Original Article

Accurate quantification standards of DNA via phosphorus measurement through microwave induced combustion (MIC)-ion chromatography

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Abstract
In the described method, quantitation of nucleic acid is carried out by measurement of the stoichiometrically existing phosphorus content by ion-chromatography as phosphate. A rapid procedure for the digestion of refractory genomic DNA based on oxygen combustion assisted by microwave radiation is applied. The advantage of this procedure is the complete decomposition of the highly refractory DNA molecule within half an hour with quantitative conversion of DNA-phosphorus to orthophosphate ion. The microwave induced combustion (MIC) based sample preparation approach coupled with ion-chromatographic measurement of the resulting phosphate using a high performance (HP) methodology provides an accurate quantitation of phosphorus mass fraction as low as 0.25 µg g⁻¹, corresponding to a DNA mass of 10 µg. The relative expanded uncertainties (% U) expressed at 95% confidence for these analyses range from 0.4 to 0.9%. The phosphorus content of the calf thymus DNA was also measured using high-performance inductively coupled plasma optical emission spectroscopy (ICP-OES), which provided independent data for comparison with the MIC–IC based approach. The method is suitable for characterizing primary calibration standards and for validating and certifying reference materials.

Keywords: Genomic DNA; DNA-phosphorus; Microwave-induced combustion; ion chromatography; Uncertainty

1. Introduction
DNA is an important analyte of molecular biology and the need for its accurate quantitation is outlined in literature [1-4]. To date; measurement by UV absorbance [5] is commonly used approach for the quantification of nucleic acids, which is based on average absorbance of 1 OD at 260 nm corresponding to a DNA mass of 10 µg/mL, respectively. However, being these values approximations, the accuracy of such measurements is somewhat limited. Measurement of fluorescence from dyes intercalated into the DNA provides a highly sensitive quantitation method [6] for DNA. However, intensity of the fluorescence depends on other parameters such as dye concentration, degree of bleaching, intensity of excitation light and additionally is interfered by quenching effects of the matrix constituents. Accuracy in quantitation of DNA can be substantially improved by providing high quality measurement standards that is traceable to international system of units (SI). The stoichiometric existence of phosphorus in the DNA backbone has been previously measured by ICP-OES [3, 4, 7] for establishing an absolute quantitation method for DNA by calibrating against a phosphorus certified reference material (CRM), thus providing a traceable methodology. Most analytical procedures developed for the determination of phosphorus in organic compounds involve the initial decomposition of the substance followed by the complete conversion of phosphorus to orthophosphate prior to instrumental detection. Among the nucleic acids, the high molecular weight genomic DNA molecule being highly refractory in nature requires 16 h of digestion prior to IC analyses [3]. Normally in classical wet chemistry, perchloric-sulfuric acid mixture [8] and thermal acidic persulfate digestion [9] are used as oxidation procedures for the conversion of phosphorus to orthophosphate ion. However, the final digest containing high concentration of sulfate and/or perchlorate creates interferences in the separation of phosphate by IC [10].

In our previous work [11] for the quantitation of DNA-phosphorus by IC, the digestion of DNA was carried out by UV digestion in the presence of nitric and hydrochloric acid, which reduced the digestion time considerably. However, the presence of large concentration of nitrate ions introduced during digestion requires gradient elution for base line resolution of the bromide. In this aspect, combustion based methods [12,13] provide an expedient alternative to decompose organic materials and the use of oxygen as an oxidant in place of nitric acid reduces the ionic load particularly for low capacity IC columns. Generally, the successful combustion techniques were
those performed in closed vessels, e.g. oxygen flask combustion [13] and combustion bombs [14]. The oxygen flask combustion had the disadvantage due to the unfavorable ratio of the vessel surface to sample; whereas the main drawback related to the combustion bombs was the contamination with metals that would create insidious problems on anion separation by IC. These drawbacks have been surmounted in recently introduced microwave induced combustion (MIC) technique [14, 15] by combining advantages of classical combustion techniques with those observed for microwave digestion in a single system. Briefly, the relatively new MIC technique, involves the organic matrix combustion in closed quartz vessel pressurized with oxygen, where the ignition step is performed by microwave radiation. As a testimony to its utility, the MIC technique has been utilized to mineralize matrices like coal [16], carbon black [17], petroleum coke [18] and various biological materials [19]. In the literature, O2-combustion and subsequent IC determination of phosphorus has been carried out for small organo-phosphorus compounds [12, 13] but none concerning the combustion of highly recalcitrant genomic DNA has been reported.

This paper reports on the effectiveness of microwave induced combustion (MIC) of a high molecular weight DNA sample prior to the determination of phosphate by ion chromatography (IC) using the “high performance methodology” [20-22] developed by the researchers at NIST to achieve low measurement uncertainty. The quantitative conversion of different phosphorus species was accomplished in the presence of microlitre quantity of dilute acidic reagent (HCl) and a 10% (v/v) hydrogen peroxide solution as an absorbing medium. The reflux step following O2-combustion converts all species of phosphorus produced during combustion to orthophosphate and produces a digest of suitable pH that is amenable to analysis by ion chromatography without further pH adjustment. The method is further applied for the quantitation of two plasmid DNA samples extracted from bacterial strains of E.coli DH5α. Inductively coupled plasma optical emission spectroscopy (ICP-OES) and UV absorbance has been employed [3, 5] to determine the phosphorus content of the DNA sample to provide independent data for comparison with the proposed microwave based combustion method.

2. Materials and Methods

2.1. Materials

Milli-Q water (18 MΩ cm) was used to prepare all samples, calibrant and reagent solutions. Hydrochloric acid (30%), hydrogen peroxide (30%) both of suprapur grades were obtained from Merck (Darmstadt, Germany). Sodium hydroxide (50% w/w, Lot 112797)) solution from Fisher Scientific (New Jersey, USA) was used to prepare eluent for IC. The CRM from which calibration solutions were prepared through serial dilution for the IC measurement was the CertiPur® reference material (phosphorus standard solution, lot no.HC934479). The certified P mass fraction is 1.00 mg g⁻¹, which was traceable to NIST SRM® 3139a, lot 060717. A stock solution (1 mg g⁻¹) of bromide was prepared by dissolving highly pure grade salt of NaBr (Suprapur®, Merck, Germany) in de-ionized water. Certified reference material of chloride (CRM 39883, TraceCERT®, lot no. BCBC2167) was obtained from Sigma-Aldrich, Switzerland which was traceable to SI unit kg and measured against NIST SRM. The certified chloride mass fraction was 1.00 mg g⁻¹ in the original stock. The genomic DNA used in this study was deoxyribonucleic acid, sodium salt, from calf thymus (Sigma, D1501). The fibrous DNA was dissolved in sterile Tris-EDTA, pH 7.5. Starting solution contained 1 mg mL⁻¹ of DNA, which was dissolved at 4°C for 26 h with slow rotation. Removal of residual RNA, protein contaminants and purification of the DNA was carried out as described by Holden et al. [3]. The density of the purified DNA stock solution (1000 µg of DNA g⁻¹) in TE buffer was found to be 0.9978 g cm⁻³.

Potassium hydrogen phthalate (C7H4KO4) pro analysis reagent grade (Merck, Germany) was used to prepare the carbon stock standard. Ammonium nitrate solution (6 mol L⁻¹) was used as igniter for the combustion procedure. Two discs of filter paper (18 mm dia., 20 mg) with low ash content (Whatman-540®, Schleicher & Schuell) were used in which the DNA sample was impregnated and which additionally aided in the combustion process. The filter paper discs were previously cleaned with 10% nitric acid for 20 min in an ultrasonic bath, rinsed well and dried in an oven for 2 h at 60°C before use.

2.2. Instrumentation

A Multiwave-3000 pressurized microwave digestion device, equipped with quartz vessels (80 mL capacity) from Anton Paar GmbH (Graz, Austria) was used for combustion, the technical details of which are described elsewhere [15]. Commercial quartz holders (Cat. no. 16427) were used to hold the paper discs that contained the DNA sample. The total organic carbon (TOC) content of the combusted DNA solution was measured using a TOC analyzer (TOC-VCPN 5000 A, Shimadzu, Japan). Total organic carbon (TOC) was measured from a calibration graph (0.50 mg L⁻¹) where potassium hydrogen phthalate (C7H4KO4) was used as a stock standard solution (1000 mg L⁻¹) for carbon. Phosphate analyses were performed using an ICS-3000 ion chromatography system (Dionex, Sunnyvale, CA, USA) equipped with a quaternary gradient pump; detector compartment containing a chromatography oven and a conductivity detector (ICS-3000 series). An anion trap column from Dionex was used to strip trace contaminants from the eluent or deionized water. The chromatography oven was utilized to help prevent baseline drift caused by temperature variation. The instrument control, data acquisition and processing were performed with Chromeleon® software (version 6.80). The detailed chromatographic conditions are described in Table 1. ICP-OES (Prodigy, High dispersion with solid state array detector, Teledyne Leeman Labs, Hudson, USA) was used for ICP-OES measurement [3] of phosphorus.

2.3. Purification of plasmid DNA from bacteria

Plasmids pBKs and pOK12 were purified from the bacterial strains of E.coli DH5α, containing, pBKs and pOK12. In order to isolate plasmid DNA from bacteria, single colony was picked up from plate and inoculated in Luria broth supplemented with appropriate concentration
and type of antibiotics (ampicillin at 100 µg/ml for pBKS and pBR322 and kanamycin at 25 µg/mL for pOK12) followed by incubation in a bacteriological incubator at 37°C with shaking (200 rpm). The overnight grown cultures were then harvested by centrifuging the culture broth at 10,000 rpm 10 min at 4°C. After harvesting the cells, plasmid purification was done using Qiagen plasmid purification kit (QIAIEN Plasmid Midi Kit). The obtained DNA pellet was re-dissolved in 1000 µl of TE buffer pH 8.0 and stored at -40°C for further use. QIAGEN plasmid purification protocols are based on a modified alkaline lysis procedure, followed by binding of plasmid DNA to QIAGEN Anion-Exchange Resin under appropriate low-salt and pH conditions. RNA, proteins, dyes, and low-molecular-weight impurities are removed by a medium-salt wash. Plasmid DNA is eluted in a high-salt buffer and then concentrated and desalted by isopropanol precipitation. The obtained plasmid DNA was run on 1.5% agarose gel to check its quality.

Table 1. Chromatographic conditions used for phosphate analysis

<table>
<thead>
<tr>
<th>Column</th>
<th>IonPac AS20 (250 mm x 4 mm), Dionex 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guard Column</td>
<td>IonPac AG20 (50 mm x 4 mm), Dionex 6</td>
</tr>
<tr>
<td>Anion Trap Column</td>
<td>ATC-4C (50 x 75 mm), Dionex 6</td>
</tr>
<tr>
<td>Eluent</td>
<td>17 mM sodium hydroxide at a flow rate of 1 mL/min</td>
</tr>
<tr>
<td>Injection Volume</td>
<td>25 µL</td>
</tr>
<tr>
<td>Detection</td>
<td>Suppressed conductivity</td>
</tr>
<tr>
<td>Suppressor</td>
<td>Dionex ASRS 500-600 mm (Thermo Scientific)</td>
</tr>
<tr>
<td>Suppressor current</td>
<td>50 mA</td>
</tr>
<tr>
<td>Quantification</td>
<td>Peak area</td>
</tr>
<tr>
<td>Data collection rate</td>
<td>3.0 Hz</td>
</tr>
</tbody>
</table>

![Filter paper disc diagram](image)

**Fig. 1** Schematic of sample loading in the quartz holder

2.4. Sample preparation for combustion

For the proposed DNA combustion procedure, two filter paper disks were taken in the quartz holder. Aliquots of DNA stock solution (10-400 µL for calf thymus, 400 µL for plasmid DNA) were taken on the filter paper by pipetting a maximum volume of 50 µL at a time followed by drying under the IR lamp. After transferring the desired quantity of DNA onto the filter paper disks, 50 µL of ammonium nitrate solution was added to the paper and dried. A schematic diagram of the DNA loading in the quartz holder is provided in Fig.1. The absorbing solutions and calibrant preparations were performed gravimetrically on a four place analytical balance. 100 µL hydrochloric acid (7.5%), 2 mL of H₂O₂, required amount of bromide stock standard (50 µg g⁻¹) and appropriate amount of DI water were weighed into a PFA beaker (20 mL capacity, Cole Parmar, USA). The solutions were diluted gravimetrically to 4 g and quantitatively transferred to the quartz vessel as absorbing solution. The quartz holder containing DNA was positioned into the quartz vessel. After the closure of the vessels and capping the rotors, they were pressurized with 20 bar of oxygen for 45 seconds by means of gas loading accessories set (Anton Paar catalogue no.17265). Then the rotors were placed inside the oven and the microwave irradiation program used for the combustion procedure was as follows: (i) 1400 W for 10 s, (ii) 0 W for 2 min and (iii) 1400 W for 25 min in the reflux (hydrolysis step) step. The microwave energy is reduced automatically in step (iii), once the reaction pressure in one of the vessels exceeds the set limit of 72 bars.

2.5. Phosphorus Analysis

A high-performance (HP) technique for ion chromatography [23] has been utilized to determine phosphorus (as orthophosphate) in the combusted sample. The high performance technique incorporates an internal standard, drift-correction methodology, gravimetric solution preparation and is based on single point calibration. Briefly, the replicates of combusted DNA samples are compared chromatographically to replicate preparations of the calibration standard, where the preparations of the calibrant are prepared to mimic the expected characteristics of the combusted DNA digest with respect to the matrix composition (chloride, nitrate), analyte (phosphate) and internal standard (bromide) mass fraction. Internal standard is used to compensate for short-term noise and a drift correction procedure [24] to correct for low frequency noise. All sample handling dilutions, addition of internal standard and reagents were performed gravimetrically.

Four preparations of each were made gravimetrically for the calibration standard and for the receiving solution of the DNA combustion process. The mass fraction of phosphorus in these solutions ranged from 0.2-6.6 µg g⁻¹. The solutions were spiked with bromide (internal standard) from a 50 µg g⁻¹ stock solution to obtain matching mass fraction with corresponding calculated phosphate mass fraction. The combusted matrix (HCl) matched calibrant and receiving solution of the DNA combustion process were run in the IC instrument in a randomized complete block sequence [21-24], which is necessary for the implementation of drift correction.

3. Result and discussion

3.1. Extent of mineralization of DNA by MIC

Genomic DNA is a highly thermally stable molecule, where even the residence time in the ICP plasma is not able to completely dissociate its polymeric backbone [3] and hence represents a challenge for O₂-combustion. In the MIC experiment, hydrochloric acid was taken in the absorbing solution along with hydrogen peroxide as an
oxidant. The use of acids however must be minimized, because the response of phosphate in suppressed conductivity detection shows a significant decrease in the presence of high concentration of chloride [25] and a highly acidic absorption solution is not suitable for use with the IC columns. Further, the conventional loading of sample in the quartz holder [18] as pressed pellets was modified to load aqueous DNA stock solution directly on ashless filter paper through sequential pipetting and drying under infrared lamp, till required quantity of DNA is transferred. Multiple combustion experiments at 10 bar of O\textsubscript{2} pressure were carried out using varying amounts of HCl (100-250 \mu L, 7.5%) and hydrogen peroxide (0.5-3mL, 30% v/v) in the absorbing solution and the mineralization efficiency was quantitated by measuring residual carbon content (RCC) [26] of the absorption solution. It was observed that lowest RCC (%) of <0.4% was obtained, when the absorbing solution contained a minimum of 100 \mu L of HCl along with 0.5 mL of H\textsubscript{2}O\textsubscript{2}. It is noteworthy, because such low RCC value was obtained for the highly recalcitrant DNA sample only with combustion without any additional reflux of the absorbing solution. After MIC, the absorption solution did not contain any unburnt black residues and was a clear colourless solution, indistinguishable from water. ICP-OES analysis [3] of this DNA preparation gave a mean phosphorus value of 3.35 \mu g g\textsuperscript{-1}. However, the phosphorus mass fraction obtained by IC was =30% of the ICP-OES value. Such a problem of non-quantitative recovery of phosphorus obtained through combustion of organo-phosphorus compounds has been observed by ion chromatographic measurements [27, 13]. Particularly, the formation of the less oxygenated species leads to negative errors when phosphate is determined by IC. Umali \textit{et al.} [13] reported quantitative recoveries of phosphorus from phenyl phosphonic acid by incorporating a post-combustion hydrolysis step at acidic pH. After MIC, introducing the refluxing step for 25 min, the phosphorus measured by IC was in the 80-90% of the ICP-OES value. These recovery values are unacceptable for high precision analysis.

![Fig-2](image)

**Fig-2** Effect of varying conc. Of hydrogen peroxide on the recovery of phosphate after MIC.

### 3.2. Influence of H\textsubscript{2}O\textsubscript{2} concentration and O\textsubscript{2} pressure on phosphorus recovery

As reported in earlier literature [13], for the hydrolysis of the combustion products, the absorption solution must be boiled at acidic pH to convert less oxygenated species of phosphorus to orthophosphate. Generally, higher concentration of acids is used for the hydrolysis of polyphosphates [13] formed during O\textsubscript{2}-combustion. However, the introduction of large concentrations of acid anions must be avoided for the determination of phosphorus as phosphate by IC. So, as the addition of 100 \mu L of HCl (7.5%) in the absorption solution provided lowest RCC value, it was decided to fix this minimum quantity of HCl in all experiments. In light of the above mentioned drawbacks, the best alternative was to further increase the oxidizing agent i.e. hydrogen peroxide in the absorption solution and oxygen pressure during combustion and introduction of a hydrolysis step. Compared to sample weights, normally taken for MIC (max.500 mg), the DNA sample weight taken in the present experiment is minute (max.400 \mu g). So, it was assumed that a pressure of 10 bar of oxygen will be sufficient. The H\textsubscript{2}O\textsubscript{2} concentration in the absorbing solution was increased keeping the O\textsubscript{2} pressure constant at 10 bar with a post combustion hydrolysis time of 25 min. Fig.2 represents the effect in percent recovery of phosphorus with varying hydrogen peroxide concentration in the absorbing solution when compared against the ICP-OES value. Each point in the graph represents the average of two combustion experiments. As can be seen, the average recovery was quantitative (=99.8%) at hydrogen peroxide concentration of 10% (v/v) or more. The precision of phosphorus determination as determined by six successive combusted DNA solution was found to be 3.4% R.S.D. While for normal phosphorus determination, these analytical performances are sufficient, they are unsatisfactory to obtain small concentration uncertainties (1% relative or better) which are assured through high measurement precision. By carrying out the combustion between 15-20 bar of O\textsubscript{2}-pressures, the precision of phosphorus determination improved (1.2% R.S.D.). So, the DNA combustion experiments were carried out at 20 bar O\textsubscript{2} pressure. In all the experiments, an increase of about 5 bars was observed during combustion. This maximum attained pressure is far below from the maximum operation pressure (80 bar) recommended by the manufacturer.

### 3.3. Matching of matrix anions

The absorption solution of combusted DNA contains chloride and nitrate at mass fractions of = 2100 and 120 \mu g g\textsuperscript{-1} respectively. It is a matter of significance to get such low concentration of nitrate in the absorption solution, when approximately 20,000 \mu g of nitrate was taken on the filter paper in the form of ammonium nitrate as igniter. The ammonium nitrate is decomposed on microwave irradiation as follows producing heat and providing ignition for the O\textsubscript{2}-combustion.

\[
2NH\textsubscript{4}NO\textsubscript{3} \rightarrow 2N\textsubscript{2} + 4H\textsubscript{2}O + O\textsubscript{2} \ (1)
\]

Further, only a small portion of nitrogen present in the base pair units of the DNA get converted to nitrate, because it has been reported [28] that organic bound nitrogen is released as N\textsubscript{2} or in the form of less soluble NO during the
combustion and the post combustion oxidation step should contain a absorption solution of basic pH for quantitative conversion of nitrogen species to nitrate. These aspects account for the much reduced concentration of nitrate found in the absorption solution compared to the total forms of nitrogen species originally present in the combustion set up. Nevertheless, the presence of such large concentration of chloride and nitrate necessitates the use of a high capacity IC column (Ion Pac AS 20,310 µeq per column) so that a bromide to nitrate ratio of ≈ 1:120 was managed by column capacity alone (Fig.3). Brennan et al. [23] reported that IC analysis of solutions containing interfering anions at significant levels could produce results with increased uncertainty. Further, it has been reported that the response of phosphate in suppressed conductivity detection shows a significant decrease in the presence of high concentration of chloride [25] and other strong anions [10]. Therefore, experiments were carried out to evaluate the effect of matrix anions on the ratio of phosphate /bromide peak area. Sets of solutions were prepared to contain identical analyte and internal standard mass fractions but with varying mass fractions of chloride and nitrate. The source of chloride and nitrate were CRM39883 (lot no.BCBC2167) and CRM 74246 (lot no.BCBC2911) standard solutions respectively from Fluka Analytical. These ratios of analyte to internal standard were compared with the results obtained when no matrix anions were present. It was observed that in the presence of matrix, the phosphate to bromide peak area ratio showed a decrease, which in consequence creates bias in the result. For example, the DNA digest that contains chloride and nitrate at mass fractions of ≈ 2100 and 120 µg g⁻¹ respectively, resulted in a 2% change in the ratio of the phosphate to bromide peak area. For a large majority of analytical applications of IC, percent level or poorer uncertainty is acceptable. In such cases, the degree of matrix matching required to compensate matrix effects is usually not rigorous. However, in the case of present IC analysis, where concentration uncertainties of <1% or better are targeted, requires close or exact matrix matching of the calibrant to ensure that the analytical sensitivity is consistent throughout the analysis. Fortunately, this is not difficult to implement for chloride, since it is introduced from HCl, added into the absorption solution and hence quantitatively well defined. However, the nitrate content found in the absorption solution is variable between different batches of the combustion experiments. To circumvent this, the calibrant solution containing HCl and H₂O₂ is also combusted in the same way by MIC using filter paper and ammonium nitrate.

3.4. Reagent blank
In the “HP” experimental design, reagents blanks were considered, because very often different phosphorus species are added as stabilizers [29] to various grades of hydrogen peroxide and even commercially available suprapur grade hydrochloric acid contains traces of bromide as a contaminant. Thus, additive bias arises for bromide and phosphate signals arising from sources other than the DNA combustion solution or calibrant is managed with blank measurement.

![Fig-3 Chromatogram showing phosphate in the absorption solution of microwave induced combustion of DNA (100 µg). Experimental details are describe in the text.](image-url)

5.5. Drift correction
The drift-correction that is part of the high-performance methodology has been described in detail by Salit et al. [21] and further elucidated by Brennan et al. [23] for IC. In the present experiment there are four preparations each for the calibrant and the DNA combusted solution. So as per randomized block sequence, the solutions are injected in a randomized sequence, until each solution has been injected once, then again in a randomized sequence, until each solution has been injected a second time, and so forth, until each preparation has been injected seven number of times. So, in all there were 56 injections, 14 per block times 4 blocks. The observed phosphate to bromide peak area for all injections (56) of the randomized block sequence is plotted against the solution run sequence. The replicate signal ratios pertaining to each solution were normalized to their mean value in this plot to allow the data for all solutions to be plotted on a common ordinate scale. A polynomial is fitted to the plotted data. The equation for the fitted polynomial is then used to correct the signal ratios for the drift.

3.6. Phosphorus mass fraction
“HP” methodology requires an initial guess at what mass fraction in the unknown DNA sample is likely to be so that matching calibration standards can be prepared. So a preliminary quantitation of phosphorus in the DNA sample is carried out by using normal ion chromatographic procedure. The combusted DNA solution and calibrant preparations are run in a randomized block sequence and the phosphate/bromide peak area ratios were recorded. The phosphate/bromide ratios were calculated and corrected for drift and the blank values were subtracted. IC analysis using “HP” methodology [21] is a relative method that compares the analyte-to-internal standard signal ratio measured in an unknown sample to those ratios measured in calibrant whose amount ratio is well known. The difference between the average analyte mass fractions of the preparations of the calibration standard and the combusted DNA solution observed chromatographically are used to calculate the phosphorus mass fraction of the DNA sample. Equation 2 is the relationship [21] that permits the calculation of phosphorus mass fraction in
DNA aliquot from the measured signal and mass ratios of the absorption solution of combusted calibrant and DNA obtained through MIC.

\[
\text{phosphorus mass fraction (µg of P g}^{-1} \text{)} = \frac{\text{phosphorus in unknown DNA}}{\text{phosphorus in calibrant}} \times \frac{\text{mass fraction in calibrant}}{\text{mass fraction in unknown DNA}}
\]

Where, \( I \) is the peak area and \( m \) is the mass of the phosphorus or bromide in the calibrant and DNA combusted solution. The calibration between peak area ratios (drift corrected and blank subtracted) and amount ratio is a straight line that passes through zero. The ratio RSDs ranged from 1.5% to 0.6% (based upon four replicate readings) for 0.2-6.6 µg g\(^{-1}\) of P, respectively.

A set of different mass fraction levels of DNA prepared from the purified DNA stock (1000 µg g\(^{-1}\)) and extracted plasmid DNA were combusted by MIC and the P mass fractions obtained from IC analyses are presented in Table 2. Consistent results were obtained with ion chromatography over a range of starting mass fractions when back calculated to the original DNA stock mass fraction. The calculated DNA mass showed relative expanded uncertainties that were in the range of 0.4 to 0.9%. There is statistically good agreement between the IC and ICP-OES [3] values for DNA mass at P mass fraction of >3, although the IC values had comparatively larger uncertainties. The plasmid DNA content obtained by IC was cross validated by UV absorbance measurement at 260 nm. The plasmid DNA concentrations by UV measurement were 7-9% higher than the calculated mass fractions obtained from the phosphorus quantitation by IC. Similar observation has been reported by Holden et al. [3] between UV absorbance and P measurement by ICP-OES for the quantitation of nucleic acid.

<table>
<thead>
<tr>
<th>Table 2 - Quantitation of the mass fraction of phosphorus and DNA in CTDN and purified plasmid DNA: Comparison to results of ICP – OES [3] and UV absorbance [5].</th>
</tr>
</thead>
<tbody>
<tr>
<td>P mass fraction measured (µg of P g(^{-1}))</td>
</tr>
<tr>
<td>CT DNA</td>
</tr>
<tr>
<td>0.332 (0.003)</td>
</tr>
<tr>
<td>1.663 (0.012)</td>
</tr>
<tr>
<td>2.453 (0.017)</td>
</tr>
<tr>
<td>4.565 (0.026)</td>
</tr>
<tr>
<td>HP-ICP-OES [3]</td>
</tr>
<tr>
<td>4.960 (0.004)</td>
</tr>
<tr>
<td>Plasmid DNA (pCK12 and pESK)</td>
</tr>
<tr>
<td>0.386 (0.007)</td>
</tr>
<tr>
<td>UV absorbance</td>
</tr>
<tr>
<td>0.038 (0.008)</td>
</tr>
<tr>
<td>UV absorbance</td>
</tr>
</tbody>
</table>

* All values in the parentheses are expanded uncertainties, see Experimental Section. * Corrected to the original DNA stock from which all the five digests were prepared.

Further, good agreement between IC and ICP-OES values, underpins that MIC based O\(_2\)-combustion and conversion of phosphorus of genomic DNA to orthophosphate ion by combustion-hydrolysis is quantitative. Additionally, good ratio precision of the phosphate to bromide peak area (0.6-1.5%) implies that conversion of different P species formed during combustion to orthophosphate is complete in the reflux step, as incomplete conversion would have resulted in erratic peak area ratios.

3.7. Uncertainty budget

The “HP” technique is readily amenable to an estimation of a complete uncertainty budget, taking into account all of the uncertainty contributions that derive from the “high-performance” protocol [20-23]. Equation 2 describes the calculation of the phosphorus mass fraction of an unknown combusted DNA solution from the measured quantities. The result is the product of two ratios: a peak area ratio and a mass ratio. Both dispersion and potential for bias contribute to uncertainty in the analyte mass fraction. The “HP” experimental protocol permits quantitative evaluation of the uncertainty from DNA sample and calibrant preparation. In IC experiments [23] variation of the peak area intensities dominates the uncertainty so the standard uncertainty can be calculated as the standard deviation.

In accordance to the recommendations from the ISO Guide [30] to the Expression of Uncertainty in Measurement (GUM), the analytical figures obtained in the HP-IC experiment were used to estimate the expanded uncertainties expressed at the 95% level of confidence. In particular, expanded uncertainties were determined for IC phosphorus measurements using the following equations:

\[
U = \sqrt{u_1^2 + u_2^2 + u_3^2 + \ldots}
\]

\[
U = k u_i
\]

\[
\%U = U \times 100/\bar{x}
\]

where \( u_i \) (\( i = 1, 2, 3, \ldots \)) represents the individual component of uncertainty, \( u_i \) is the combined uncertainty, \( k \) is the coverage factor, \( U \) is the expanded uncertainty, and \( x \) is the observed measurement of phosphorous mass. Propagated components of uncertainty include observed measurement repeatability, observed variability in the determination of IC sensitivity, uncertainty due to blank variability, and uncertainties in the known values for the phosphorus CRM standard. The expanded uncertainties are based on the measured standard uncertainty where the coverage factor \( k = 2.56 \).

4. Conclusions

The proposed MIC procedure represents combined features of oxygen combustion and microwave-assisted wet digestion for the digestion of highly recalcitrant genomic DNA sample. Further, the sample digestion is achieved within ≈ half an hour, compared to a hot plate digestion time of 16 h. The MIC based sample preparation approach coupled with the ion chromatographic measurement of phosphate using high performance (HP) methodology provides an accurate quantitation of phosphorus mass fractions as low as 0.25 µg g\(^{-1}\), corresponding to a DNA mass of 10 µg. The relative expanded uncertainties (% \( U \)) expressed at 95% confidence for these analyses range from 0.4 to 0.9% for the DNA standard, where as in the case of plasmid DNA preparations, the uncertainties were
relatively higher between 1.4-1.9%. The method is more suitable for characterizing primary calibration standards and for validating and certifying reference materials than for routine measurements, particularly for those samples that are limited in volume.

References

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