packet generates an 'image current' each time it comes into proximity with the detector plate. This image current decays over time as the ion packet loses coherence. The frequency of this transient current is characteristic of the m/z ratio of the ions. The frequency information, and thus the mass-to-charge ratios of ions present, is extracted by performing a Fourier transformation on the time-domain signal, producing frequencies of ions that are converted to m/z via a straightforward, 2nd order calibration equation.

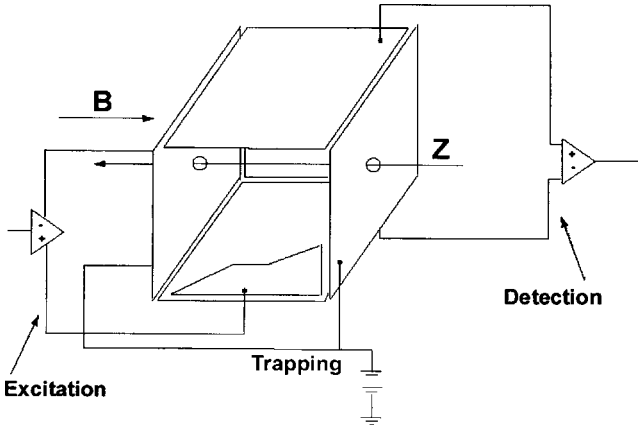

Fig. 1. Schematic representation of an ion cyclotron resonance ion trap. Ions are trapped in the x–y-plane by the magnetic field (B) and held in the z-plane by a trapping potential applied to the end plates. Excitation occurs by applying broadband radio frequency voltage pulse to the transmitter plates, followed by detection of the image current on the same plates. Figure adapted from Fig. 7 in [7].

Four mass spectral parameters that are particularly relevant to organic phosphorus speciation in natural environments are discussed briefly here. First, mass resolving power, R, increases linearly with increasing magnetic field [6]. High mass resolving power is necessary to analyze ions in complex mixtures where several ions per nominal mass may be present. It has also been observed that peak coalescence decreases quadratically with increasing magnetic field. Peak coalescence is a phenomenon in which ions of similar cyclotron frequencies couple, appearing in the mass spectrum as a single peak. This makes unique determination of the exact mass of an ion impossible, even when tandem mass spectrometry is utilized. Thirdly, the number of ions that can be trapped during one time event increases quadratically with increasing magnetic field, increasing signal-to-noise ratios. Finally, greater ion kinetic energy can be achieved, thus improving fragmentation efficiency in tandem mass spectrometry experiments.

Fourier-transform ion cyclotron resonance mass spectrometry at 9.4 T now offers the highest resolving power and mass accuracy of any currently available mass spectrometry technique [7,8]. For biogeochemical studies of dissolved organic matter, resolving powers of 500,000 have been achieved with mass accuracies of less than 1 ppm [9]. FT-ICR at 9.4 T is now referred to as 'ultrahigh resolution' mass spectrometry to distinguish it from current high-resolution double focusing and time-of-flight instruments, and is the only technique capable of resolving and identifying individual organic compounds in complex environmental matrices (Fig. 2).

2.4. Multi-stage mass spectrometry

The combination of two or more discrete mass analysis stages results in a very powerful technique referred to as multi-stage mass spectrometry. In tandem mass spectrometry, two mass analyzers are combined, with a reactor region inserted between them where chemical reactions are allowed to occur. The first analyzer acts as a mass filter, allowing in principle only a single molecular ion to be transferred to the reaction section. Here the original *precursor ion* is fragmented by any one of a number of processes, and the resulting fragments, or *product ions*, are then transferred to the second analyzer where mass analysis is carried out. This type of tandem mass spectrometry is often referred to as MS/MS. Instruments in which this process is repeated, with product ions from the first tandem serving as precursor ions for an additional tandem spectrometer, is referred to as MS/MS/MS/MS [10].

An experiment similar in principle to the tandem mass spectrometry just described can be carried out by FT-ICR mass spectrometry. The technique is not truly tandem, but better described as two-dimensional (in time) because the entire process of precursor isolation, precursor reaction and product mass analysis can be done in a single cell.

Two-dimensional FT-ICR MS begins with application of the stored waveform inverse Fourier-transform (SWIFT) excitation that removes all but a single chosen ion from the ion trap [11]. After removal of all ions except that chosen, this single *precursor ion* is fragmented and the *product ions* produced from fragmentation mass analyzed as previously described. Fig. 3 demonstrates the FT-ICR two-dimensional mass spectrometry experiment with spectra of a fulvic acid molecule and its collision-induced dissociation products.

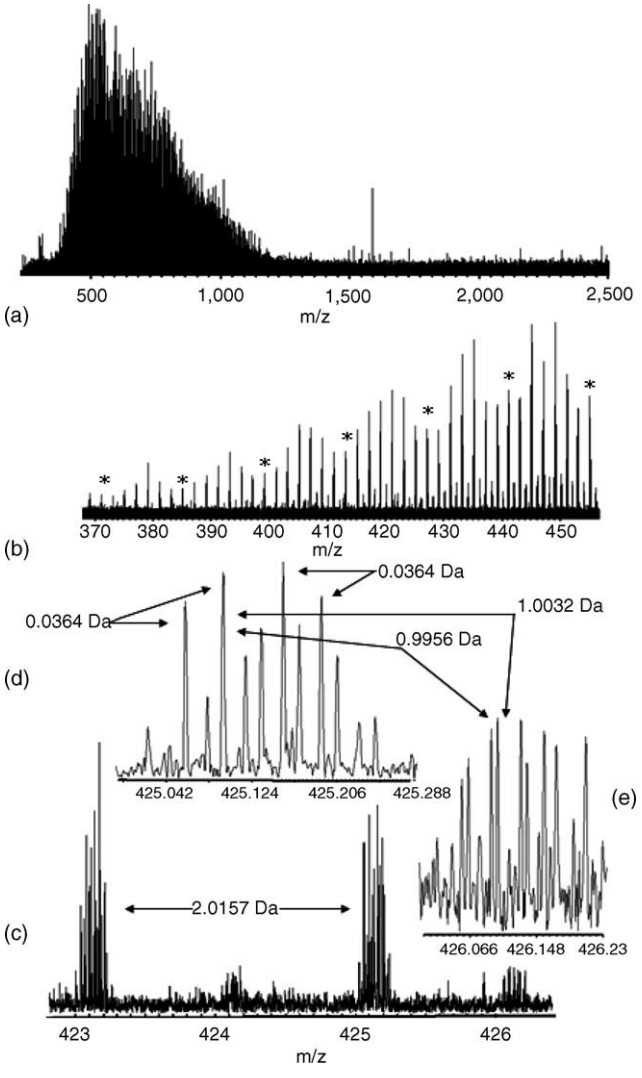

Fig. 2. Positive mode electrospray ionization Fourier-transform ion cyclotron resonance mass spectrum of Suwannee River fulvic acid mixture. (a) Entire spectrum over 225–3000 *m/z* region; (b) and (c) after scale expansion to highlight the 370–450 and 423–426 *m/z* regions; (d) and (e) after further expansion to highlight 0.3 mass windows. Reprinted from [3] with permission from the American Chemical Society.

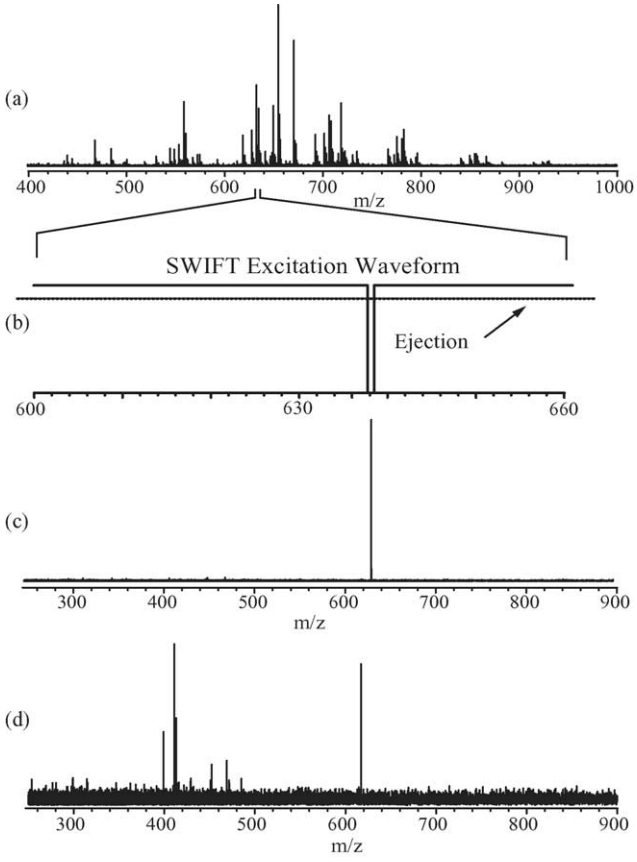

Fig. 3. Series of electrospray ionization Fourier-transform ion cyclotron resonance mass spectra obtained in a two-dimensional mass spectrometry experiment. (a) Full mass spectrum of fulvic acid mixture; (b) stored waveform inverse Fourier-transform (SWIFT) waveform ejection from the ion cyclotron resonance cell of ions of all but a narrow *m/z* range; (c) the resulting isolated parent ion mass spectrum; (d) the product ion mass spectra produced by collision-induced dissociation. Reprinted from [4] with permission from the American Chemical Society.

3. Ionization

Much of the motivation for the rapid improvements in mass spectrometry technology that have occurred over the past 10–15 years was the result of the interest in characterizing large, polar biomolecules. Unfortunately, traditional electron impact and chemical ionization methods can only produce intact, gas-phase ions of volatile and semivolatile molecules below about 300 Da. However, within a short period, both matrix assisted laser desorption ionization (MALDI) and electrospray ionization (ESI) were introduced and found widespread applications in biomolecular mass spectrometry. Both are soft ionization techniques that result in little molecular fragmentation. It should be noted that the importance of these breakthrough technologies in biomolecular mass spectrometry were recognized by Nobel Prize Awards in 2002 [12] to their developers; John Fenn (electrospray ionization) [13] and Koichi Tanaka (matrix assisted laser desorption ionization) [14].

To date no matrix entirely suitable for organic phosphorus compounds has been developed, and electrospray is now the ionization method of choice for organic phosphates. ESI mass spectra are characterized by intact molecular ions that often have multiple charges. High charge states are desirable because they reduce the mass-to-charge ratios of large molecules because of the high z. Then, mass analyzers with limited mass ranges can be used. Unfortunately, phosphates are not very efficiently ionized in an ESI source, particularly in complex mixtures (e.g. natural DOM) where other molecules that more effectively compete for charge are present [15].

The inductively coupled plasma ion source accomplishes exactly the opposite of the two soft ionization methods just described. Molecules are reduced to their atomic (i.e. elemental) components through the application of intense energy. Masses corresponding to elements of interest (e.g. 30.974 for P) are then specifically monitored.

The ICP has become the ionization method of choice for elemental mass spectrometry. At typical plasma temperatures of 5000–10,000 °C, virtually all elements on the periodic chart are also atomized and ionized to varying degrees. Elemental mass spectrometry normally requires only low-resolution analyzers because unit mass resolution is typically required; i.e. the mass difference between elements, which is always equal to or greater than 1 Da. However, there are some elements which can be obscured by molecular fragments commonly present in IC plasmas and thus require higher resolution mass analyzers. Phosphorus is just such an element; high resolution and a large abundance sensitivity is necessary for phosphorus analyses because of interferences from $^{15}N^{16}O^{+}$ (30.9950) and $^{14}N^{16}O^{1}H^{+}$ (31.0581), both of which are present in the atmosphere and in a standard plasma and which are very near the P^{+} peak at 30.974 Da.

4. Quantitative phosphorus analysis by ICP ionization with high-resolution mass spectrometry

There are many environments where total phosphorus levels are very low but where small changes in P concentrations can and are having pronounced effects. The formerly oligotrophic Florida Everglades is just such an ecosystem [16–18]. The pristine Everglades, with typical total P concentrations of ∼10 μg/L, are dominated by sawgrass fields and wet prairies. However, agricultural and domestic runoff with P concentrations exceeding 200 μg/L have caused significant taxonomic shifts in certain areas, with dense stands of cattails now dominating in these impacted areas. Analytical methods capable of detecting low and subtle changes in phosphorus concentrations, including various forms of organic phosphorus that often comprise up to 50% of the total P present [19], are therefore required to fully understand the community-level changes induced by phosphorus loading in such oligotrophic systems.

4.1. Quantitative reliability of ICP-HRMS

As noted previously, high resolution and a large abundance sensitivity is necessary for phosphorus analyses because of interferences from $^{15}N^{16}O^{+}$ and $^{14}N^{16}O^{1}H^{+}$ which are present in a standard plasma and which are very near the P^{+} peak at 30.974 Da. These interferences can be overcome by using a high-resolution mass analyzer. Fig. 4 is a mass spectrum demonstrating this approach. This spectrum was acquired on the Finnigan MAT Element ICP-MS system located in the Geochemistry Laboratory at the National High Magnetic Field Laboratory. The Element is a double

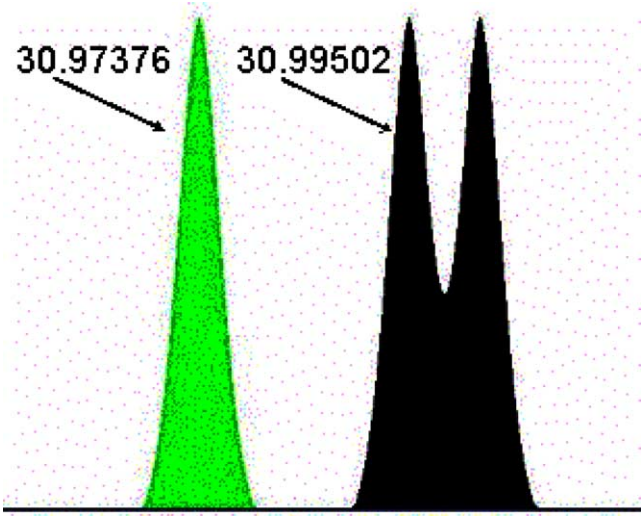

Fig. 4. Medium-resolution mass spectrum ($R = 3000$) demonstrating separation of the P^{+} peak at 30.97376 Da from the $^{15}N^{16}O^{+}$ peak at 30.99502 Da. This spectrum obtained on the Finnigan Element ICP-MS system in the Geochemistry Laboratory at the National High Magnetic Field Laboratory, Tallahassee, FL USA.

focusing sector instrument with reversed Nier–Johnson (BE) geometry with high resolution (>8000 resolving power) and excellent abundance sensitivity [20]. Abundance sensitivity is the ability to resolve and quantify a very small peak adjacent to a large matrix or background peak. For quantitative P analyses, only the medium resolution capability ($R > 3000$) of the Element is required.

It should be noted that there are other approaches to removing these interferences, including the use of a reaction cell to convert P^+ to its stable oxide, PO^+, a species that is generally free from such intereferences. Bandura et al. [21], for example, described such a reaction cell that included application of an axial field for focusing these oxide ions. Detection limits for phosphorylated proteins with their system approached 1 μg-P/L. Another technique for suppressing the NO and NOH interferences included the use of a hexapole collision and reaction cell in front of the mass analyzer [22]. Both of these techniques have the advantage that low-resolution mass spectrometers (e.g. quadrupoles) can be used.

The quantitative reliability of ICP-HRMS for low-level phosphorus analyses was demonstrated by comparing it to the standard total dissolved phosphorus (TDP) batch method. That method includes a persulfate oxidation step to convert all forms of phosphorus to the ortho-phosphate form that is then suitable for the molybdenum blue reaction. Using the same set of standards, calibration curves for both methods were developed over a range of 0–200 μg-P/L. Figures of merit for the two methods are included in Table 1. The ICP-MS method is clearly superior to the classical method over this concentration range.

The two methods were also compared using actual data on field samples from various sites in the Everglades. Fig. 5 is a plot in which results of the two methods are regressed against each other, with a regression line of unit slope included. This data supports previous conclusions that the colorimetric method overestimates P concentrations at low levels due to interferences from silica, germanium and arsenic [23], and underestimates higher concentrations due to incomplete reactions and color development [24].

Table 1
Analytical parameters for comparison of the ICP-MS and colorimetric methods of phosphorus determinations

Figure of merit	ICP-MS method	Colorimetric method
Calibration sensitivity (m)[a]	38.1 ± 0.152	0.004 ± 0.0004
Analytical sensitivity (γ)[b]	316	1.70
Limit of detection (LOD)[c]	~0.4 μg-P/L	~3.5 μg-P/L
Limit of quantitation (MQL)[d]	~1.7 μg-P/L	~12 μg-P/L

[a] Slope of the calibration curve.
[b] Slope of the calibration curve by the S.D. of the measurement.
[c] The concentration that produces a signal equal to the average plus 3 S.D. of the blank signal.
[d] The concentration that produces a signal equal to the average plus 10 S.D. of the blank signal.

4.2. High-resolution separation methods with ICP-HRMS detection

In addition to providing superior quantitative analyses of total dissolved phosphorus, ICP with high-resolution mass analysis is an ideal detector for separation methods in which various forms of phosphorus are first physically resolved. Indeed, in situations like the Everglades where the total phosphorus levels are low, an ICP-HRMS is the only detector with sufficient sensitivity and selectivity to detect individual phosphorus-containing compounds. The selectivity of ICP-HRMS detection is especially important in oligotrophic wetlands where organic carbon is up to 1000× greater in abundance than organic phosphorus. With P-specific MS detection, organic compounds without phosphorus are essentially invisible to the detector.

One of the most powerful separation methods now available is CE. CE with ICP-MS detection is potentially a very powerful combination for determining the speciation of many elements, not just phosphorus. However, interfacing the very low flow CE column to the ICP torch is quite challenging, and normally some degradation of the CE separating capacity results [25,26]. Nevertheless, CE-ICP-MS is finding increasing use for element speciation studies [27].

The electropherogram in Fig. 6 demonstrates the potential of CE-ICP-MS for organic phosphorus speciation. This is a "phosphogram" showing the presence of individual organic phosphorus compounds in an Everglades treatment wetland. We refer to this as a "phosphogram" because the eluent from the CE column is being detected by the Element ICP-HR-MS system using selected ion monitoring at $m/z = 30.974$ Da. Peaks in the phosphogram represent individual organic phosphorus compounds which have not yet been qualitatively identified.

High performance liquid chromatography does not provide the separation power of CE, but is much more easily interfaced to an ICP-MS for P-specific detection since the mass of analyte introduced to the mass spectrometer is much greater and flow artifacts that degrade resolution in CE are not

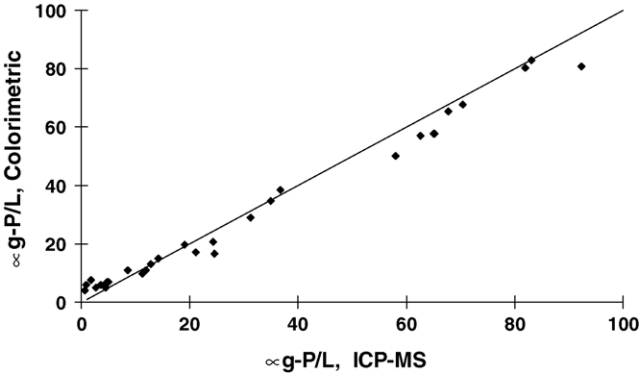

Fig. 5. Comparison of total phosphate data determined by inductively coupled plasma mass spectrometry (ICP-MS) and colorimetric methods; solid line is linear regression, indicating a 1:1 relationship.

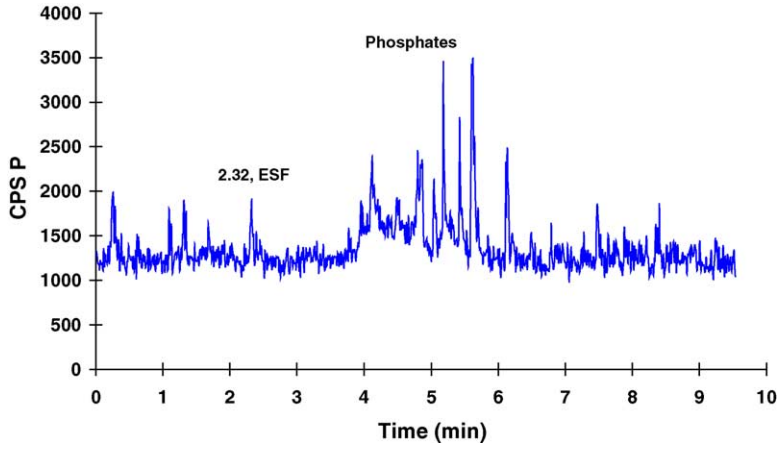

Fig. 6. Capillary electropherogram with P-specific ICP-HRMS detection ("phosphogram"). The peak at 2.32 min is the electroosmotic flow marker; peaks between 4 and 7 min are organic phosphorus compounds.

present. However, since phosphates are ionic in aqueous solutions, simple reversed phase HPLC cannot effectively separate them due to lack of partitioning into the organic stationary phase. By adding an appropriate ion-pairing reagent to the mobile phase, e.g. tetrabutylammonium chloride, the normally hydrophobic stationary phase becomes coated with the relatively hydrophobic cation (tetrabutylammonium). Phosphate anions can then form ion-pairs with the exposed positive charges on the stationary phase surface, providing an ion-exchange type mechanism [28,29]. This technique is referred to as ion pairing high performance LC (IP-HPLC).

Fig. 7 is an IP-HPLC separation of a mixture of organic phosphate standards, demonstrating the capability of IP-HPLC with P-specific MS detection to distinguish broad classes of org-P compounds. The phosphograms in Fig. 8, obtained using this IP-HPLC system, are of the same organic phosphorus extracts from the Everglades treatment wetland as previously analysed by CE-ICP-HRMS (Fig. 6). Fig. 8(a) is a sample from the inflow to the wetland after phosphorus was selectively concentrated (see Section 5), while Fig. 8(b) is an analogous sample from the outflow. These IP-HPLC phosphograms taken together demonstrate the conversion of phosphorus from essentially all ortho-phosphate to a variety of organic phosphates, indicating biological uptake of the inorganic P. This is, of course, the exact purpose of the treatment wetland.

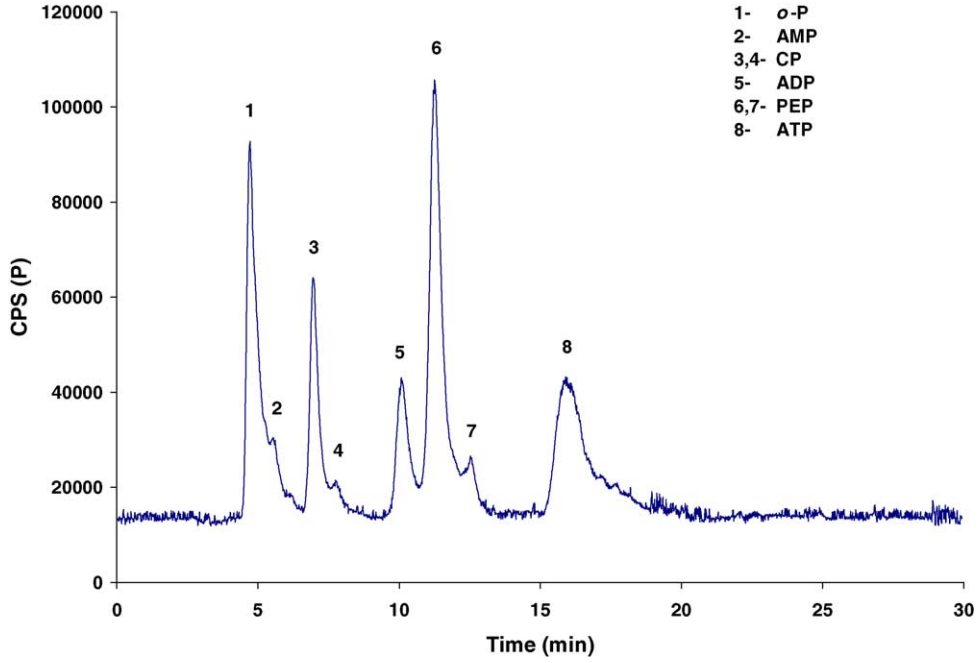

Fig. 7. Optimized separation of organic phosphate standards by ion pair chromatography with direct, on-line with P-specific ICP-HRMS detection (AMP, ADP, ATP = adenosine mono-, di- and triphosphate; CP = creatinephosphate; PEP = phosphoenolpyruvate). The peak at ~5 min is ortho-phosphate.

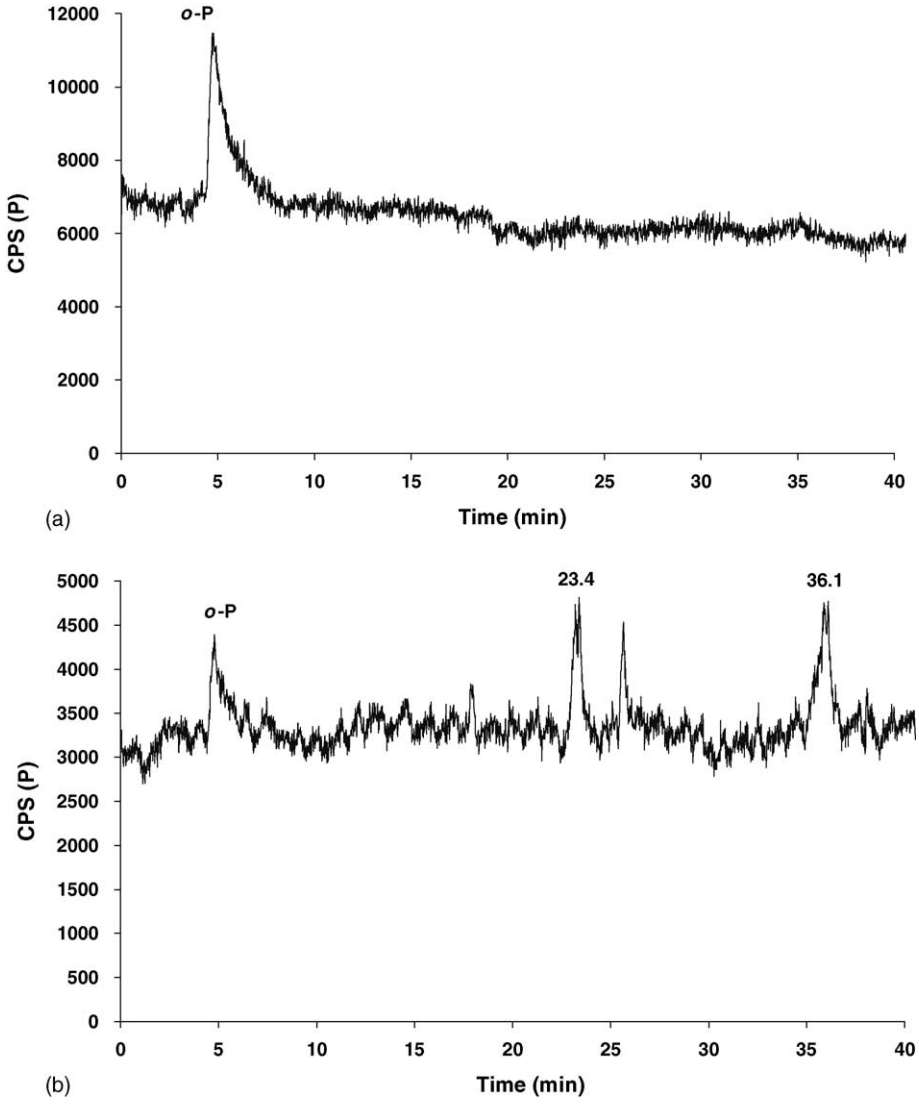

Fig. 8. Ion pair HPLC separation of organic phosphorus compounds with P-specific ICP-HRMS detection from (a) treatment wetland inflow and (b) treatment wetland outflow.

5. Qualitative analysis of organic phosphorus by electrospray ionization and ultrahigh resolution FT-ICR mass spectrometry

5.1. Concentration and isolation of DOP

We have already discussed the difficulty in trying to quantify organic phosphorus compounds that are present at relatively low levels in an overwhelming background of dissolved organic carbon molecules. Qualitatively identifying individual org-P compounds in an oligotrophic system such as the Everglades, where total DOP is ∼1000× lower in concentration than total DOC, is a similarly difficult challenge. Therefore, before any molecular identifications of individual DOP compounds in the Everglades can be accomplished, preconcentration of DOP from sub-ppb levels must be achieved.

We use a combination of tangential cross flow filtration (CFF) and phosphorus precipitation to get DOP fractions that are sufficiently concentrated and relatively free of interfering DOM. CFF is a popular method for fractionating natural dissolved organic matter into molecular weight fractions [30–32]. CFF can also concentrate organic species retained by the membrane (i.e. the “retentate”) by up to 20-fold. An additional step that further concentrates DOP and isolates it from the high-level DOM background was also developed [15]. This method, which was an adaptation of one used by Anderson and McKercher to isolate inositol penta- and hexaphosphates from soil [33,34], involves precipitation with barium acetate followed by reconstitution in distilled water and removal of excess barium by ion-exchange. The effectiveness of this isolation can be appreciated by comparing the spectra in Fig. 9. Reducing the number of other non-phosphorus containing ions in the analyzer cell significantly improves signal-to-noise ratios and mass resolution and allows the determination of unambiguous molecular formulas for many compounds [15]. While the question of alteration of phospho-

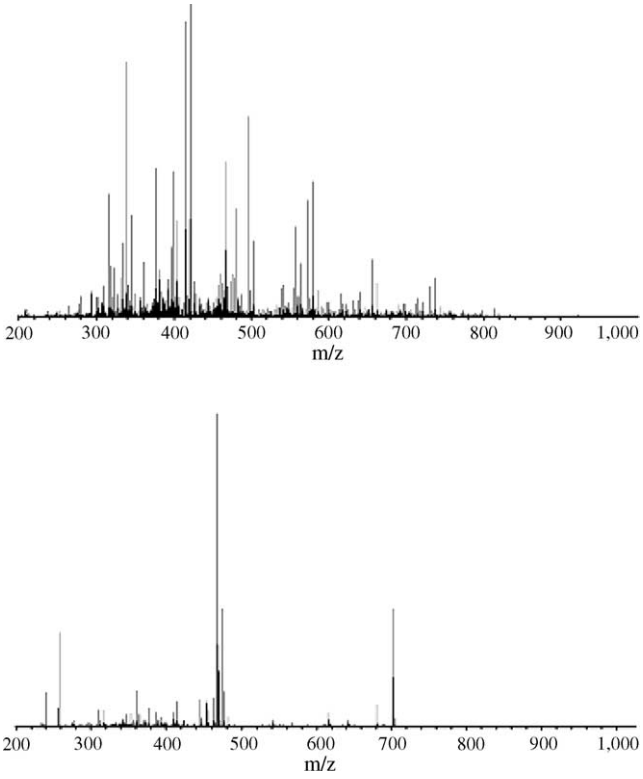

Fig. 9. Electrospray ion cyclotron resonance mass spectra of dissolved organic matter isolated from experimental nutrient removal wetland outflow before (top) and after (bottom) selective organic phosphorus concentration. Reprinted from [15] with permission from the American Chemical Society.

rus speciation by the CFF and phosphorus pre-concentration steps cannot be answered at present, it should be noted that organic phosphorus standards carried through the entire procedure were observed unaltered in FT-ICR mass spectra [15].

5.2. Chemical formulas of organic phosphorus compounds from exact mass measurements

Very low mass errors are required if organic compounds are to be identified by chemical formulas based solely on mass as determined from a mass spectrum. Table 2 lists measured and theoretical masses of five standards used to validate the phosphorus isolation procedure, as determined by ultrahigh resolution FT-ICR mass spectrometry. With these mass accuracies of less than 1 ppm, unequivocal chemical formulas can be assigned. It should be noted that the ultrahigh mass accuracy of FT-ICR MS could be realized only when the number of different ions in the cell is minimized through a selective isolation and pre-concentration procedure such as that we used.

Table 2
Measured and theoretical masses of organic phosphorus standards

Measured mass (Da)	Theoretical mass (Da)	Elemental composition	Mass error (ppm)[a]
547.9668	547.9666	$2[C_5H_9O_8PNa_2]$	−0.36
369.0451	369.0451	$C_{10}H_{13}N_5O_7PNa$	0.00
347.0630	347.0631	$C_{10}H_{14}N_5O_7P$	+0.29
273.9833	273.9833	$C_5H_9O_8PNa_2$	0.00
261.0402	261.0402	$C_9H_{12}NO_6P$	0.00

[a] Mass error (in ppm) = $(\Delta m/m) \times 10^6$, where Δm is the uncertainty in the determined mass m.

Table 3
Exact masses and elemental compositions of organic phosphorus molecules observed in an everglades treatment wetland inflow

Measured mass (Da)	Theoretical mass (Da)	Elemental composition	Mass error (ppm)[a]
478.1186	478.1190	$C_{13}H_{24}N_6O_{10}PNa$	+0.82
474.3182	474.3184	$C_{24}H_{43}N_4O_4Na$	+0.44
443.3248	443.3249	$C_{24}H_{45}NO_6$	+0.22
415.2934	415.2936	$C_{22}H_{41}NO_6$	+0.34

In our studies of organic phosphorus speciation in an Everglades treatment wetland, we concentrated organic matter by CFF and further isolated DOP from surface waters at four sites. These sites represent a gradation in water residence time within the wetland and include the inflow and integrated outflow. The concentrates were then analyzed by ultrahigh resolution FT-ICR with electrospray ionization. Detailed mass analysis of each peak in these spectra allowed several phosphorus-containing compounds to be identified. Table 3 lists the org-P compounds identified in the inflow alone. Calculation of elemental compositions based on mass was made possible by the relatively low mass errors. For org-P compounds, only C, H, N, O, P, Na, S, and Fe could be included in calculated formulas. Because the number of chemical formulas possible within a given mass window increase with increasing mass, it is impossible to make unambiguous assignments much beyond ∼600 Da from this data alone.

Table 4 includes a list of formulas of organic compounds containing phosphorus that were identified in surface waters at all four sampling sites. These would appear to be part of a refractory high-molecular weight DOP fraction that is nonlabile to mono- and di-ester phosphatase enzymes [35]. We believe that the compounds we have identified by FT-ICR MS are associated in some way with high-molecular weight humic-like molecules, and this association renders them unavailable to microorganisms. This conclusion is based on the

Table 4
Exact masses and elemental compositions of organic phosphorus molecules identified at all sites in the everglades treatment wetland

Mass (Da)	Elemental composition
592.15044	$C_{18}H_{30}N_6O_{13}PNa$
577.20237	$C_{22}H_{33}N_7O_8PNa$
518.13138	$C_{21}H_{23}N_6O_8P$
451.10521	$C_8H_{19}N_{11}O_8PNa$
444.11291	$C_{11}H_{17}N_{12}O_6P$
369.09506	$C_{12}H_{16}N_7O_5P$
364.12881	$C_{15}H_{25}O_8P$

fact that these org-P compounds were isolated with a 1000 Da CFF membrane.

In addition to the compounds listed in Table 4, there were a number of org-P compounds found only in water from the treatment wetland outflow. It might be reasonable to assume that these were compounds formed within the treatment areas, although further quantitative experiments would be needed to verify this. Unfortunately, there are too few compounds identified to draw any meaningful conclusions regarding the chemical properties of org-P formed within the wetland vs. that which is present in the inflow and is refractory during its residence.

5.3. Structural studies of organic phosphorus compounds by two-dimensional FT-ICR MS (FT-ICR MS/MS)

While formula assignments by FT-ICR mass spectrometry represent a significant advance in understanding organic phosphorus speciation, even more information is required to better understand the structures of the phosphorus compounds so far identified. We therefore carried out a series of MS/MS experiments on selected ions thought to contain P. One of our primary goals was to develop a mass spectrometry method that would quickly identify molecules that contained phosphorus by observing product ions which had "neutral losses" consistent with P ($\Delta m = 31$), PO_3 ($\Delta m = 79$), or H_3PO_4 ($\Delta m = 98$) [36].

Fig. 10 includes a SWIFT isolated spectrum of one ion observed in the Everglades treatment wetland, along with the spectrum of this same ion after collision induced dissociation (CID) fragmentation. From the product ion spectrum it is possible to reconstruct the precursor molecule and verify its original chemical formula assignment. This approach is useful for determining the principle labile functional groups on a molecule, as well as for assigning unequivocal chemical formulas for which the exact mass is insufficient to make such an assignment (e.g. too many formula possibilities). For example, the molecular mass of the parent ion in Figure 21 is ~453 Da, and at this mass even high-resolution FT-ICR MS with a mass uncertainty 1 ppm cannot provide an unequivocal formula for this compound. However, we can calculate very precisely the masses of fragments lost from the parent ion, and this allows us to determine the exact formulas of the fragments. In this case we see losses that correspond to H_2O, $C_6H_{13}NO$, and $C_{12}H_{22}N_2O_3$ fragments, and we can then put these fragments back together to get the formula of the original parent molecule.

Unfortunately, CID fragmentation did not result in neutral losses from any of the ions we analyzed which could be related to phosphorus or phosphorus-containing fragments. We then carried out a similar experiment with an adenosine monophosphate standard, and again did not observe any recognizable P fragments. It thus appears that C–O–P bonds in organophosphates are too strong to be broken by these fragmentation techniques. Work is currently underway in our lab to find a fragmentation method that will break these C–O–P bonds and thus readily identify organic phosphorus compounds.

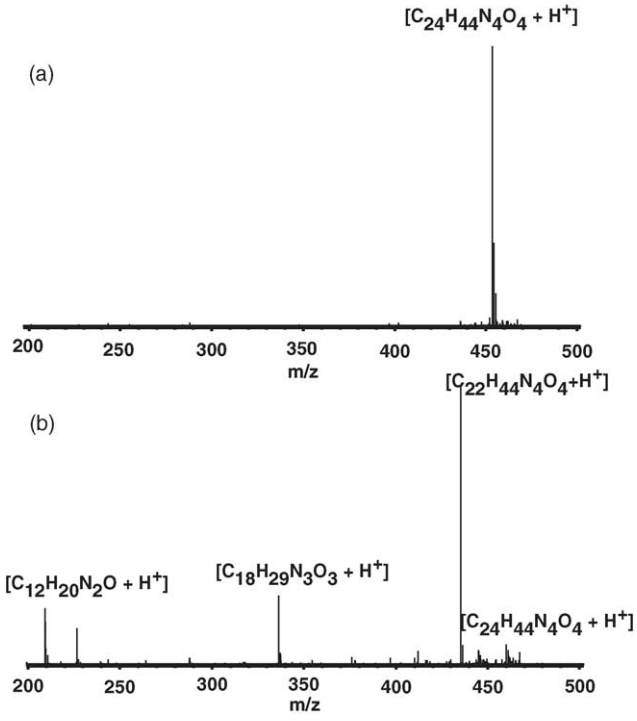

Fig. 10. (a) Spectrum of a stored waveform inverse Fourier-transform isolated ion at nominal mass 453 *m/z*. This molecule is present in dissolved organic matter at the treatment wetland outflow. (b) Spectrum of this ion and resulting products after fragmentation by collision induced dissociation.

6. Discussion

In this article, we have summarized our work on organic phosphorus speciation in an oligotrophic wetland. We believe it is the first attempt at using modern, high-resolution mass spectrometry techniques for such a study. While certainly not comprehensive, our data demonstrate that molecular speciation information on individual DOP compounds can be obtained, in spite of the very low levels (<1 μg-P/L) at which they are found and amidst the large background of other natural organic carbon compounds.

High-resolution MS with ICP ionization is now a proven technique for measuring phosphorus at the same levels as the conventional batch colorimetric test. More importantly, ICP-HRMS is useful as a P-specific detector for liquid chromatography and capillary electrophoresis, an application that may well prove to be invaluable in quantitative org-P speciation studies.

Qualitative identification of individual organic phosphorus compounds now appears possible with electrospray ionization and high-resolution mass spectrometry. While we have used FT-ICR mass analysis, other high-resolution mass analyzers would also be appropriate. However, it is obvious

that, regardless of the analyzer used, these studies will always require concentration and very selective isolation of target phosphorus compounds. Finally, one particularly important development that would significantly advance this field would be a fragmentation technique that would result in product ions with phosphorus signals; e.g. loss of P, PO_3, or H_3PO_4.

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