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Phosphorus and osmium as elemental tags for the determination of global DNA methylation—A novel application of high performance liquid chromatography inductively coupled plasma mass spectrometry in epigenetic studies

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ABSTRACT

The hyphenation of high performance liquid chromatography with inductively coupled plasma mass spectrometry (HPLC–ICP-MS) is proposed in this work as a novel approach for the evaluation of DNA methylation, defined as the ratio between methylated cytosine and total cytosine bases in DNA. In the first part, reversed phase separation of 5-methyl-2′-deoxycytidine monophosphate (5mdCMP) and four deoxynucleotides with specific ICP-MS detection on ^{31}P had been explored. In further development, selective labeling of 5-methylcytosine in ssDNA was carried out using potassium osmate (K_2OsO_4) in the presence of strong oxidant ($K_3Fe(CN)_6$) and *N,N,N′,N′*-tetramethylethylenediamine (TEMED). The sample was then cleaned up and introduced to size exclusion chromatography–ICP-MS for specific detection at ^{31}P and ^{189}Os and for evaluation of the molar ratio between Os and P eluted in DNA molecular mass fraction. The quantification of the two elemental tags was achieved by external calibration with phosphoric acid and Os(VI)-TEMED, respectively. The amount of Os in DNA fraction corresponded to methylated cytosines, while P signal was directly proportional to the total amount of DNA and could be recalculated to the amount of cytosine bases. The two procedures were tested by analyzing salmon testes DNA and a commercial oligonucleotide of known composition. For comparative purposes, these same samples were digested to deoxynucleosides and analyzed by reversed phase HPLC with spectrophotometric detection (DAD) at 280 nm. The results obtained using two procedures based on ICP-MS detection were in good agreement and also in agreement with the results obtained by HPLC–DAD procedure. In conclusion, ICP-MS specific detection at internal or external element tags seems to be an interesting alternative for the evaluation of global DNA in epigenetic studies.

1. Introduction

Epigenetic component of gene regulation consists of alterations in chromatin structure in such a way that in its condensed form genes are inactivated and they are potentially expressed when chromatin is open (active). These two dynamic chromatin states are controlled by reversible epigenetic patterns of DNA methylation and histone modifications [1,2]. DNA methylation occurs at carbon 5-position in cytosine pyrimidine ring and generally speaking, methylated cytosines favor condensed chromatin state, while unmethylated cytosines promote relaxed structure. Of special interest are CpG-islands in the promoters of genes, where methylated cytosines are associated with gene silencing [3]. Epigenetic events contribute to the normal process of cell differentiation and development, however they can be misdirected and as such have been implicated in oncogenesis and other types of pathologies [4]. Therefore, the analysis of actual DNA methylation status is gaining strength in the fields of epigenetics, cancer risk assessment, diagnosis and therapy monitoring [5,6].

In molecular biology, site-specific sequence techniques are in use to detect subtle differences in the methylation status of individual genes [7–10]. On the other hand, global methylation of DNA has often been evaluated as an indicator of large-scale epigenetic effects [11–17]. This parameter is defined as the ratio between methylated cytosines and total amount of cytosine bases in DNA. Several analytical procedures have been reported, based on different DNA digestion protocols followed by chromatographic or electrophoretic separation [11,12,14,18–23]. The detection tools employed in those studies include UV/vis

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[12,14,22], molecular mass spectrometries [11,20,21] and measurements of laser-induced fluorescence [23].

Inductively coupled plasma mass spectrometry (ICP-MS) is a well established element-specific detector in quantitative analysis of variety of biomolecules [24–26]. In particular, the determination of deoxyribonucleotides in DNA digests has been carried out by liquid chromatography or capillary electrophoresis with ICP-MS detection at phosphorus, which is naturally present in these compounds [27,28]. Similar methodological approach has also been reported in studies on DNA adducts with metal-based drugs [29,30] and carcinogenic agents [31,32], however the application of phosphorus as internal elemental tag for the evaluation of DNA methylation has not been explored yet.

The use of external elemental tags and their specific ICP-MS detection has emerged as a novel strategy in proteomics and, more general, in cellular biology applications [24,33–35]. Such an approach enables for enhanced quantification of labeled biomolecules, based on exceptional features of elemental mass spectrometry in terms of sensitivity, selectivity, multielemental and isotopic capabilities [36,37]. In the context of this work, the use of osmium tetroxide in nucleic acid research should be highlighted. First, it was demonstrated that osmium can be bound to nucleobases pyrimidine in the presence of tertiary nitrogen donor ligands [38] and, in further studies, the selectivity of reaction toward cytosine and thymine as well as toward methylated versus non-methylated cytosine has been studied [39,40]. In particular, under appropriate pH, oxidative conditions, temperature and time of reaction, the selective labeling on 5-methylcytosine can be achieved [41,42]. The electroactivity of osmium labels, the feasibility of electroanalytical techniques and fluorimetric detection for DNA/RNA probing have been extensively studied [39,41,43–48]. Such labeling reactions would be also of interest in the field of ICP-MS applications.

The goal of this work was to explore a potential of ICP-MS detection in the evaluation of global DNA methylation. Two analytical procedures are proposed, based on different DNA preparation, liquid chromatography separation and ICP-MS detection. The use of phosphorus as internal elemental tag required DNA digestion to deoxynucleotides (including 5-methyl-2′-deoxycytidine monophosphate (5mdCMP)) prior to their reversed phase separation. When osmium was selectively attached to 5-methylcytosine in ssDNA, no further hydrolysis was needed and the separation was accomplished by size exclusion chromatography. The quantification of Os and P in DNA column fraction enabled to calculate the percentage of methylated cytosines. The commercial oligonucleotide and salmon testes DNA were analyzed by the two procedures and the percentages of global methylation evaluated were in agreement. For accuracy checking, these same samples were also analyzed by liquid chromatography separation of nucleosides with UV detection [14]. The results obtained in this work provide a proof of the concept that ICP-MS could be a useful, complementary tool in epigenetic studies [49].

2. Material and methods

2.1. Apparatus

An Agilent series 1200 liquid chromatographic system equipped with a quaternary pump, a well plate autosampler, a column oven, a diode array detector, and a ChemStation (Agilent Technologies, Palo Alto, CA, USA) was used. The chromatographic columns were Luna C18 (250 × 4.6 mm, 5 μm) and BioSep-SEC-S 3000 (300 × 7.8 mm, 5 μm) from Phenomenex. For specific detection on phosphorus and osmium, the column effluent was on-line introduced to inductively coupled plasma mass spectrometry system via the short-length Teflon tubing.

Table 1
HPLC–ICP-MS instrument operating conditions.

Reversed phase liquid chromatography (separation of nucleotides)	
Column	Luna C18 (250 × 4.6 mm, 5 μm) with a guard column
Mobile phase	30 mM HCOOH, 10 mM $HCOONH_4$, 0.1% of TEA:MeOH (97:3), pH 3.0
Elution	Isocratic
Temperature	Ambient
Flow	1.0 mL min^{-1}
Injection volume	20 μL
Size exclusion chromatography (ssDNA)	
Column	BioSep-SEC-S 3000 (300 × 7.8 mm, 5 μm)
Mobile phase	25 mM CH_3COONH_4, pH 5.1
Elution	Isocratic
Temperature	Ambient
Flow	1.0 mL min^{-1}
Injection volume	100 μL
ICP-MS detection	
Forward power	1500 W
Nebulizer gas flow	0.9 L min^{-1}
Make-up gas	0.1 L min^{-1}
Nebulizer	MiraMist Teflon®
Spray chamber	Peltier-cooled chamber, 2 °C
Sample and skimmer cones	Platinum
Sample depth	8 mm
Channels monitored	^{31}P, ^{189}Os
Acquisition mode	Time-resolved analysis
Dwell time	100 ms
Collision/reaction cell	3.5 mL min^{-1} He

A model 7500ce ICP-MS (Agilent Technologies, Tokyo, Japan) was used with a MiraMist Teflon® nebulizer. A Peltier-cooled chamber was operated at 2 °C. Tuning procedure was performed daily using diluted Agilent solution (Li, Y, Tl, Ce, 1 μg L^{-1} each). The chromatographic and ICP-MS instrumental operating conditions are given in Table 1.

2.2. Reagents and solutions

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

The deoxynucleosides (dC – 2′-deoxycytidine, dA – 2′-deoxyadenosine, dG – 2′-deoxyguanosine, T – thymidine) were obtained from Sigma (St. Louis, MO, USA). Deoxyribonucleic acid sodium salt from salmon testes was from Sigma and the oligonucleotide (28 bases, dA:dC:dT:dG (1:2:2:2) with 2% 5mdC) was from Integrated DNA Technologies, Inc., Coralville, IA, USA. Other two oligonucleotides from this same company were also used, namely 24 bases 5mdC:dA:dG (1:2:1) and 24 bases dC:dA:dG (1:2:1).

Aldrich reagent potassium osmate and the following Sigma analytical grade reagents were also used: *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), potassium hexacyanoferrate(III), EDTA, ammonium acetate, ammonium phosphate dibasic, triethylamine (TEA), formic acid, ammonium formate, phosphoric acid, hydrochloric acid, sodium acetate, zinc sulfate, nuclease P1 and Tris/hydrochloride. Calf intestinal alkaline phosphatase (CIAP) was from New England Biolabs.

DNA extracts from onion and tomato plant tissues were analyzed as the real world samples.

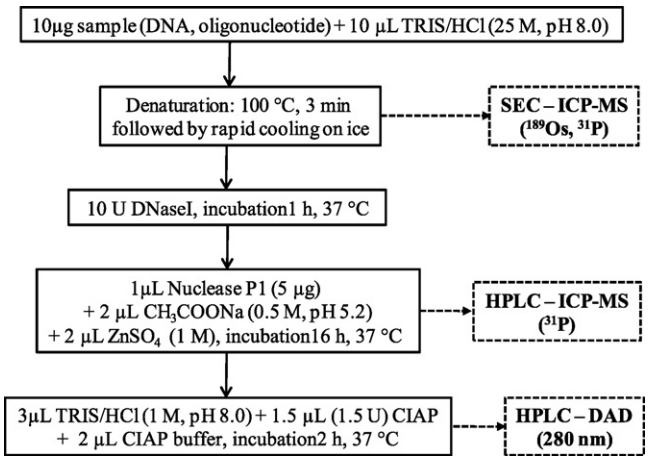

Fig. 1. Scheme of DNA digestion, indicating at which point of the procedure the samples were taken for ssDNA, deoxynucleotides or deoxynucleosides separation.

2.3. Procedures

2.3.1. Sample preparation

Commercial oligonucleotide and DNA from salmon testes, onion and tomato were analyzed by three procedures that required three different sample preparation protocols. In Fig. 1, a scheme of DNA digestion is presented [50,51], indicating at which point the samples were taken for size exclusion chromatography with ICP-MS detection at ^{189}Os and ^{31}P (ssDNA), for reversed phase chromatography with ICP-MS detection at ^{31}P (deoxynucleotides) and for reversed phase chromatography with DAD detection (deoxynucleosides). Taking 10 μg of oligonucleotide or DNA, the final volume of the sample containing deoxynucleotides was brought to 50 μL and that containing deoxynucleosides to 100 μL. For HPLC–ICP-MS (deoxynucleotides) the sample was diluted with the mobile phase 1:20 and for HPLC–DAD (deoxynucleosides) 1:10.

High molecular mass genomic DNA was extracted from onion and tomato plant tissues using the DNeasy system (Qiagen) [14].

2.3.2. Reversed phase separation of nucleotides with ICP-MS detection at ^{31}P

The diluted oligonucleotide or DNA digest was injected to HPLC–ICP-MS system. The chromatographic and detection conditions are given in Table 1. External calibration was performed by injecting phosphoric acid at the concentration levels 0, 0.2, 1.0, 5.0, 10, 25, 50 μmol L^{-1}. For peak area measurement mode, background equivalent concentration (BEC) was 0.46 μmol L^{-1}; r^2 >0.9998 and the detection limit evaluated based on six standard deviations of baseline measured in the elution region of the analyte was 0.04 μmol L^{-1}.

2.3.3. Size exclusion chromatography of Os-labeled ssDNA with ICP-MS detection at ^{189}Os, ^{31}P

The denaturalized DNA or oligonucleotide was labeled with osmium at 5-methylcytosine groups, as described elsewhere [41,42]. In brief, the sample aliquot (Fig. 1) was added to the ice-cooled mixture containing 200 μL Tris/HCl 0.3 mol L^{-1} (pH 7.0), 10 μL $K_3Fe(CN)_6$ 0.2 mol L^{-1}, 10 μL K_2OsO_4 10 mmol L^{-1} and TEMED 75 mmol L^{-1}. The mixture was then treated by the Wizard DNA column clean-up system (Promega). The purified DNA was eluted with 100 μL of deionized water, diluted 1:10 with the mobile phase and introduced to SEC–ICP-MS. The chromatographic and detection conditions are given in Table 1. External calibration was performed by injecting phosphoric acid (0–50 μmol L^{-1}) and seven concentration levels of osmium (0, 0.01, 0.02, 0.05, 0.10, 0.25, 0.50 μmol L^{-1}) in the solution used for DNA-labeling (Tris/HCl, $K_3Fe(CN)_6$, TEMED). For phosphorus, essentially these same analytical parameters were obtained as for the reversed phase separation, while for Os the obtained values were as follows: BEC 1.54 nmol L^{-1}, r^2 >0.9998, LOD 0.84 nmol L^{-1}.

Table 2
Analytical figures of merit evaluated for the reversed phase separation of 2′-deoxycytidine MP (dCMP), 5-methyl-2′-deoxycytidine MP (5mdCMP), 2′-deoxyadenine MP (dAMP), 2′-deoxyguanosine MP (dGMP) and thymidine MP (dTMP) with ICP-MS detection at ^{31}P.

Analyte	$T_{ret} \pm$ SD, min (n = 5)	k	LOD, μmol L^{-1}
dCMP	4.01 ± 0.09	0.60	0.051
5mdCMP	5.30 ± 0.08	1.09	0.066
dAMP	8.60 ± 0.11	2.47	0.101
dGMP	10.4 ± 0.1	3.20	0.130
dTMP	12.5 ± 0.1	4.04	0.150

k: retention factor; and LOD: detection limit evaluated based on six standard deviations of baseline measured in the elution region of the analyte.

2.3.4. Reversed phase separation of nucleosides with DAD detection

The procedure reported previously was used [14]. In brief, deoxynucleosides were obtained by calf intestinal alkaline phosphatase treatment of nucleotides (Fig. 1), the final volume was brought to 100 μL and, after dilution 1:10 with the initial mobile phase, the separation was accomplished on Luna C18 (250 × 4.6 mm, 5 μm) column in gradient elution (phosphate buffer mobile phase, pH 4.0, methanol gradient from 0 to 15%), at total flow rate 1 mL min^{-1} and column temperature 40 °C. The spectrophotometric detection was at 280 nm and the quantification was based on external calibration using respective deoxynucleosides.

3. Results and discussion

3.1. Reversed phase separation of nucleotides with ICP-MS detection at ^{31}P

The original idea of this approach was to apply reversed phase liquid chromatography with on-line ICP-MS detection at ^{31}P for the determination of 5-methyl-2′-deoxycytidine monophosphate, 2′-deoxycytidine monophosphate and other deoxynucleotides in order to evaluate the percentage of methylated DNA cytosines. In Fig. 1, the procedure used for DNA digestion is presented [50,51]. The analytical challenge was to achieve baseline separation of five compounds in relatively short time and to set instrumental conditions for sensitive and interference-free ICP-MS detection of mono-isotopic phosphorus. In the previous studies [27–32], the separation of deoxynucleotides and respective adducts with cisplatin, styrene oxide or melphalan, was accomplished with the mobile phases containing acetate buffer (pH 5–5.8) and methanol (up to 30%). Gradient elution was preferred over isocratic conditions and the separation time varied from 13 min to 1 h. In this work, it was decided to increase the acidity of the mobile phase, add triethylamine to improve peak symmetry and thus lower the requirement for organic modifier. Formate buffer was used instead of acetate, which enabled to explore lower pH region. The chromatographic conditions finally selected are listed in Table 1. In brief, using isocratic elution with 30 mmol L^{-1} HCOOH, 10 mmol L^{-1} $HCOONH_4$, 0.1% TEA (pH 3.0):MeOH (97:3) and a total flow rate 1 mL min^{-1}, the five compounds of interest were separated within 14 min in the following elution order: dCMP, 5mdCMP, dAMP, dGMP, dTMP. This elution order was assessed by injecting the digests of three oligonucleotides of different compositions, namely dA:dC:dT:dG (1:2:2:2) with 2% 5mdC, 5mdC:dA:dG (1:2:1) and dC:dA:dG (1:2:1). The retention times with respective standard deviations and the values of retention factors are given in Table 2.

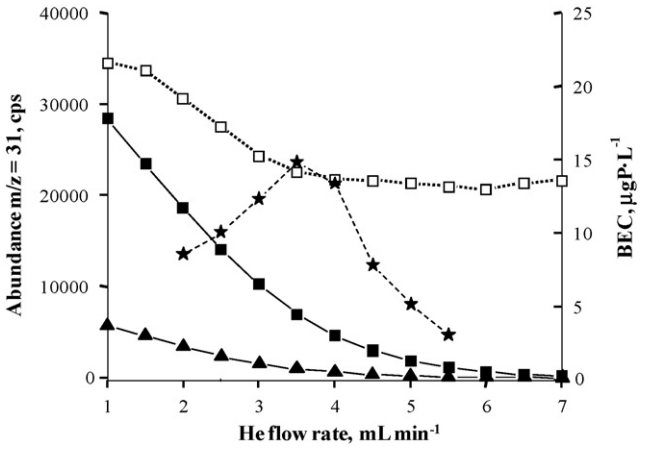

Fig. 2. ICP-MS detection at ^{31}P with collision/reaction cell: selection of helium flow rate. (···□···) BEC, μgP L^{-1}; (—■—) H_3PO_4, 100 μgP L^{-1}; (—▲—) mobile phase; (–★–) S/N.

In order to control polyatomic interferences from $^{15}N^{16}O^+$, $^{14}N^{16}O^1H^+$ and $^{12}C^1H_3{}^{16}O^+$ ions potentially occurring at $m/z = 31$, the octopole reaction/collision cell was used in kinetic energy discrimination mode. The selection criterion for gas flow rate was the highest possible signal to noise ratio. As shown in Fig. 2, for different He flow rates (1–7 mL min^{-1}), the signal at $m/z = 31$ was measured for phosphorus standard (phosphoric acid, 100 μgP L^{-1}) and for blank (mobile phase) solutions. The value 3.5 mL min^{-1} was selected for further experiments. The detection limits for five compounds evaluated from peak area measurements were in the range 0.051–0.150 μmol L^{-1} (Table 2). These values were higher with respect to LOD obtained for phosphoric acid eluting in the dead volume (0.042 μmol L^{-1}). Moreover, higher LOD values were obtained for deoxynucleotides eluting later, which should be ascribed to peak broadening. On the other hand, the obtained values were similar to LOD = 0.14 μmol L^{-1}, reported for reversed phase separation of deoxynucleotides by Profrock et al. [27] and lower than those reported in other studies [29–32]. It should be noted however, that 5mdCMP had not been considered in any of the above cited studies. Since the main goal of this work was to evaluate global DNA methylation, it is important that the LOD values for dCMP (0.051 μmol L^{-1}) and for 5mdCMP (0.066 μmol L^{-1}) were very similar and also close to the LOD of inorganic phosphorus standard (0.042 μmol L^{-1}).

The analysis of deoxynucleotides was carried out in Sigma reagent salmon testes DNA, in the commercial oligonucleotide of known composition and in DNA digests from tomato and onion plants. For comparative purposes, deoxynucleosides were obtained from these same samples (Fig. 1) and determined by HPLC–DAD procedure reported previously [14]. As an example, the chromatograms obtained by the two procedures in the analysis of salmon DNA digests are presented in Fig. 3. The concentrations

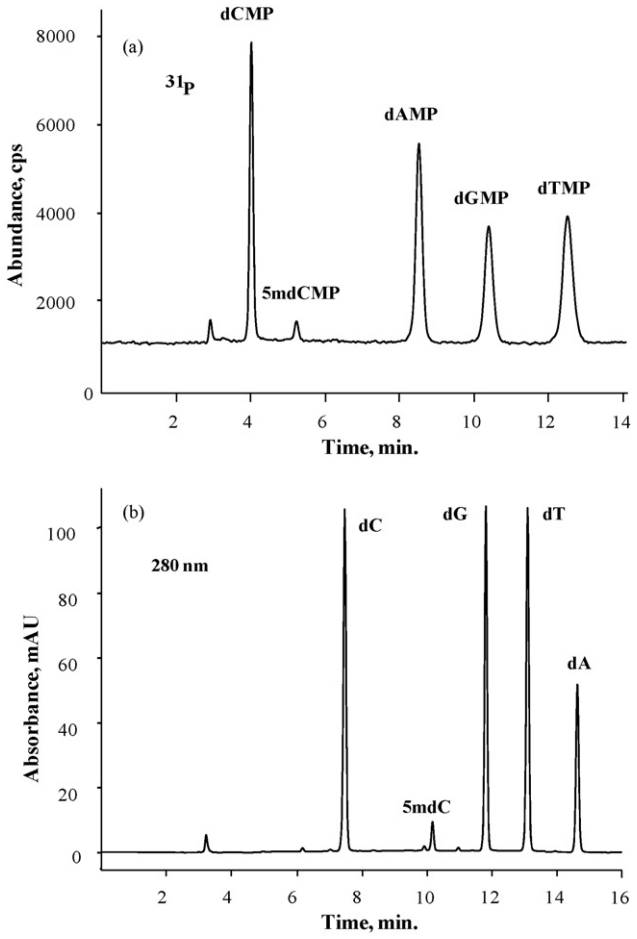

Fig. 3. Reversed phase chromatograms obtained in the analysis of salmon testes DNA digests: (a) separation of deoxynucleotides, ICP-MS detection at ^{31}P; (b) separation of deoxynucleosides, DAD detection at 280 nm [14].

of five compounds found in salmon testes DNA and oligonucleotide are summarized in Table 3. It is important to note that 10 μg of the sample were taken for any procedure and then, for HPLC–ICP-MS the final volume was brought to 50 μL and the sample was diluted 1:20, while for HPLC–DAD the final volume was 100 μL and the dilution factor 1:10. Therefore, the molar concentration levels found in the injected solutions for deoxynucleotides should be essentially the same as those determined for deoxynucleosides and indeed, no statistically significant differences were observed between the results obtained by the two procedures (ANOVA, $p < 0.05$). For oligonucleotide, procedure recovery was evaluated as the ratio between the sum of analyte masses derived from the concentration values obtained in each procedure and the initial sample mass (10 μg). The percentage recovery for HPLC–ICP-MS procedure was 99.3% and for HPLC–DAD procedure 99.0%.

Table 3
Quantification results obtained in the analysis of oligonucleotide and salmon testes DNA digests by the proposed procedure (HPLC separation of deoxynucleotides with ICP-MS detection at ^{31}P) and by the procedure previously reported (HPLC separation of deoxynucleosides with DAD detection at 280 nm). In each case, the mean concentration in the injected solution and respective SD values are presented, based on triplicate analysis.

Analyte	HPLC–ICP-MS, mean ± SD, μmol L^{-1}		HPLC–DAD, mean ± SD, μmol L^{-1}	
	Oligonucleotide	Salmon testes DNA	Oligonucleotide	Salmon testes DNA
dC	9.03 ± 0.09	6.31 ± 0.08	9.11 ± 0.10	6.50 ± 0.07
5mdC	0.20 ± 0.01	0.48 ± 0.01	0.20 ± 0.01	0.49 ± 0.01
dA	4.41 ± 0.06	9.60 ± 0.16	4.38 ± 0.05	9.93 ± 0.15
dG	9.12 ± 0.15	6.53 ± 0.19	9.16 ± 0.09	7.01 ± 0.16
dT	9.25 ± 0.20	9.30 ± 0.38	9.31 ± 0.17	9.85 ± 0.29

Oligonucleotide composition: dA:dC:dT:dG (1:2:2:2) with 2% 5mdC.

Table 4
Percentage of global DNA methylation, evaluated from the results obtained by three independent procedures.

Sample	HPLC–DAD	HPLC–ICP-MS (^{31}P)		SEC–ICP-MS (^{31}P, ^{189}Os)
	Calibration with deoxynucleosides	Calibration with H_3PO_4	Without calibration[a]	Calibration with H_3PO_4, Os-TEMED
Oligonucleotide	2.15	2.17	2.25	2.20
Salmon DNA	7.01	7.07	7.03	6.72
Onion DNA	16.3	15.6	15.7	–
Tomato DNA	12.8	13.0	13.00	–

[a] Based on 5mdCMP and dCMP peak area measurements.

Finally, the molar ratio dA:dC:dT:dG found in the commercial oligonucleotide of known composition (2:1:2:2 with 2% mdC) was 2.09:1.00:2.07:2.10 with 2.17% of mdCMP based on HPLC–ICP-MS results and 2.13:1.00:2.09:2.12 with 2.14% of mdC based on HPLC–DAD.

An important advantage of ICP-MS in bio-molecular chemistry is its capability to provide compound-independent sensitivity, based on specific detection at internal or external elemental tags [24,26]. In this sense, the global methylation in the analyzed DNA samples was evaluated in this work directly from 5mdCMP and dCMP peak area measurements and the results obtained are presented in Table 4. This same parameter was also calculated from respective values of molar concentrations, based on external calibration with phosphoric acid. For comparative purposes, the global DNA methylation was also determined by HPLC–DAD procedure [14]. As can be observed in Table 4, very good agreement was obtained between the results from two independent procedures (HPLC–ICP-MS and HPLC–DAD) and also between the results obtained while using two different quantification approaches in HPLC–ICP-MS procedures.

The results presented above indicate that the procedure proposed in this work provides reliable quantification of deoxynucleotides based on external calibration with inorganic phosphorus (phosphoric acid). On the other hand, global DNA methylation can be evaluated directly from the peak area measurements, eliminating the need for calibration.

3.2. Size exclusion chromatography of Os-labeled ssDNA with ICP-MS detection at ^{189}Os and ^{31}P

As described in Section 1, osmium compounds have been extensively used in nucleic acid research, yet the procedure proposed in this work is the original contribution exploring the feasibility of ICP-MS detection at Os for the evaluation of DNA methylation. It was considered that osmium would be a good candidate for external elemental tag, because of its relatively high ionization efficiency (about 80%), no isobaric interferences expected at isotope ^{189}Os and, in consequence, low detection limit. Furthermore, osmium is practically absent in biological samples. The combination of osmate (K_2OsO_4), with $K_3Fe(CN)_6$ as an activator and TEMED as a reaction accelerating ligand enabled for selective attachment of external elemental tag to methylated cytosine in ssDNA, as shown in Fig. 4 [41,42]. The reaction mixture was then fractionated by size exclusion chromatography with on-line ICP-MS detection at ^{189}Os and ^{31}P. The quantification of the two elements in DNA-containing column fraction was accomplished by peak area measurements and two independent external calibrations with Os-TEMED and phosphoric acid, respectively. It was assumed that the molar amount of Os ([Os]) would correspond to the number of methylated cytosines ([5mdC]), while phosphorus ([P]) would provide data on total DNA. Based on the percentage CG pairs in specific DNA (%CG), the number of phosphorus moles can be easily recalculated to that of total cytosine bases ([5mdC + dC]). Doing so, the global methylation of DNA can be evaluated using the following equation:

$$\text{Global DNA methylation} = \frac{[5\text{mdC}]}{[5\text{mdC} + \text{dC}]} \times 100\% = \frac{[\text{Os}]}{0.5(\%\,\text{CG})[\text{P}]} \times 100\%$$

In Fig. 5a, the SEC–ICP-MS chromatogram obtained for salmon testes DNA is presented. The elution of two poorly resolved Os-containing fractions can be observed in high molecular mass region. Since the excess of labeling reagent was used, the formation of Os-containing particles could be responsible for this effect. It was

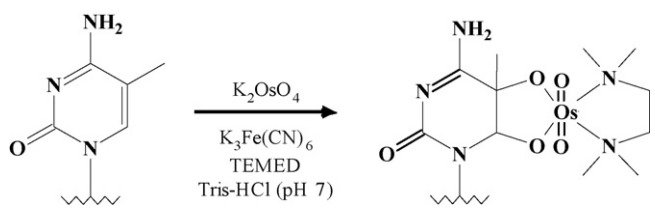

Fig. 4. Labeling reaction scheme for specific attachment of osmium to methylated cytosine groups in DNA [41,42].

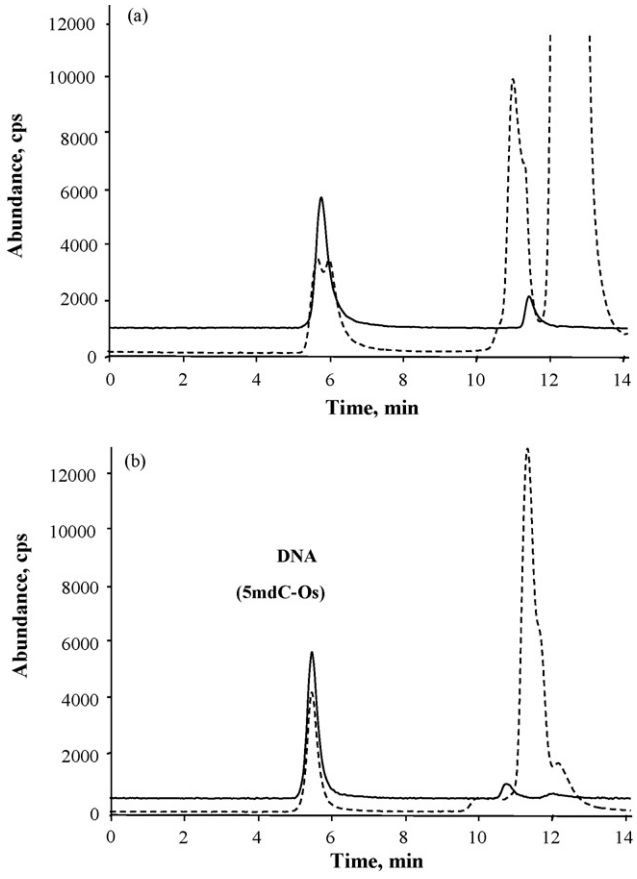

Fig. 5. SEC–ICP-MS chromatograms of salmon testes ssDNA labeled with Os: (a) direct injection of the reaction mixture; (b) after sample clean-up. ((—) ^{189}Os, (—) ^{31}P).

decided to clean-up the reaction mixture prior to its introduction to SEC–ICP-MS system. The commercial DNA column clean-up system was used for this purpose, as described in Section 2.3 and the chromatogram obtained is shown in Fig. 5b. Perfect co-elution of osmium and phosphorus in DNA fraction can be observed. The recovery of DNA in the procedure was estimated based on phosphorus quantification (24.6 μmolP L^{-1} eluted in DNA fraction), the molecular mass of salmon DNA (1.6×10^6 g mol^{-1}) and the number of base pairs (2000) [52]. Since 10 μg of the sample were taken for the analysis, the recovery obtained was 80%. It is also known that salmon testes DNA contains 41.2% of CG pairs [53], which enabled to evaluate global DNA methylation. The value obtained was 6.72%, in agreement with the results of other two procedures, as presented in Table 4. Using this same approach, the methylation of cytosines obtained in the commercial oligonucleotide was 2.20%, also in agreement with the results provided by other two procedures (Table 4). It should be stressed that incomplete recovery of DNA (about 80%) would not affect the methylation results obtained, since this parameter is calculated from the relation between Os and P in this same DNA-containing SEC fraction.

4. Conclusions

The feasibility of ICP-MS as element-specific detector in epigenetic studies has been explored in this work, focusing on the evaluation of global DNA methylation. Two novel analytical approaches were proposed, based on different DNA preparation, liquid chromatography separation and ICP-MS detection at phosphorus and osmium as internal and external elemental tags, respectively. In the first procedure, DNA was digested to deoxynucleotides and analyzed by HPLC–ICP-MS, using phosphorus as natural tag. It was demonstrated that quantification of 5mdCMP and four deoxynucleotides can be achieved, based on external calibration with phosphoric acid. More importantly, the results obtained indicate that global DNA methylation can be evaluated directly from 5mdCMP and dCMP peak area measurements, with no need for calibration. The simplicity is an important feature of the second procedure proposed. In fact, only denaturation step was required. The ssDNA was then selectively labeled with osmium at methylated cytosine groups and, after clean-up step, the sample was fractionated by size exclusion chromatography. ICP-MS detection was carried out at phosphorus and osmium in order to get information of total amount of DNA and the amount of methylated cytosines. This approach required external calibration for the two elemental tags and enabled for the evaluation of global DNA methylation.

The two procedures were tested using salmon testes DNA and commercial oligonucleotide. For accuracy checking, these same samples were also analyzed by liquid chromatography separation of deoxynucleosides with UV detection [14]. The results obtained provide a proof of concept that ICP-MS could be a useful, complementary tool in epigenetic studies [49].

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