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Quantification of phosphorus in DNA using capillary electrophoresis hyphenated with inductively coupled plasma mass spectrometry

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A B S T R A C T

We have analyzed phosphorus in an enzymatically digested DNA molecule using capillary electrophoresis (CE) hyphenated with inductively coupled plasma mass spectrometry (ICP-MS). The DNA concentration was quantified by the phosphorus value obtained in the CE-ICP-MS analysis. The CE-ICP-MS measurement, for which the interface device AIF-01 equipped three layered nebulizer was adopted, was achieved with limited μL/min nebulizing without loss of sample in the vaporizing chamber. The samples of nucleotides and free phosphate were separated well in the CE-ICP-MS measurement, and the calibration curve (0.1–10 μg/mL) of the phosphorus showed a linear (R^2 = 0.999) increase in intensity. After digestion of the 100-bp double-strand DNA sample to deoxyribonucleotide-5′-monophosphates (dNMPs) by phosphodiesterase-I, phosphorus was detected by CE-ICP-MS without further purification steps. In this study, we applied two calculation schemes of DNA analysis using a dNMP concentration obtained from CE-ICP-MS. Comparative CE-ICP-MS analysis with DNA digested to dNMPs showed that the assay gave an equal value obtained from the total DNA quantification using fluorescence detection. The detection limits of the DNA sample obtained from these species and phosphorus in nucleotides using CE-ICP-MS were 3.1–26 ng/mL. These LOD values were equal to the conventional fluorescence determination of DNA.

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1. Introduction

A demand for the quantification of nucleic acids, such as DNA and RNA, has been increased in many areas, such as clinical diagnosis, food analysis, microbial analysis, and molecular biology. These nucleic acids have conventionally been determined with a photometric method such as UV adsorption (OD 260 nm) or fluorescence detection. The analytical results obtained from these photometric analyses were different depending on these analytical and sample conditions such as salt levels and pH. Though these methods essentially require a well-characterized or quantified reference material of nucleic acid, a well-established calibrant such as certified reference materials for quantification of nucleic acids does not exist. Although an accurate analysis of nucleic acid determined by phosphorus content in acid-digested nucleotides has developed, accurate quantification of each molecule of nucleic acids and intact DNA sequence has not been achieved yet [1]. DNA is composed of deoxynucleotides (dNMPs), which consist of deoxyribose, a base, and phosphate. Therefore, the determination of phosphorus is one of the most effective methods to quantify nucleotides. The determination of phosphorus can be used for the quantification of macromolecules, such as DNA, due to the fixed stoichiometry of these elements in that molecule. The quantification of a DNA sample using inductively coupled plasma optical emission spectrometry (ICP-OES) on the basis of the precise measurement of the phosphorus content of DNA has been reported [2–4]. Although the ICP-OES method is effective for the analysis of total phosphorus in a nucleic acid sample, care is required to avoid contamination of the materials that include phosphorus. Furthermore, this method requires a large volume of sample material. On the other hand, capillary electrophoresis (CE) has the advantages of achieving good separation and requiring small volume of samples; numerous studies have used CE methods for the analysis of dNMPs, which are easily separated due to their negative charge in a widely pH range [5]. In the case of the quantification of DNA, which was digested to dNMPs, the quantification value is obtained from each dNMP. Therefore, the base composition, which was not obtained in photometric analysis such as UV or fluorescence determination, is even obtained from sequence unknown sample of DNA. The composition of bases is available for the monomeric analysis such as guanine and cytosine (GC) content [6–9] and modification of bases [10,11]. And then, the phosphorus measurement provides a highly accurate quantification of mass for both dNMPs and DNA using higher order reference material of phosphate (e.g. distributed from the Japan Calibration Service System (JCSS) and NIST). Moreover, the contaminants including phosphorus are also separated using CE, and inductively coupled plasma mass spectrometry (ICP-MS) represents a highly sensitive technique for the determination of

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an element. Then, we have developed an interface device for CE hyphenated with ICP-MS [12]. There are several reports on the interface device for CE hyphenated with ICP-MS [13–16]. These reports demonstrate the advantages regarding the high efficiency of the sample injection and ease of handling for the maintenance of the interface device. Although a few studies on dNMP analysis using CE-ICP-MS have been reported [12,16], quantitative DNA analysis was not realized in these reports. In the present article, to improve the accuracy of phosphorus quantification for DNA analysis, we applied the quantification of phosphorus in nucleotides obtained from an enzymatically digested DNA molecule using CE-ICP-MS equipped with our interface device. The content of nucleotides content in DNA sample was separated by CE, and the concentration of DNA was then obtained from the measured value of phosphorus in each nucleotide without intact DNA reference materials. Moreover, the composition of each nucleotide and impurity in DNA sample can be obtained in this method.

2. Materials and methods

2.1. Chemical reagents

The free acid forms of deoxyadenosine-5′-monophosphate (dAMP), deoxyguanosine-5′-monophosphate (dGMP), deoxycytidine-5′-monophosphate (dCMP), and thymidine-5′-monophosphate (dTMP) were purchased from Sigma–Aldrich Japan (Tokyo, Japan) at >98% purity. The nitric acid solution was adjusted to 0.1% by dissolving an appropriate amount in ultra-pure water. Potassium hydroxide (1.25%) was prepared by dissolving potassium hydroxide in methanol. A phosphate ion standard solution (Kanto, Tokyo, Japan), assured by JCSS, was used as the phosphorus calibration solution. Selenium and cesium were purchased from Kanto (Tokyo, Japan). Cetyltrimethylammonium bromide (CTAB) and ammonium acetate were purchased from Wako (Osaka, Japan). Citric acid and γ-aminobutyric acid (GABA) were purchased from Sigma–Aldrich, Japan.

2.2. Sample preparation

A different dNMP stock solution (about 1 g/L) was gravimetrically prepared by dissolving an appropriate amount of deoxyribonucleotide in Tris–HCl (10 mM, pH 8.0). Each test solution was prepared by diluting the stock solution using ultra-pure water. These solutions were used for the assignment of the nucleotide peaks in the electropherograms. A phosphate ion standard solution was gravimetrically diluted with ultra-pure water.

2.3. dsDNA amplification

A double-stranded DNA (dsDNA) was adopted in this study. The dsDNA along a sequence of the NF-106, the sequence of which includes *M. musculus* DNA as a collagenase promoter (Gene bank accession number: X82000) [17] and whose theoretical size and GC content were 100 bp and about 50%, respectively, was amplified using PCR. The template sequence and primers for the PCR were synthesized and purified using HPLC through a commercial service (TaKaRa Bio, Shiga, Japan). Sequences of the template (TEMP) and primers (P) were as follows: TEMP-f: 5′-agttgagggg actttcccag gcaccccaaa gtggtgactt gtcactatca tgctataaaa tagaagatga ttgccctggg aaggagttga ggggactttc-3′, TEMP-r: 3′-tcaactcccc tgaaagggtc cgtggggttt caccactgaa cagtgatagt acgatatttt atcttctacg aacgggaccc ttcctcaact cccctgaaag-5′, P-f: 5′-agttgagggg actttccc-3′, P-r: 5′-gaaagtcccc tcaactcc-3′. PCR was performed using TaKaRa Taq HS (TaKaRa Bio, Shiga, Japan) on a TaKaRa thermal cycler Dice TP600 (TaKaRa Bio, Shiga, Japan). Amplified PCR products were doubly purified using first a Microcon YM-30 (Millipore, Tokyo, Japan) filter device and then a MinElute PCR purification kit (Qiagen, Tokyo, Japan). A single band of approximately 100 bp was observed using conventional microchip based gel-electrophoresis (data not shown, 2100 Bioanalyzer, Agilent, Tokyo, Japan).

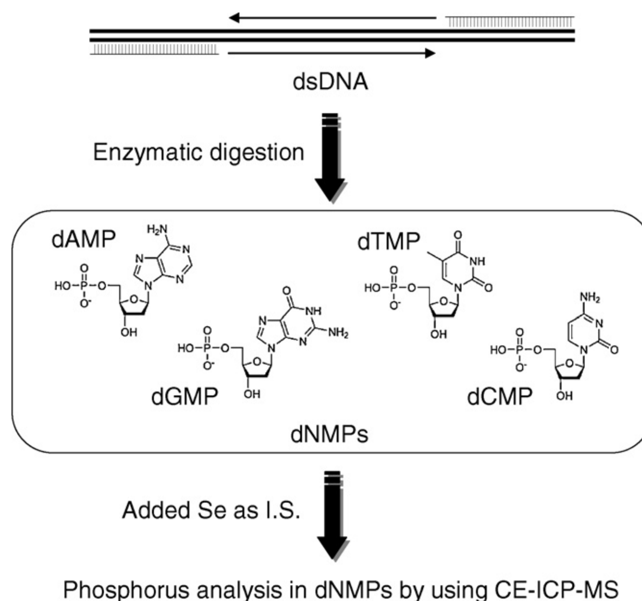

Fig. 1. Schematic diagram of the analytical procedure of a DNA sample.

2.4. Enzymatic DNA digestion

The DNA samples were enzymatically digested using phosphodiesterase I (PD-I; Sigma, USA), which is a highly purified enzyme adopted in some reports [11,18–20], and deoxyribonuclease I (DNase I; Sigma, USA) [11] in a 1× PCR buffer. The DNA samples were treated with 5 mU of PD-I and 5 mU of DNase I for a 490 ng sample of DNA for 60 min at 37 °C (U denotes units). The DNA concentration was obtained from the result of fluorescence-based analysis using microchip-based gel-electrophoresis. The enzymes were inactivated by heating for 20 min at 95 °C in the thermal cycler. The digested sample was directly injected into the CE-ICP-MS without any purification step (Fig. 1).

2.5. Apparatus

The instrumental set-up in all experiments for CE-ICP-MS consisted of a P/ACE MDQ™ capillary electrophoresis system (Beckman Coulter, Tokyo, Japan) attached with a fused silica capillary (ID: 50 μm, OD: 375 μm; GL Science, Tokyo, Japan) using an external detector assay and Agilent 7500a ICP-MS (Agilent International, Tokyo, Japan). Both instruments were connected via the interface device AIF-01 (S.T. Japan, Tokyo, Japan). Details of the instrument were reported in a previous study [12]. The operation condition of the ICP-MS system was as reported in Table 1. The sheath solution was flowed using natural drawing to obtain stable electrophoretic conditions and minimize the introduction of the sheath flow into the plasma.

2.6. Separation condition for CE-ICP-MS

A 0.1% HNO_3 solution was used as the sheath liquid. A spike of 5 μg/mL of Se was used as the internal standard in the sample solution, and 0.1 μg/mL of Cs in the sheath solution was used as the monitoring material to observe the stability of the nebulization dur-

Table 1
Instrumental and operating conditions of ICP-MS.

ICP-MS system:	Agilent 7500a
Plasma conditions:	
Incident Rf power	1.5 kW
Reflected power	<1 W
Outer gas flow rate	Ar 15 L/min
Intermediate gas flow rate	Ar 0.9 L/min
Carrier gas flow rate	Ar 1.00 L/min
Make-up gas flow rate	Ar 0.25 L/min
Sampling depth:	7 mm from work coil
Data acquisition:	
Scanning mode	Peak hopping
Data points	1 points/peak
Dwell time	10 ms/point
Measured *m*/*z*:	^{31}P, ^{82}Se, ^{133}Cs

ing all experiments. Before use, the capillary was pre-conditioned by washing with 1.25% of KOH in methanol for 3 min and 0.1 M NaOH for 1 min followed by a flushing period of 1 min with ultrapure water. To clearly flush the sample solution including the enzyme protein from the capillary, the concentration of the KOH solution was set higher than that in a previous report [12]. After each run, the capillary was washed with 0.1 M NaOH (3 min, 20 psi), water (3 min, 20 psi), and an electrophoresis buffer (3 min, 20 psi). The electrophoresis eluent consisted of 5 mM of CTAB and 5 mM of a citric acid buffer (pH 3.3 adjusted by GABA). The sample was injected with 0.5 psi for 10 s with pressure. The separation mode was micellar electrokinetic chromatography (MEKC) in the reverse mode, and the applied voltage was set at 30 kV. The operation condition of the ICP-MS system was as reported in Table 1. The samples containing dNMPs (dAMP, dTMP, dGMP, and dCMP) were analyzed using CE-ICP-MS and calibrated using a standard phosphate solution.

3. Results and discussion

3.1. Phosphorus analysis

Phosphorus determination using CE-ICP-MS was performed to investigate the ability to quantify the phosphorus in nucleotides. The interface achieved nebulizing at 4.5 μL/min and effective introduction of the sample to ICP-MS. Using ICP-MS, the following elements were measured in this experiment: ^{31}P, ^{82}Se, and ^{133}Cs. The element of ^{133}Cs was used for the monitoring of the flowing of the sheath liquid. The electropherograms of the phosphorus analysis monitoring ^{31}P and ^{82}Se as used for the internal standard in CE-ICP-MS are shown in Fig. 2. These elements were separated with an eluent consisting of 5 mM of CTAB and 5 mM of a citric acid buffer (pH 3.3 adjusted by GABA) using the MEKC reverse mode at 30 kV. No influence of the detergent in the eluent for the detection in ICP-MS was observed. In this experiment, a 0.1% HNO_3 solution was used as the sheath liquid. The influence of the sheath liquid for the measurement of mass ^{31}P was not observed. When more concentrated HNO_3 solution (e.g. 1%) was used, interference caused by the polyatomic ion (NO^+ and NOH^+) on mass ^{31}P was observed (data not shown). The RSD of the migration time and peak area of the phosphorus analysis in CE-ICP-MS was 1.34% and 1.81% ($n = 3$), respectively. The calibration curve (0.1–10 μg/ml) of phosphorus shows linearity ($R^2 = 0.999$) increasing in intensity (Fig. 3). The sample volume of this experiment was calculated to be approximately 10 nL per analysis. The detection limit and absolute detection limit of phosphorus were 0.9 μg/L and 0.009 pg, respectively. This value was two orders lower than that in a previous report [16]. The three-layered nebulizer performed highly efficient

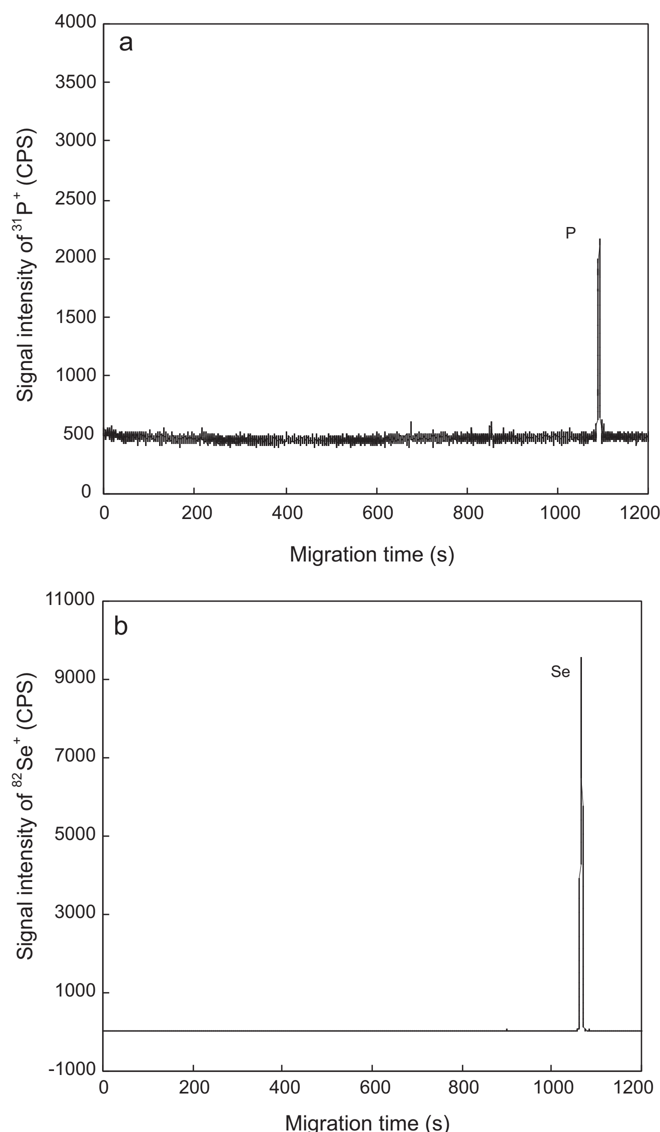

Fig. 2. Electropherograms of phosphorus analysis in CE-ICP-MS. Phosphate solution (Se as internal standard) measured on the mass of ^{31}P (a) and ^{82}Se (b).

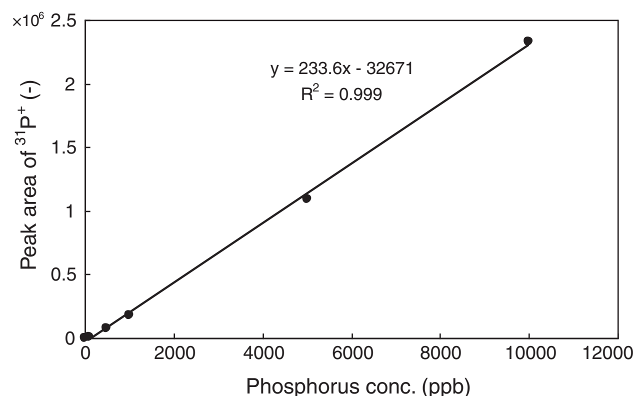

Fig. 3. Linearity of phosphorus analysis in CE-ICP-MS. A phosphorus standard solution was serially diluted and determined for linearity and sensitivity of detection. Concentration of phosphorus (ppb) plotted against the peak area of $^{31}P^+$ detected.

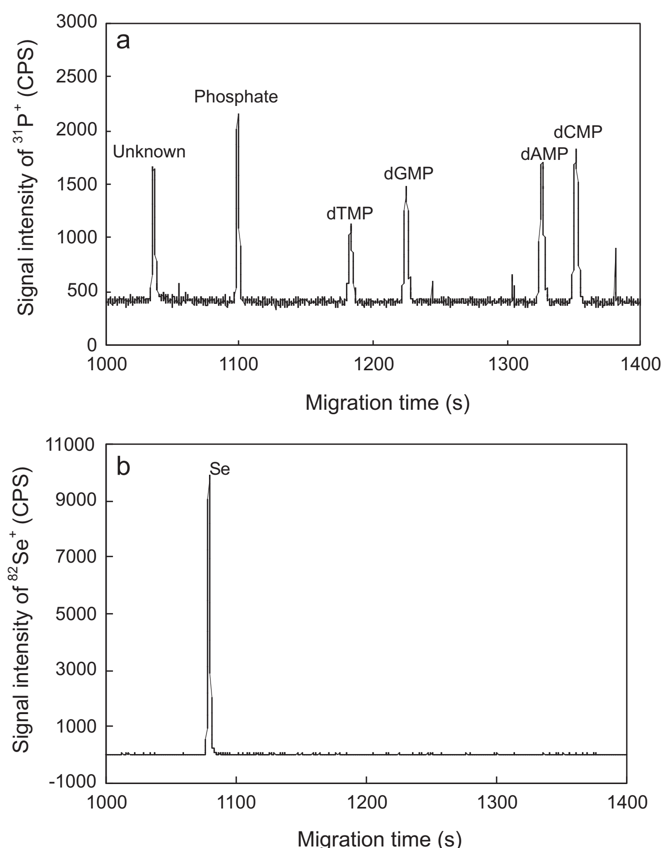

Fig. 4. Electropherograms of dNMP analysis using CE-ICP-MS. Enzymatically digested DNA sample (Se as internal standard) measured on the mass of ^{31}P (a) and ^{82}Se (b).

nebulizing. These factors, therefore, might be expected to lead to the improvement of sensitivity.

3.2. DNA quantification

To evaluate the possibility of determining phosphorus in DNA samples, the purified 100-bp amplicon of NF-106 was enzymatically digested to dNMPs and separated under the former CE conditions without further purification steps. Using ICP-MS, the following elements were measured in this experiment: ^{31}P, ^{82}Se, and ^{133}Cs. The element ^{133}Cs was used for the monitoring of the flowing of the sheath liquid. The electropherograms of dNMP analysis of digested DNA monitoring ^{31}P and ^{82}Se in CE-ICP-MS are shown in Fig. 4. These peaks in the electropherogram were clearly assigned to dTMP, dGMP, dAMP, dCMP, and free phosphate, and one unknown peak was observed. In this study, a high concentration of KOH (1.25%) in the methanol solution was adopted in the flushing procedure in the CE conditions. When the concentration of the KOH solution was low, the separation and detection of phosphorus obtained from dNMPs were not performed well (data not shown). As shown in Fig. 4b, ^{82}Se is detected clearly, and the element serves effectively as an internal standard in phosphorus analysis. The sample volume of this experiment was calculated to be approximately 10 nL per analysis, demonstrating that this analytical method is effective for samples of small volume. The concentration of the intact DNA sample was calculated by the concentration of dNMPs calibrated using the former calibration curve of phosphorus. Two concentrations of intact DNA are shown in Table 2. Table 2a shows the concentration of dNMPs obtained from the phosphorus analysis and theoretical number of dNMPs in DNA sequence. The total value of the dNMP concentration represents the concentration of intact DNA (30.0 μg/ml). Although the concentration of dTMP was an unsuitable result compared with theoretical value, the concentration of other nucleotides was almost fit in theoretical number. The variation in the concentration of these nucleotides may be caused by under- or over-reaction of the enzymatic digestion. The concentrations of DNA obtained from each kind of dNMPs were calculated with molecular weight and composition number in the DNA molecule of each dNMP (Table 2b). The average of these values also means the concentration of intact DNA (28.6 μg/ml). These values were equal in the standard deviation. If the DNA was digested to dNMPs perfectly, the concentration of dNMPs and DNA obtained from each kind of dNMPs should be almost the same value in this sample. However, these results have shown some difference in each dNMP. The differences in the concentration of each kind of dNMPs resulted from an excessive or insufficient digesting reaction of DNA. The differences are shown as phosphate and unknown peaks in the electropherogram (Fig. 4) and especially, the unknown peak was derived from impurities of the enzyme because the DNA sample has no monomeric impurities in other chromatographic analysis (data not shown); thus, the measurement using CE-ICP-MS would be effective for the evaluation of the digesting reaction and the contamination of the materials including phosphorus. Furthermore, the concentration of the intact DNA sample employed in this analysis was calculated at 24.5 μg/ml using fluorescence measurement in advance. These values were almost equal in these measurements. The DNA concentration was obtained basically from the analytical values of nucleotide in this study, and the RSD of the dNMPs concentration was 3.6% (Table 2a). The coefficients of variation for the assay of the DNA quantification using fluorescence detection such as SYBR green I were ranged from 4.6 to 11% in the previous report [21]. Therefore, the DNA quantification using phosphorus analysis is found to be a more accurate quantitative method than conventional method. The detection limits of the DNA sample obtained from these species and phosphorus in nucleotides using CE-ICP-MS were 3.1–26 ng/mL. These LOD values were even equal to the conventional fluorescence detection when the PicoGreen quantification of DNA was used [22]. Therefore, this analytical method is useful for the quantification of DNA samples in the pre- or post-PCR steps. Although the LOD value is not mentioned in a previous study [16], we assume that the LOD value in this study has been improved

Table 2
Results of dNMP analysis of digested DNA. (a) Concentration of dNMPs obtained from phosphorus analysis using CE-ICP-MS and theoretical number of dNMPs in DNA sequence; (b) concentration of DNA obtained from each kind of dNMPs using CE-ICP-MS.

(a)	Concentration of dNMPs (μg/ml)	Concentration of dNMPs (μM)	Theoretical number in DNA sequence	(b)	Concentration of DNA (μg/ml) dAMP
dAMP	8.6	26.0	52	dAMP	30.9
dTMP	5.3	16.4	51	dTMP	20.1
dGMP	7.7	22.2	49	dGMP	28.0
dCMP	8.4	27.3	48	dCMP	35.4
Total	30.0	–	–	Ave.	28.6
SD	1.1	–	–	SD	6.1
RSD (%)	3.6	–	–	RSD (%)	21.4

by two orders or more because of the use of an effective interface device to the ICP-MS.

4. Conclusions

CE hyphenated with ICP-MS using the developed interface has proved to be a suitable technique for the separation and element-specific determination of phosphorus in dNMPs. Phosphorus was determined with good repeatability and linearity in intensity with CE-ICP-MS equipped with the interface device. And four kinds of dNMPs, which were enzymatically digested from DNA, were separated at the baseline with CE and detected as phosphorus in ICP-MS. In this study, we applied two calculation schemes of DNA analysis using the dNMP concentration obtained from CE-ICP-MS without intact DNA reference materials. These schemes were effective to improve the accuracy of the DNA measurement using phosphorus analysis. The LOD value of DNA analysis was almost equal to the conventional assay using fluorescence detection. And base compositions and impurity in DNA sample, which were not possible to obtain in photometric analysis, were also obtained. These results are promising for the future investigation of phosphorus in the pre- or post-PCR process and other elements in real samples, such as gDNA and RNA.

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