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# Traceable Phosphorus Measurements by ICP-OES and HPLC for the Quantitation of DNA

**Marcia J. Holden,* Savelas A. Rabb, Yadu B. Tewari, and Michael R. Winchester**

*Chemical Science and Technology Laboratory, National Institute of Standards and Technology, 100 Bureau Drive, Gaithersburg, Maryland 20899-8311*

**Measurement of the phosphorus content of nucleotides and deoxyribonucleic acid (DNA) offers an approach to the quantitation of nucleic acids that is traceable to the SI. Such measurements can be an alternative to the commonly used spectroscopic tools that are not traceable. Phosphorus measurements of thymidine 5′-monophosphate (TMP) and acid-digested plasmid and genomic DNA preparations were made using high-performance inductively coupled plasma optical emission spectroscopy (HP-ICP-OES) and high-performance liquid chromatography (HPLC) and compared for bias and uncertainty. A prerequisite for quality measurement is the purity of the materials. Quantitation with the two platforms was comparable for the TMP. However, the HPLC values had larger uncertainties and were all statistically different from the gravimetric values at the 95% confidence level. When using ICP-OES, the digestion of the nucleotide monophosphate can be eliminated, thus simplifying the procedure. The differences between the results obtained by using the two platforms, when measuring genomic or plasmid DNA, were dependent on the mass fraction of the digest. ICP-OES measurement of phosphorus provides a highly accurate quantitation for both nucleotide monophosphates and DNA with expanded uncertainties of less than 0.1%. Currently, ICP-OES requires a significant sample size restricting its usefulness for the quantitation of DNA but represents a valuable tool for certification of reference materials. HPLC requires smaller amounts of material to perform the analysis but is less useful for certification of reference materials because of lower accuracy and 10-fold higher expanded uncertainties.**

The accurate determination of the amount of DNA isolated from a biological material is not trivial. The methods in common use are spectroscopic measurements. Measurement of absorbance of nucleic acids in the ultraviolet (260 nm) has been a common laboratory technique for 60 years since Warburg and Christian[1] introduced the idea of quantitating DNA that contaminated protein preparations. However, this method is not sensitive enough for small quantities of DNA. Interference by copurified protein and some reagents used in DNA isolation, such as phenol, lead to incorrect assessments of the quantity of DNA present. Furthermore, the method does not distinguish between ribonucleic acid (RNA) and DNA.

More recently developed fluorescent methods depend on a signal generated upon binding of a dye that either intercalates between DNA bases or binds in the minor groove of DNA. Two dyes in common use are PicoGreen and Hoechst 33258.[2,3] These methods are more sensitive for the quantitation of small amounts of DNA, and some dyes can distinguish between DNA and RNA. While protein does not interfere in the measurements, some other chemicals can. Kit manufacturers frequently provide the fluorescent dyes and the DNA for calibration, which has been quantitated by absorbance at 260 nm, so that in the end the accuracy is again dependent on absorbance.

There is a continuing interest in finding accurate ways to quantitate DNA. Other approaches currently under investigation include mass spectrometry and nuclear magnetic resonance. Enzymatic digestion to individual nucleotides followed by chromatography and isotope dilution mass spectrometry has been used to quantitate a 20-mer oligonucleotide.[4] Measurement of all four nucleoside and nucleotide components demonstrated that the ratios of measured components matched the theoretical based on the known sequence of the oligonucleotide.

The production of nucleic acid Certified Reference Materials (CRMs) will require an accurate and traceable measurement for the certification of the mass fraction of material in a preparation. Long-established methods in common laboratory use suffer from the above-mentioned interferences and lack good uncertainty estimates. The use of phosphorus measurements performed on purified DNA and incorporating the use of an appropriate CRM as a calibrant would provide a traceable methodology for the certification of DNA quantity. The use of phosphorus content to quantitate DNA was introduced by Chargaff and Zamenhof[5] in 1948 and was the original method for determining an extinction coefficient for the quantification of DNA.[6]

Phosphorus measurements have previously been made using ICP-MS[7−16] and ICP-OES[17−19] in the determination of biomolecules

* To whom correspondence should be addressed. E-mail: marcia.holden@nist.gov.

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(e.g., proteins, nucleotides, and DNA). ICP-MS is usually preferred to ICP-OES due to higher P sensitivity and lower detection limits, in particular for analytical separations. On-line separation techniques are being used to detect the degree of phosphorylation in proteins[8,9] and the formation of DNA fragments and adducts.[14−16] These processes could result in low P concentrations, making low detection limits a necessity for quantitative analyses. Edler et al.[15] monitored the presence of DNA adducts in the presence of styrene oxide with HPLC−ICP-MS. Results showed adduct formation occurred in 3.6 nucleotides for every 1000 (3.3 $\mu$g mL$^{-1}$ P). This observation was well above the detection limit of 3 modified nucleotides for every $10^7$ (0.28 ng mL$^{-1}$ P). Profrock et al.[20] evaluated the determination of P in monophosphates and DNA using an octopole reaction system-ICP-MS in conjunction with either capillary electrophoresis or HPLC. Detection limits ranged from 3 to 90 $\mu$g L$^{-1}$ P for the individual nucleotides. Interferences can often plague phosphorus ICP-MS measurements, particularly in quadrupole instruments.[9,15,20] However, these interferences can be overcome with the utilization of reaction cell, collision cell, or sector field MS systems. Notably, none of the cited ICP-MS publications focused on evaluations of analytical bias and uncertainty associated with quantitative determinations.

ICP-OES can also be used to conduct measurements on biomolecules, provided the analyses are not limited by the sample quantity or P concentration. Donald et al.[18] found that ICP-OES results were within 1.8% of those of isotope dilution mass spectrometry for an enzyme-digested oligonucleotide. The relative uncertainty for ICP-OES measurements was 4.6% (95% confidence interval). Yang et al.[17] also evaluated the mass of oligonucleotides and achieved relative uncertainties on the order of 1%. English et al.[19] utilized ICP-OES measurements to compare the accuracy of DNA quantitation approaches by absorbance and fluorescence. Biases were observed (8−38%) in the absorbance and fluorescence measurements due likely to contaminants and changes in the structural orientation of the DNA.

At the National Institute of Standards and Technology (NIST), we are interested in developing methodologies that can be used to provide accurate measurements of DNA and nucleic acid mass that are traceable to the SI. One of our long-term goals is to provide Standard Reference Materials (SRMs) having certified values for DNA mass with small uncertainties. This paper reports on a study regarding the implementation of ICP-OES and HPLC determinations of P in DNA and nucleic acid samples for such purposes. A systematic approach was applied to this study by quantitating the amount of phosphorus in digested and undigested nucleic acid preparations, including thymidine 5′-monophosphate (TMP), plasmid DNA, and genomic DNA from calf thymus. An ICP-OES approach referred to as high-performance ICP-OES (HP-ICP-OES) was utilized for these analyses. It is a ratio-based technique that incorporates a drift correction.[21] Expanded uncertainties on the order of 0.1% have been observed using HP-ICP-OES for single-element solutions. HP-ICP-OES was designed to make traceable measurements with small uncertainties.[22,23] However, large sample sizes (10 mL of 50 $\mu$g of DNA mL$^{-1}$) are usually required, making the HP-ICP-OES technique less useful for routine analyses of nucleic acids, in particular DNA. Analysis by HPLC can be done with a much smaller sample size. For these reasons, we decided to undertake a study to compare HPLC and ICP-OES measurements in terms of accuracy and precision on representative samples.

## EXPERIMENTAL SECTION

**Preparation of Nucleotides and Nucleic Acids.** The nucleotide monophosphate used in the study was TMP (free acid, Sigma Chemical Co., T-9758). [Identification of commercial products in this paper was done in order to specify the experimental procedure. In no case does this imply endorsement or recommendation by the National Institute of Standards and Technology.] Determination of the mass fraction of water[24] in TMP was carried out using a Metrohm model 633 automatic Karl Fischer titrator and a model 665 Dosimat. The sample vessel was enclosed in a Plexiglas box, which was maintained under positive pressure with a flow of dry nitrogen gas. The solvent used for the titrations was a mixture of methanol and Hydranal Composite 2 solution. The end point was determined coulometrically with an appropriate correction for drift. The Karl Fischer titrator was calibrated with water-saturated 1-octanol solution. The reported mass fraction moisture content of 4.55% (±0.28%) at the 95% confidence level is based on the solubility of water in 1-octanol at 25 °C.[25]

The purity of the TMP sample was examined by using an Agilent HPLC model HP1100 equipped with an UV detector and Alltech ODS2 (150 mm × 4.5 mm) column thermostated at 35 °C (Table 1 for complete operating conditions). Samples were run at two wavelengths (210 and 260 nm), and only a single peak was observed. Based on this chromatographic analysis, we judge that the TMP was at least 99% pure (mass).

For the second HPLC analysis of TMP, a Nucleogen DEAE (125 mm × 4.0 mm) column was used (Table 1). Samples were run at two wavelengths (210 and 260 nm), and again, only a single peak was observed. Based on this chromatographic analysis, we


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**Table 1. Operating Conditions of Agilent HPLC Model HP1100 Equipped with a Variable-Wavelength UV Detector for Purity Check of TMP**

| | | |
|---|---|---|
| column | Alltech ODS2 (150 mm × 4.5 mm) | Nucleogen DEAE (125 mm × 4.0 mm) |
| temperature | 35 °C | 35 °C |
| wavelength | 210 and 260 nm | 210 and 260 nm |
| mobile phases | (I) 0.025 M $KH_2PO_4$ (pH 3.5) | (I) 0.01 M $KH_2PO_4$ (pH 3.0) |
| | (II) methanol | (II)0.1 M $K_2HPO_4$(pH 7.3) |
| gradient | $t = 0$ min; $\Phi(I) = 0.95$; $\Phi(II) = 0.05$ | $t = 0$ min; $\Phi(I) = 0.90$; $\Phi(II) = 0.10$ |
| | $t = 20$ min; $\Phi(I) = 0.70$; $\Phi(II) = 0.3$ | $t = 15$ min; $\Phi(I) = 0.20$; $\Phi(II) = 0.80$ |
| flow rate | 0.8 mL $min^{-1}$ | 1.0 mL $min^{-1}$ |
| retention time | TMP = 11.3 min | TMP = 3.1 min |

again judge the TMP to be at least 99% pure (mass).

The mass of the TMP was determined using a calibrated five decimal place balance (readability to 0.01 mg). True masses were obtained from the apparent masses in air by making buoyancy corrections. Density corrections were $\rho$(TMP) = 1.7 g $cm^{-3}$, $\rho$(air) = 0.0012 g $cm^{-3}$. The TMP was dissolved in 5% (vol) HCl. All solutions were made in Teflon bottles. Samples that were to be digested were placed in a 120 °C oven for 14 h. The mass fraction of phosphorus in TMP is 0.09613.

Plasmids were purified from *Escherichia coli* cellular lysates and further purified using CsCl/ethidium bromide equilibrium gradient centrifugation (Beckman L7−65 ultracentrifuge, VTI-65.2 rotor, 58K rpm, 24 h) to remove contaminating *E. coli* genomic DNA.[26] Ethidium bromide was removed from the DNA by chromatography on Dowex AG50W-X8, and the DNA was precipitated using 7.5 mol $L^{-1}$ ammonium acetate and 100% ethanol.[26] The purified plasmids were washed free of small molecules using centrifugal concentrators (10 or 30 kDa cutoff). A quantitative PCR assay (TaqMan chemistry) was used to monitor for any residual *E. coli* genomic DNA.[27] The target for amplification was a conserved region of the 23S rRNA gene.

The genomic DNA used in this study was deoxyribonucleic acid, sodium salt, from calf thymus (Sigma, D1501). The DNA was dry and in the form of fibers, which were dissolved in sterile TE buffer (10 mmol $L^{-1}$ Trizma base (2 amino-2-hydroxymethyl-1,3-propanediol), 1 mmol $L^{-1}$ ethylenediaminetetraacetic acid (EDTA), tetrasodium salt, pH 7.5). Starting solutions contained 1−2 mg $mL^{-1}$ of DNA, which was allowed to dissolve at 4 °C for at least 24 h with slow rotation of the tubes. Any residual RNA was digested with 0.1−0.5 mg $mL^{-1}$ of RNase at room temperature for 90 min. Protein contaminates were removed by incubation with Proteinase K (0.4 mg $mL^{-1}$) for 90 min at 37 °C. The DNA was extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1 by volume) and then twice with chloroform. Following these extractions, the DNA was precipitated with ammonium acetate and ethanol. The precipitated DNA was collected with a glass rod, washed three times with 70% (vol) ethanol and allowed to dry. Finally, the DNA was dissolved in sterile, RNase-free, DNase-free, pure water, and washed using centrifugal concentrators to remove any residual small molecules.

Electrophoresis of plasmid and genomic DNA to determine fragment size was done on 0.8% mass agarose gels in TBE buffer (90 mmol $L^{-1}$ boric acid, 90 mmol $L^{-1}$ Trizma base, 20 mmol $L^{-1}$ EDTA) and visualized with ethidium bromide. The most common fragment size in the cleaned genomic DNA preparation was the same as the DNA prior to cleaning (25−50 kb).

Absorbance of DNA in TE buffer was measured on a Beckman DU650 spectrometer over a range of 220−320 nm. DNA concentration estimates were based on an extinction coefficient for absorbance at 260 nm (1 cm path length) of 0.02 $\mu$g $mL^{-1}$ or DNA with an absorbance of 1 = 50 $\mu$g $mL^{-1}$. DNA preparations were checked for any residual protein using the BioRad protein assay with bovine serum albumin as a standard. The density of the DNA solution in TE buffer (600 $\mu$g of DNA $g^{-1}$) was measured using a DMA 35 density meter (Mettler/Paar, Graz, Austria) and was determined to be essentially the same as water (0.998 versus 0.997 g $cm^{-3}$ for pure water at 23 °C).

HCl was added gravimetrically to plasmid and calf thymus genomic DNA preparations so that concentrated HCl constituted 5% (mass) of the preparations, and the DNA was digested at 120 °C for 16 h in screw-capped, thick-walled Teflon bottles. Filled bottles were weighed before and after heat treatment to monitor any loss of water vapor through the seal.

**Phosphate Analysis. HPLC.** Analysis of phosphate[28] was carried out using a Dionex DX 500 ion chromatograph with an ED 50 conductivity detector (Table 2). The retention time of phosphate ion was 7.0 min. The phosphate peak was well separated from the chloride and the nitrate ion peaks, which had retention times less than 4 min.

For quantitative analysis, a standard phosphate solution was injected before and after each injection of the dilute digested phosphate sample solution. The mass fraction of phosphate in the standard and in the unknown sample were kept very close to each other. The mass fraction of the phosphate in the digested sample solution was determined from the average response factor (mass fraction/area) of the standard phosphate solution and the area of the dilute digested sample solution. The reported mass fractions are an average of at least five measurements. The samples were analyzed blindly, with the analyst given only the approximate mass fraction of the material so that suitable working solutions could be prepared.

**Phosphorus Measurements. ICP-OES.** HP-ICP-OES was used to determine P in the nucleic acid samples. Briefly, HP-ICP-OES[21−23] employs a careful experimental comparison of a sample with a calibration standard that is prepared to mimic the expected nature of the sample, in terms of both matrix and the analyte mass fraction. Any observed difference between the sample and standard is used to compute the analyte mass fraction in the

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**Table 2. Operating Conditions of Dionex DX 500 Ion Chromatograph with an ED 50 Conductivity Detector for Analysis of Phosphate**

| | |
|---|---|
| column | Dionex AS11 anion exchange (250 mm × 4 mm) |
| guard column | Dionex AG11 anion exchange (50 mm × 4 mm) |
| temperature | column set at 35 °C |
| detector | ED 50 conductivity cell set at $T = 35$ °C; temperature compensation, 1.7 °C$^{-1}$ |
| current | self-regenerating suppressor current was set at $i = 300$ mA |
| mobile phase | (I) water; (II) KOH generated by Dionex EG-40 eluent generator cartridge |
| gradient | $t = 0$ to 1 min Φ (I) = 0.90 |
| | $t = 1$ to 10 min Φ (I) = 0.90 to Φ (I) = 0.60 |
| | $t = 10$ to 14 min Φ (I) = 0.60 |
| | $t = 14$ to 17 min Φ (I) = 0.60 to Φ (I) = 0.10 |
| | $t = 17$ to 20 min Φ (I) = 0.10 |
| | $t = 20$ to 21 min Φ (I) = 0.10 to Φ (I) = 0.90 |
| flow rate | 1.0 cm$^3$ min$^{-1}$ |
| retention time | phosphate 7.00 min |

sample. An internal standard spike is used to help correct for high-frequency noise, and a drift correction procedure[29] is incorporated to correct for low-frequency noise (i.e., drift). In many cases, HP-ICP-OES can provide elemental determinations with expanded uncertainties, corresponding to 95% confidence intervals, that are better than a few parts per thousand.[21,22,23]

A Perkin-Elmer 3300DV ICP-OES instrument (Norwalk, CT) was used for these determinations. The operating conditions can be found in Table 3. These conditions rendered a robust plasma with a Mg(II) 280.270 nm/Mg(I) 285.213 nm intensity ratio of 8.4. This value has been corrected for the differing Echelle grating diffraction efficiencies at the two wavelengths by multiplying the observed ratio (4.5) by 1.85.[30] The selection of the analyte and internal standard wavelengths was based on similar excitation energies as well as intensity ratio precision (0.2−0.3% for five replicate measurements). The signals were integrated using a segmented-array charge-coupled device detector. Integration parameters were set manually to ensure simultaneous acquisition of the signals.

Four preparations were typically made gravimetrically for both the calibration standard and the nucleic acid samples. The mass fraction of P in these solutions ranged from 3 to 6 $\mu$g g$^{-1}$. The solutions were spiked with a solution that contained Ge at a mass fraction of 100 $\mu$g g$^{-1}$ to obtain a final Ge mass fraction of ∼20 $\mu$g g$^{-1}$. The spike solution was prepared from a 10 000 $\mu$g mL$^{-1}$ Ge solution CRM (Inorganic Ventures, Lakewood, NJ). A single P determination, requiring between 1 and 2 h, consisted of five repeat measurements on the series of dilute solutions (random order). Five separate determinations were generally used to calculate the average P mass fraction for a given unknown sample. However, small sample size (∼10 mL) limited the plasmid DNA analyses to a single experiment. The samples were analyzed blindly, with the analyst given only the approximate P mass fraction of the material so that suitable solution preparations could be performed.

**Standard Reference Material and Expanded Uncertainties.** The CRM from which calibration solutions were prepared for both the HPLC and ICP-OES methods was NIST SRM 3139a Phosphorus Standard Solution, Lot No. 890607. The certified P mass fraction is 9.99 mg g$^{-1}$, and the expanded uncertainty corresponding to a 95% confidence interval is 0.04 mg g$^{-1}$ (https://srmors.nist.gov/view_detail.cfm?srm=3139A). It should be noted that the use of SRM 3139a as the calibration standard provides traceability to the SI for both the HPLC and HP-ICP-OES measurements. This is because SRM 3139a is prepared from and certified (using HP-ICP-OES) against SRM 194 Ammonium Dihydrogen Phosphate. The P mass fraction of SRM 194 was certified using coulometry, a "primary direct" method as defined by the Comité Consultatif pour la Quantité de Matière (CCQM, Consultative Committee for Amount of Substance).[31,32]

**Table 3. ICP-OES Operating Conditions**

| | |
|---|---|
| plasma gas (L min$^{-1}$) | 15 |
| auxiliary gas (L min$^{-1}$) | 0.5 |
| nebulizer gas (L min$^{-1}$) | 0.5 |
| power (kW) | 1.5 |
| viewing | axial |
| sample uptake (mL min$^{-1}$) | 0.17 |
| nebulizer | glass concentric |
| spray chamber | cyclone |
| analyte wavelength (nm) | P I 213.617 |
| reference wavelength (nm) | Ge I 209.426 |
| on-chip integration time (ms) | 128 or 256 |
| total read time (s) | 8.196 |

HPLC measures total phosphate, and ICP-OES measures phosphorus. In this work, there is an assumption that the proportion of standard to unknown used to calculate mass fraction is equivalent whether phosphorus or phosphate is measured.

All uncertainties shown for the data consist of expanded uncertainties, which are 95% confidence intervals calculated according to the principles of the ISO *Guide to the Expression of Uncertainty in Measurement (GUM)*.[33] Expanded uncertainties were calculated for the phosphate/phosphorus measurements as well as the gravimetric preparations of thymidine monophosphate. Propagated components of uncertainty include observed variability of measurement replication, observed variability in the determination of the ICP-OES and HPLC instrument sensitivities, and uncertainties in the known values for the ICP-OES and HPLC calibration solutions. Uncertainties in the gravimetric preparation

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**Table 4. Mass Fraction of Phosphorus in Digested TMP Solutions[a]**

| $\mu$g P g$^{-1}$ of solution | | | % difference from gravimetric | |
|---|---|---|---|---|
| gravimetric | ICP-OES | HPLC | ICP-OES | HPLC |
| 96.60 (0.51) | 95.86 (0.07) | 93.30 (0.93) | −0.8 | −3.4 |
| 48.35 (0.26) | 48.42 (0.03) | 50.40 (0.56) | +0.1 | +4.0 |
| 9.85 (0.05) | 9.902 (0.007) | 9.35 (0.10) | +0.5 | −5.5 |

[a] All values in parentheses are expanded uncertainties; see Experimental Section.

**Table 5. Mass Fraction of Phosphorus in Undigested TMP Solutions[a]**

| $\mu$g P g$^{-1}$ of solution | | % difference from gravimetric |
|---|---|---|
| gravimetric | ICP-OES | ICP-OES |
| 96.45 (0.51) | 96.66 (0.05) | +0.2 |
| 48.23 (0.26) | 48.38 (0.03) | +0.3 |
| 21.99 (0.12) | 21.93 (0.01) | −0.3 |
| 9.84 (0.05) | 9.925 (0.005) | +0.9 |

[a] All values in parentheses are expanded uncertainties; see Experimental Section.

**Table 6. Quantitation of the Mass Fraction of Phosphorus and DNA in a Preparation of Genomic DNA: Comparison to Results of Measurements of Absorbance at 260 nm[a]**

| P mass fraction | | DNA mass fraction |
|---|---|---|
| measured ($\mu$g of P g$^{-1}$) | corrected[b] ($\mu$g of P g$^{-1}$) | $\mu$g of DNA g$^{-1}$ |
| ICP-OES | | |
| 56.41 (0.03) | 59.22 (0.03) | 590.7 (0.3) |
| 29.62 (0.02) | 59.30 (0.04) | 591.5 (0.4) |
| 6.022 (0.005) | 59.28 (0.05) | 591.3 (0.5) |
| HPLC | | |
| 56.25 (0.46) | 59.03 (0.48) | 588.8 (4.8) |
| 29.32 (0.85) | 58.70 (1.70) | 585.5 (17.0) |
| 3.75 (0.05) | 36.95 (0.49) | 368.5 (4.9) |
| Absorbance ($\mu$g DNA mL$^{-1}$)[c] | | 642 (2) |

[a] All values in parentheses are expanded uncertainties; see Experimental Section. [b] Corrected to the original stock material from which all three digests were prepared. [c] DNA is expressed as concentration as per tradition, but the mass fraction value is essentially equivalent. The density of a solution of DNA (600 $\mu$g of DNA mL$^{-1}$) in water differs from water by no more than 0.1% (0.998 g cm$^{-3}$ versus 0.997 g cm$^{-3}$ for pure water at 23 °C).

of TMP include those associated with the mass of TMP, the mass of the 5% HCl solution, Karl Fischer determination of the water content of TMP, and possible undetected TMP impurities.

## RESULTS AND DISCUSSION

Initial measurements of the phosphorus content of nucleic acids were made on solutions of a nucleotide monophosphate, TMP, a small molecule and one of the four common base components of DNA. The purity of the TMP was evaluated using two different HPLC methods, and no impurities were detected. The water content of the TMP was measured using Karl Fischer analysis and found to be 4.55% (±0.28%). Gravimetric preparations of TMP consisting of a range of mass fraction levels were made in 5% (vol) HCl. These solutions were either analyzed by ICP-OES without further treatment or were digested at 120 °C for 14 h and then analyzed by both ICP-OES and HPLC. Table 4 shows the results for digested TMP. The expanded uncertainties from the mean for ICP-OES were less than 0.1%, which is typical for the HP-ICP-OES method.[21] The differences of the HP-ICP-OES values from the gravimetric values ranged from 0.1 to 0.8% of the mass fraction. The HPLC values had larger uncertainties and differed more significantly from both the gravimetric and ICP-OES values, with differences from gravimetric ranging from 3.4 to 5.5% of the mass fraction. For both methodologies, the level of agreement with the gravimetric values was independent of the starting mass fraction of phosphorus in the solutions.

For ICP-OES analysis of TMP, it was not necessary to digest the TMP solution prior to analysis as indicated by the results shown in Table 5. Residence time in the plasma was sufficient to completely dissociate the TMP molecule. Agreement of the ICP-OES results with the gravimetric values was in the same range as for the digested TMP samples. The use of undigested TMP is not appropriate for HPLC analysis of the phosphate mass fraction.

Of the ICP-OES results reported in Tables 4 and 5, five of the seven values statistically agree with the gravimetric values at the 95% confidence level. Two others (Table 4, value 1; Table 5, value 4) statistically disagree, but the differences are quite small. It is likely that there are possible sources of error that have not been accounted for as yet.

Measurements were made of the phosphorus/phosphate content of acid-digested calf thymus genomic DNA by ICP-OES and HPLC. The source of the genomic DNA was commercial (Sigma, D-1501, Type 1, fibrous preparation). The DNA was cleaned prior to analysis. Biological materials generally contain substantial amounts of phosphorus-containing organic compounds, in addition to nucleic acids. These include phosphoproteins as well as small molecules. So to convert the measurement of phosphorus to an accurate value of starting DNA mass, it is important to remove extraneous sources of phosphorus. The DNA was treated with both RNase and proteinase K prior to extraction and precipitation. The final DNA preparation was extensively washed to remove small molecules.

Digestion of the acidified DNA solution at 120 °C was required for phosphorus/phosphate analysis of genomic DNA. Comparison of digested versus undigested DNA using ICP-OES showed that the level of measured phosphorus of undigested DNA was ~25% of that seen when the DNA was digested. Residence time in the plasma was insufficient to completely dissociate the DNA. A digestion experiment was initially conducted, varying the concentration of hydrochloric acid and the time of the heat treatment, to determine the appropriate conditions (5% (vol) HCl, 16 h at 120 °C) for complete digestion. HPLC analysis was used to detect any undigested nucleotides (data not shown).

Experiments consisted of the analysis of several different mass fraction levels of DNA prepared by dilution from the same stock of purified DNA and then digested. Table 6 shows the results on one such series. Very consistent results were obtained with ICP-OES over a range of starting mass fractions when back calculated to the original DNA stock mass fraction. The results showed small uncertainties as was previously seen with the analysis of TMP.

**Table 7. Quantitation of the Mass Fraction of Phosphorus in Plasmid DNA Preparations: Comparison of Results Obtained by Using HPLC, ICP-OES, and Absorbance at 260 nm[a]**

| sample 1 | | sample 2 | |
|---|---|---|---|
| P mass fraction ($\mu$g of P g$^{-1}$) | DNA mass fraction ($\mu$g of DNA g$^{-1}$) | P mass fraction ($\mu$g of P g$^{-1}$) | DNA mass fraction ($\mu$g of DNA g$^{-1}$) |
| ICP-OES | | | |
| 4.748 (0.009) | 47.36 (0.09) | 6.01 (0.01) | 59.94 (0.10) |
| HPLC | | | |
| 3.26 (0.11) | 32.52 (1.10) | | |
| Absorbance ($\mu$g of DNA mL$^{-1}$) | 53.2 (3.8) | | 66.5 (2.1) |

[a] All values in parentheses are expanded uncertainties; see Experimental Section.

Reasonable correlation of HPLC measured mass fractions with those of ICP-OES were seen with more concentrated samples. A shoulder was consistently seen on the phosphate chromatography peak when dilute samples (~5 $\mu$g of P g $^{-1}$) were analyzed that was not seen with more concentrated samples. The results of the HPLC analysis of the most dilute sample gave reduced values when related to the original stock. Investigations showed that this was not due to incomplete digestion of the nucleotides, but likely the result of the formation of an unknown compound. This limits the utility of HPLC measurements to more concentrated solutions. The estimation of DNA concentration in TE buffer determined from absorbance at 260 nm differs from the mass fraction determination by phosphorus analysis (ICP-OES and HPLC), by 9% (Table 6).

Plasmids are circular pieces of double-stranded DNA that can be propagated in bacteria and purified to provide a source of well-defined DNA of specific size and sequence. The usual isolation procedures for bacterially derived plasmid preparations results in some contamination by bacterial genomic DNA. To eliminate this, the plasmid was purified on CsCl gradients set up by ultracentrifugation. Residual genomic DNA contamination, measured using a real-time quantitative PCR assay for the 23S rRNA gene from *E. coli*, constituted ~1% of total DNA. Table 7 shows the ICP-OES analysis of the phosphorus content of two purified plasmid samples. One sample was also analyzed by HPLC; the value obtained was smaller than that determined by ICP-OES (3.26 vs 4.75 $\mu$g of P g $^{-1}$). The same shoulder artifact was seen in HPLC chromatograms of the plasmid DNA as was seen with the genomic DNA at a similar low mass fraction. This resulted in an underestimate of the phosphorus content.

The calculated values of the mass fraction of DNA in both plasmid preparations, as determined by ICP-OES, show the same correlation with the absorbance measurement at 260 nm as was seen with genomic DNA. The concentration was ~10% higher than the calculated mass fractions from the phosphorus quantitation.

In 1948, Chargaff and Zamenhof[5] quantitated the phosphorus content of purified DNA preparations using a colorimetric method and determined an extinction coefficient for the absorbance at 260 nm based on the phosphorus values [$\epsilon$(P) = 6100]. Subsequently, others reported $\epsilon$(P) values that ranged widely from 6000 to 11 000 as reviewed by Beaven et al.[6] The variation was likely due to the quality of the DNA preparations and the phosphorus measurements. Because of the large size of cellular DNA and the fact that the isolation and purification results in varying degrees of fragmentation, the extinction coefficient for DNA is expressed in units of micrograms per milliliter rather than molar. The most often used coefficient is 0.02 ($\mu$g of DNA mL$^{-1}$)$^{-1}$ cm$^{-1}$ or an absorbance of 1 = 50 $\mu$g DNA mL$^{-1}$. This corresponds to an $\epsilon$(P) of 6200. In this study, the mass fraction of the plasmid and genomic DNA based on phosphorus was consistently 10% lower than the DNA concentration as determined by absorbance at 260 nm using the above-mentioned relationship (absorbance of 1 = 50 $\mu$g of DNA mL$^{-1}$). It would be of value to revisit the issue of the extinction coefficient for DNA now that more precise and accurate quantitation of phosphorus is possible and improved methods for the isolation and purification of DNA are available.

## CONCLUSIONS

Phosphorus measurements by HP-ICP-OES will be a valuable tool for certification of future nucleic acid CRMs. Results showed that accurate results with very small uncertainties (0.1%) can be obtained with ICP-OES on digested DNA as has been seen with single-element solutions.[21] It is suitable for any size of nucleic acid from nucleotides to genomic DNA. Measurement of phosphorus requires that the DNA be cleaned of impurities. Digestion of the DNA with acid and heat releases the phosphorus. Residence time in the plasma (ICP-OES) will compensate if digestion is not 100% complete or if an artifact is formed. Analysis of individual nucleotide species can be accomplished without digestion. The most important advantage of the phosphorus analysis is the provision of a complete uncertainty estimate and traceability provided by the use of CRMs, a NIST SRM in this study. HPLC is less useful for certification of CRMs but could be used for quantitation in other cases with the proviso that the limitations are understood.

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