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Quantification of 5-methyl-2′-deoxycytidine in the DNA

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Methylation at position 5 of cytosine (Cyt) at the CpG sequences leading to formation of 5-methyl-cytosine (m^{5}Cyt) is an important element of epigenetic regulation of gene expression. Modification of the normal methylation pattern, unique to each organism, leads to the development of pathological processes and diseases, including cancer. Therefore, quantification of the DNA methylation and analysis of changes in the methylation pattern is very important from a practical point of view and can be used for diagnostic purposes, as well as monitoring of the treatment progress. In this paper we present a new method for quantification of 5-methyl-2′deoxycytidine (m^{5}C) in the DNA. The technique is based on conversion of m^{5}C into fluorescent 3,N^4-etheno-5-methyl-2′deoxycytidine (εm^{5}C) and its identification by reversed-phase high-performance liquid chromatography (RP-HPLC). The assay was used to evaluate m^{5}C concentration in DNA of calf thymus and peripheral blood of cows bred under different conditions. This approach can be applied for measuring of 5-methylcytosine in cellular DNA from different cells and tissues.

Key words: DNA methylation, 3,N^4-etheno-5-methyl-2′deoxcytidine, fluorescence, RP-HPLC

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INTRODUCTION

Gene expression during development of Eukaryotes is regulated by complex processes (Kim *et al.,* 2009). One of them is DNA methylation that relies on the covalent attachment of a methyl group to cytosine, which results in formation of 5-methyl-cytosine (m^{5}Cyt) (Jones *et al.*, (2001). This reaction is catalysed by a group of several enzymes, called DNA methyltransferases (DNMT), whose activity depends on the stage of development. Methylation of DNA occurs mainly within the so-called CpG islands located at gene promoter regions (but sometimes also in the body of genes). The methylation pattern is unique to every individual and is maintained by DNA methyltransferase 1 (DNMT1) throughout life, during every cell division (Law *et al.*, 2010; Hermann *et al.*, 2004). Cytosine methylation level is generally stable in somatic cells, however it decreases somewhat with ageing of an organism as well as a result of certain diseases (especially cancer) (Feinberg & Vogelstein, 1983; Feinberg & Tycko, 2004; Fraga *et al.*, 2007; Kulis & Esteller, 2010). The discovery of a relationship between changes in the pattern of DNA methylation and development of pathology was a breakthrough for understanding the etiologic basis of many diseases and has opened new possibilities for early diagnosis and monitoring of treatment progress. Both hypomethylation of the whole DNA (global) and site-specific hypermethylation have been observed in common human chronic degenerative diseases and cancer (Ballestar, 2011; Jones & Baylin, 2002; Laird & Jaenisch, 1994; Jones, 1996; Liu *et al.*, 2003).

Linking the methylation status of DNA with different diseases has created a need for developing a reliable method for its level determination in the DNA.

Current methods of DNA methylation analysis can be divided into: *i)* global and *ii)* gene-specific (Oakeley, 1999). For determination of non-specific, large-scale genome-wide changes in cytosine methylation, three different approaches have been applied. The commonly used methods are high-performance liquid (HPLC) or gas chromatography (GC) and capillary electrophoresis (CE) with UV detection or coupled to electrospray ionisation mass spectrometry (ESI–MS) (Fraga *et al.*, 2002; Yanez Barrientos *et al.*, 2013; Armstrong *et al.*, 2011; Friso *et al.*, 2002; Ma *et al.*, 2009; San Romerio *et al.*, 2005). The second approach is based on radioactive [^{32}P]-postlabelling of nucleotides in hydrolysed DNA followed by TLC separation or attachment of [^{3}H] labeled CH_3 group to non-methylated CpG followed by scintillation counting (Wilson *et al.*, 1986; Barciszewska *et al.*, 2007). Methods based on HPLC require about 2–10 μg of the starting material, while for radioactive analysis a 10 times smaller quantity is sufficient. However, despite high sensitivity of the radioactive methods they allow only determination of changes between the samples being compared and results may differ from experiment to experiment (Magaña *et al.*, 2008). The third approach is based on an immunoassay with the use of a monoclonal antibody directed against m^{5}Cyt detected by fluorescence measurement of fluorescein isothiocyanate-linked secondary antibody (Oakeley *et al.*, 1997; Kremer *et al.*, 2012). However, this strategy, like the previous one, allows only detection of changes in methylation level between the samples being compared in a given series (Magaña *et al.*, 2008).

Herein we report a new method for the quantification of m^{5}C in DNA. The method is based on the formation of 3,N^4-etheno-5-methyl-2′deoxycytidine (εm^{5}C) and fluorimetric analysis of its concentration. The method

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Abbreviations: Cyt, cytosine; m^{5}Cyt, 5-methyl-cytosine; m^{5}C, 5-methyl-2′deoxycytidine; εm^{5}C, 3,N^4-etheno-5-methyl-2′deoxycytidine; C, 2′-deoxycytidine; G, 2′-deoxyguanosine; A, 2′-deoxyadenosine; T, thymidine; εAde, 1,N^6-etheno-adenine; εGua, 1,N^2-etheno-guanine; CAA, 2-chloroacetaldehyde; CIAP, calf intestinal alkaline phosphatase; NP1, nuclease P1 from *P. citrinum*; MNase, micrococcal nuclease from *S. aureus*; SPII, phosphodiesterase II from bovine spleen; DNMT1, DNA methyltransferase 1; RP-HPLC, reversed-phase high-performance liquid chromatography; ESI–MS, electrospray ionisation mass spectrometry; FLD, fluorescence detectors.

permits lowering of the detection limit of m^5C several times as compared to UV absorbance measurement. HPLC separation additionally improves the selectivity of m^5C analysis.

MATERIALS AND METHODS

Instruments and reagents. A Waters 600E HPLC chromatograph equipped with a diode array detector Waters 2996 and a scanning fluorescence detector Waters 474 Satin was used in this work. The nucleoside mixture was separated at a room temperature on the columns: *Atlantis* C18 (3.0×150 mm×5 μm), Hypersil BDS-C18 (125 mm×4 mm×5 μm) and X-Bridge (125 mm×4 mm×5 μm) purchased from Waters Milford, MA, USA). All chemicals were of analytical reagent grade. Deionised water (18.2 MX cm, Labconco, USA) and HPLC grade acetonitrile (Merck) were used throughout.

The deoxynucleoside standards (2'-deoxycytidine, 5-methyl-2'-deoxycytidine, 2'-deoxyguanosine, 2'-deoxyadenosine, thymidine) and 2-chloroacetaldehyde, calf intestinal alkaline phosphatase (CIAP), nuclease P1 (NP1) from *P. citrinum* and micrococcal nuclease (MNase) from *S. aureus* and phosphodiesterase II (SPII) from bovine spleen, ammonium phosphate dibasic, phosphoric acid, hydrochloric acid, sodium acetate, zinc sulphate and Tris were purchased from Sigma-Aldrich. Calf thymus DNA was purchased from Merck, T4 polynucleotide kinase (PNK) from Pharmacia and [γ-^{32}P]ATP from Hartmann Analytic, Germany.

Synthesis of etheno-derivative standard compounds and modification of DNA. Synthesis of etheno-nucleoside derivative was carried out using 11 mM of nucleoside (m^5C, C, A, G) dissolved in 50 μL of solution containing 200 mM sodium citrate buffer pH = 3.5 (C, m^5C) or pH = 4.5 (A, G) and 1.0 M 2-chloroacetaldehyde (CAA) and incubated for 30 h at 37°C (Kochetkov *et al*., 1971; Biernat *et al*., 1978). Afterwards, the obtained modified nucleosides were dried out using SpeedVac.

Modification of DNA. For modification, commercial DNA from calf thymus or DNA isolated from *B. taurus* blood was used. DNA from *B. taurus* was extracted according to the method described in ref. (Miller *et al*., 1988). In a typical experiment, 20 μg of ctDNA were reacted with CAA for 30 h at pH=3.5 and temp.=37°C. Modified DNA was ethanol precipitated and dried out.

Hydrolysis of DNA. In a typical experiment, 2 μg of DNA were dissolved in 24 μl of water, denatured at 95°C for 2 min and cooled, after that 4 μl 0.3 M sodium acetate (pH=5.3), 1 μl of 10 mM $ZnCl_2$, 2 μl NP1 and 1 μl (10 u) CIAP were added and incubated for 4 h at 37 °C. Afterwards, the mixture of nucleosides was dried out using SpeedVac. The hydrolysed samples were stored at –20°C until analysis.

HPLC/FLD analysis. The separations of standard nucleosides and their etheno-derivatives were carried out at 30°C in a gradient of acetonitrile in 0.01 M phosphate buffer at pH=3, according to the following program: 5 min isocratically in 0.01 M sodium phosphate buffer at pH=3 followed by linear increment of acetonitrile up to 5% for 5-60 min, at a flow rate 0.5 mL×min^{-1}. Detection by UV diode array was carried out at λ=270 nm. Fluorescence measurement was made using λ_{Ex}=290 nm and λ_{Em}=340 nm. Nucleosides were identified by ESI-MS and absorption spectra were compared with respective standards. Calibration curve was prepared by using different amounts of εm^5C ranging from 3.4 pg to 5.6 ng.

[^{32}P]-postlabelling analysis of m^5C. A sample of DNA (1 μg) was hydrolysed to nucleotides with MNase and SPII, and then labelled with [γ-^{32}P]ATP and T4 kinase. The obtained mononucleotide diphosphates were stripped of 3'-phosphate and separated with two-dimensional TLC chromatography (Wilson *et al*., 1986; Barciszewska *et al.,* 2007). A clearly separated pattern of spots was scanned with a phosphoimager. The $^{32P}R(\%)$ coefficients were calculated according to the formula $^{32P}R(\%) = [m^5C]\times100/[m^5C+C+T]$ where $[m^5C]$ and [C] are spot intensities for single nucleotides obtained from phosphoimager measurement.

RESULTS AND DISCUSSION

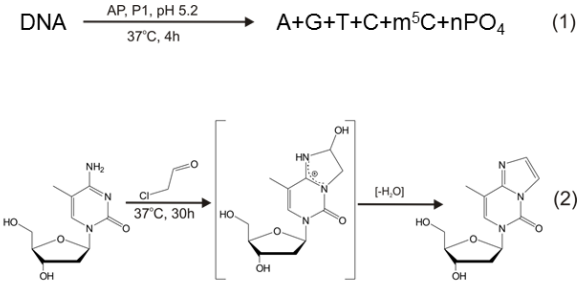
Synthesis of εm^5C

3,N^4-etheno-5-methyl-2'deoxcytidine (εm^5C) was prepared *via* the reaction of m^5C with 2-chloroacetaldehyde (CAA) as presented in Scheme 1. The reaction is known to be not specific and lead to derivatisation of other nucleosides (except of T) (Kochetkov *et al*., 1971; Barrio *et al*., 1972; 1976; Spencer *et al*., 1974; Bedell *et al*., 1986; Krzyzosiak *et al*., 1981; Kusmierek & Singer 1982; Oakeley *et al*., 1999). The yield of the reaction of individual nucleoside with CAA depends on pH of the reaction medium. To establish the optimum conditions for εm^5C synthesis, different pH values and temperatures were tested. The highest yield of the reaction conducted at 37°C was observed at pH=5 after 18 h (Fig. 1a). However, the yield of the reaction carried out at pH=3.5 reached the same level (ca. 98% yield) after 30 h (Fig. 1b). Under the same conditions (pH=3.5, 37°C, 30 h) conversion of C into εC amounted to about 86%. Thus, for the purpose of this approach to minimize the efficiency of 2'-deoxyadenosine and 2'-deoxyguanosine ethenylation, the reaction was carried out at pH=3.5. It should be noted that under the conditions applied formation of 1,N^6-etheno-adenine (εAde) and traces of 1,N^2-etheno-guanine (εGua) are observed. The procedure described also allows for modification of oxidized derivatives of m^5C as it was proved for 5-hydroxymetyl-2'deoxycytidine.

For the study of m^5C derivatisation in a native sample, a commercially available calf thymus DNA was used. Two ways of ctDNA modification were tested: *i*) DNA was modified with CAA, ethanol precipitated and dried out (this step allows for partial removal of CAA), after that DNA was dissolved in water and subjected to hydrolysis to nucleosides with nuclease P1 and CIAP; *ii*) the enzymatic hydrolysis was performed first and then free nucleosides were modified with CAA. The first method was found to be less efficient, probably due to the loss of part of the material during precipitation and less efficient hydrolysis of modified DNA. A typical experiment was described in the experimental section.

HPLC analysis

To determine the correct retention time of each nucleoside and their etheno-derivatives, the standards of C, m^5C, A, G, T, εAde, εGua, εC and εm^5C, were synthesised and subjected to separate analyses (Table 1). Several factors were found to be critical for HPLC analysis of etheno-nucleosides. These include the content and pH of the mobile phase and the type of column. The maximum intensity of fluorescence for εm^5C and εC was observed at pH=3, while for εA at pH=7 and it is reduced



Scheme 1. Method of m^{5}C analysis in DNA:
1) enzymatic hydrolysis with calf intestine alkaline phosphatase (AP) and nuclease P1 from *Penicillium citrinum* (P1); 2) modification of 5-methyl-2′-deoxycytidine with 2-chloroacetaldehyde (CAA); A — 2′-deoxyadenosine, G — 2′-deoxyguanosine, T — deoxythymidine, C — 2′-deoxycytidine, m^{5}C — 5-methyl-2′-deoxycytidine.

by 2/3 with pH decreased to 3 (Barrio *et al.*, 1976; Bedell *et al.*, 1986). Low pH, necessary for the fluorescence measurement, affects the retention times of separated nucleosides, particularly adenosine, as observed earlier (Wagner & Capesius 1981; Johnston *et al.*, 2005; Gehrke *et al.*, 1984). Nucleosides are relatively polar as evidenced by the calculated n-octanol-water partition coefficient ($-1.23<\log P<1$), so to obtain good performance, a mobile phase containing more than 90% of water was used. To get the best separation, different C18 columns i.e. Hypersil BDS, X-Bridge and Atlantis were tested (Table 1) (Bezy *et al.*, 2005). Comparison of elution profiles indicates that each column gave a good peak resolution for εm^{5}C. However, Hypersil BDS is prone to reduce peak resolution for other etheno-derivatives (co-elution of εC and εGua) (Table 1).

The best chromatographic separation of enzymatically hydrolysed calf thymus DNA modified with CAA was achieved by using Atlantis C18: in 0–5 min isocratically in 0.01M sodium phosphate buffer (pH=3) followed by linear increment of acetonitrile for up to 5% at a flow rate 0.5 mL × min^{-1} (Fig. 2). Under the proposed chromatographic conditions, the peaks corresponding to C, m^{5}C, A, G and their etheno derivatives were significantly separated from biological noise. Chromatogram presented in Fig. 2a,b showed significantly separated peaks corresponding to εAde, traces of εGua, εC and εm^{5}C.

The UV spectra of each etheno-derivatives are shown as inlets in Fig. 2b. Correct assignment of εm^{5}C position on the elution profile was confirmed by spiking with a synthetically obtained standard (Fig. 2c). ESI-MS analyses of fractions eluted at 26.3 and 42.8 min from Atlantis C18 show ions having m/z 252.1 and 266.2 which agree with the calculated m/z values for εC and εm^{5}C (Fig. 2d, e).

Calibration curve and detection limit

For quantitative determination of εm^{5}C , a calibration curve was made. To plot a calibration curve, a solution was prepared from a weighted portion of purified εm^{5}C and serially diluted so that samples containing various amounts (3.7–560 pmol) of εm^{5}C were obtained and analysed by HPLC-FLD. The lower limit of εm^{5}C detection was found to be 0.02 pmol. In the concentration range of 1–50 μM the calibration curve is linear (Fig. 3e). The linearity was evaluated *via* determination of the regression coefficient which amounted $R^2=0.9999$.

Analysis of DNA methylation level in blood samples

DNA samples isolated from peripheral blood of cattle from different breeding farms were subjected to analysis by the proposed method. Three different samples of 2 μg of DNA were digested with NP1 and AP, modified with CAA and subjected to HPLC separation (Fig. 3a–c). Peak areas for εm^{5}C of each sample were calculated and the corresponding concentrations were determined from the calibration curve. The resulting concentrations of m^{5}C amounted 0.53, 0.67 and 5.2 μM, respectively

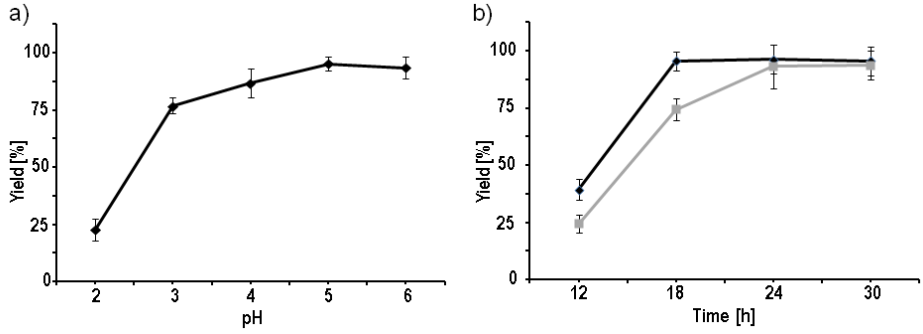


Figure 1. Yield of 3,N^4-etheno-5-methyl-2′-deoxycytidine synthesis carried out at 37°C *vs* pH of the reaction mixture (a) and progress of the reaction proceeding at pH = 5 black, pH = 3.5 grey (b).

Table 1. Retention times of nucleosides and their etheno derivatives measured for three chromatographic columns

Compound	Retention time (min)			Compound	Retention time [min]		
	Hypersil BDS	X-Bridge	Atlantis		Hypersil BDS	X-Bridge	Atlantis
C	3.03	3.32	18.4	εC	11.09	17.55	26.4
m^{5}C	6.11	6.2	17.6	εm^{5}C	17.52	31.79	42.3
A	10.54	11.9	24.2	εAde	9.95	8.36	13.9
G	12.65	12.4	23.9	εGua	9.94	11.62	21.9
T	12.07	16.7	26.7	–	–	–	–

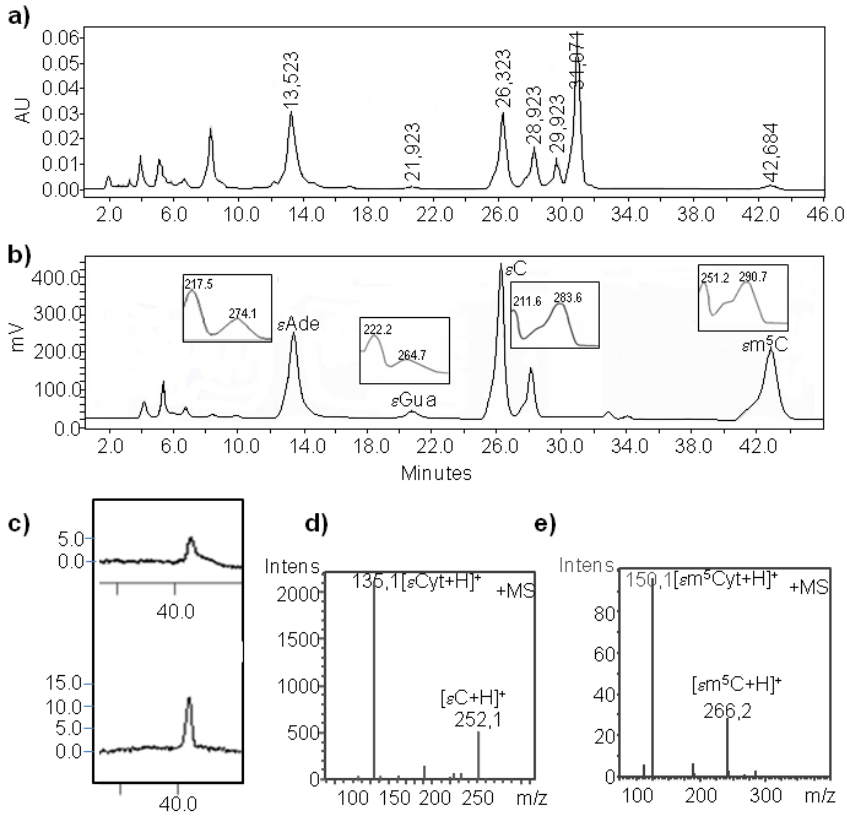


Figure 2. HPLC with UV (a) and fluorimetric detection (b) of enzymatically hydrolyzed calf thymus DNA modified with CAA separated on Atlantis C18 column. Inserts show continuous UV spectra of analyzed ethenonucleosides; c) HPLC-UV chromatograms for the analysis of ε-m^{5}C (upper) hydrolyzed calf thymus DNA, sample spiked with a standard (lower); ESI/MS spectra of compounds eluted at r.t. = 42.9 min (d) and r.t. = 26.4 min (e)

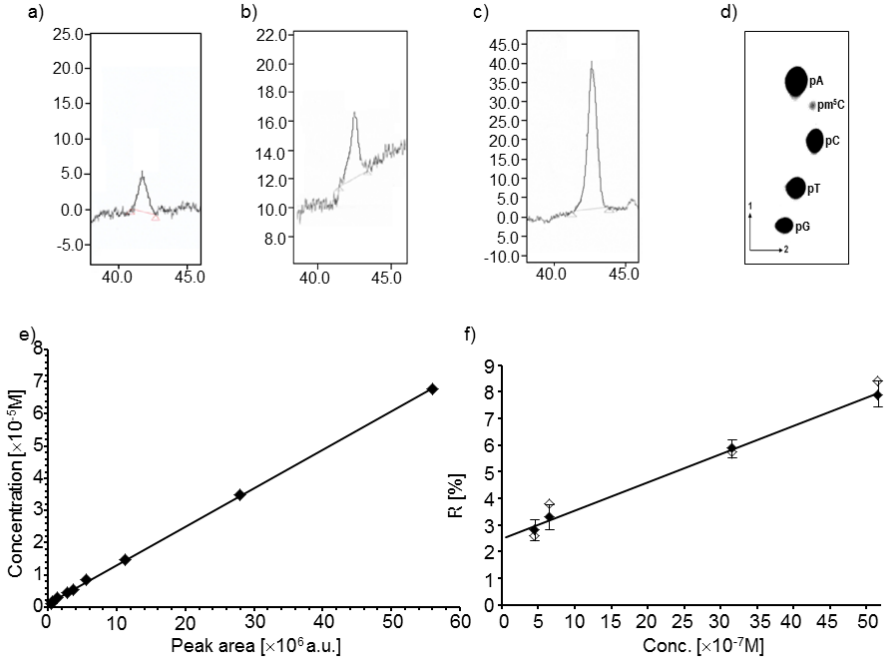


Figure 3. (a–c) Analysis of 5-methyl-2′-deoxycytidine in DNA isolated from blood of *Bos taurus*. Hydrolyzed, modified separation HPLC/FDL of εm^{5}C from DNA of three different individuals; (d) example of 2-dimensional thin layer chromatography of a sample obtained from DNA digests using the [^{32}P]-postlabeling technique; (e) calibration curve for the HPLC/FDL analysis of εm^{5}C; (f) comparison of R(%) coefficients determined by [^{32}P]-postlabeling technique followed by TLC separation ^{32P}R(%) — open diamonds and calculated from HPLC analysis ^{F}R(%) — black diamonds. ^{F}R(%) was plotted against concentration of m^{5}C determined by a fluorimetric method.

Table 2. Concentration of m5dC in DNA from different samples.

No.	Conc. [×10^{-6}M]	^{F}R(%)	^{32P}R(%)
1	0.53	2.8	2.6
2	0.67	3.3	3.8
3	5.2	7.9	8.4
ctDNA	3.2	5.9	5.8.

^{F}R(%) coefficient calculated based on HPLC/FDL analysis; ^{32P}R(%) coefficient calculated using [^{32}P]-post-labelling technique.

(Table 2). For comparison, the concentration of m^{5}C in a commercially available calf thymus DNA amounts to 3.2 μM. To verify the obtained results, the same samples were examined by using [^{32}P]-postlabelling method (Fig. 3d) (Barciszewska *et al.*, 2007) (see experimental section). The obtained values of ^{32P}R(%) coefficients are collected in Table 2. For comparison, ^{F}R(%) coefficients were calculated on the basis of the fluorescence measurement. ^{F}R(%) values were calculated by using a modified equation $^{F}R(\%) = m^5C \times 100/[m^5C+C]$ since concentration of T could not be determined by using the fluorimetric method.

In the calculations of ^{F}R(%) coefficients it was assumed that the concentration of εm^{5}C measured corresponds to the value of the m^{5}C concentration (functionalization was nearly quantitative), while the peak area for εC was corrected taking into account that the yield of C to εC functionalization amounted to 86%. Calculated values of ^{F}R(%) coefficients are collected in Table 2. The plot of ^{F}R(%) values *vs* concentration of m^{5}C is linear, which indicates a good data correlation. The observed differences between ^{F}R(%) and ^{32P}R(%) values are within the experimental error (Table 2; Fig. 3f).

CONCLUSION

In conclusion, the presented method of quantification of m^{5}C in genomic DNA shows several advantages. The limit of εm^{5}C detection by fluorescence measurement is 0.02 pmol. The sensitivity of the method proposed is higher than that of the methods based on UV detection. The synthesis of etheno derivatives was carried out at 37°C and separation of the reaction mixture without additional purification step significantly shortened the time of analysis. The method proposed allows direct quantification of m^{5}C (by using calibration curve) as well as calculation of the R coefficient, while [^{32}P]-postlabelling assay allows only the determination of R values indicating the percentage share of m^{5}C in relation to other pyrimidines.

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