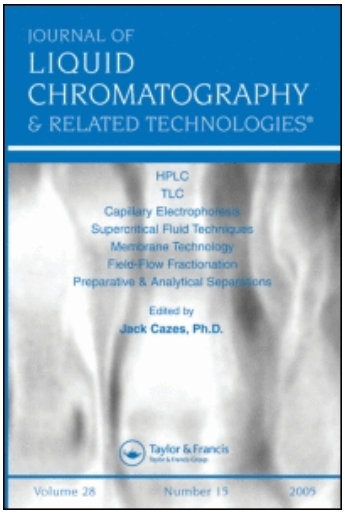

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AN IMPROVED SAMPLE PREPARATION METHOD FOR THE QUANTITATIVE HPLC DETERMINATION OF 5-METHYL-DEOXYCYTIDINE IN ANIMAL TISSUE DNA

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ABSTRACT

Techniques are presented for the purification of DNA from mammalian tissues, its enzymatic hydrolysis to deoxyribonucleosides and the separation and quantification of these by high pressure liquid chromatography (HPLC). The method is used to quantify 5-methyldeoxycytidine (5MdC) and deoxycytidine (dC) in DNA. From this data the molar %5MdC, i.e. 100 x 5MdC/(dC + 5MdC), is calculated for DNA. The precision of the method matches or exceeds that of other published HPLC methods for quantifying the %5MdC. The DNA obtained is extremely clean, and the enzymatic hydrolysis provides deoxyribonucleosides without contamination so there are no extraneous peaks in the region of interest, and peaks that are obtained have sufficient area to eliminate errors from variation in integration. The %5MdC is a quantitative and absolute measure of the genome wide DNA methylation. This method is suitable to quantify small changes in mammalian enzymatic DNA methylation due to age, diet, drugs or carcinogens.

INTRODUCTION

Measurement of 5-methyldeoxycytidine (5MdC) content in DNA by enzymatic digestion and HPLC is an excellent technique with a number of advantages. The data generated are both absolute and quantitative, and the measurement of both 5MdC and deoxycytidine (dC) is made from the same chromatogram and thus requires no internal standard. It is a convenient choice because the method uses standard laboratory equipment and nontoxic, nonradioactive reagents and enzymes.

A number of effective techniques have been described for quantifying 5MdC by HPLC.[1-5] In particular, the techniques of Kuo *et al.*[1] and Gehrke *et al.*[3] are often used. Here we describe a combination of techniques for careful purification, quantification and hydrolysis of animal tissue DNA which produces extremely uniform peaks, and eliminates extraneous peaks, in chromatograms. The method described avoids a number of variables often encountered with DNA preparation, digestion and chromatography and gives precise quantification of 5MdC in animal tissues.

Enzymatic DNA methylation as 5MdC has essential roles in the organization and control of the mammalian genome from genetic imprinting and embryonic development[6-10] through adulthood and aging. Changes in adult mammalian 5MdC occur with manipulation of methyl metabolism (e.g. S-adenosylmethionine and S-adenosylhomocysteine)[11-12] carcinogenesis[11-15] and aging.[16-20] The DNA of vertebrate tissues contains between 2% and 10% 5MdC.[1,21] The sensitivity of the method presented here allows for the determination of the %5MdC from just a few micrograms of mammalian DNA. The precision of the method is suitable to follow changes in genome wide %5MdC occurring with carcinogenesis, aging and manipulation of methyl metabolism.

MATERIALS AND METHODS

Reagents and Enzymes

Reagent or molecular biology grade chemicals were used throughout. Deoxyribonucleosides used for external standards in HPLC were from Sigma Chemical, St. Louis, MO (5MdC # M-3155; dC # M-3897). NaOH, used for base treatment of DNA, and acetic acid, used to titrate NaOH before DNA digests, were commercially standardized solutions of 1.0 M NaOH and 1.0 M acetic acid (Mallinckrodt Chemical, Paris, KY). HPLC grade methanol was

from J.T. Baker, Phillipsburg, NJ (# 9093-03). Enzymes were from Sigma Chemical, St. Louis, MO (proteinase K # P-0390) and from Boehringer Mannheim Biochemicals, Indianapolis, IN (RNase, DNase free, # 1119915; P1 nuclease # 236225; alkaline phosphatase # 713023).

Isolation of Nuclei from Frozen Tissue

Frozen tissue (e.g. 300 mg of liver at -70°C) was crushed and ground to a powder with liquid nitrogen and a pestle in a ceramic mortar. Subsequent nuclear isolation steps were performed on ice with solutions at ~4°C. Nuclei were isolated by an adaption of conventional means.[22] Briefly, the powdered tissue was transferred to a Potter homogenizer to which was added 20 volumes of "nuclear isolation buffer" (250 mM sucrose, 5 mM $MgCl_2$, 20 mM Tris-HCl pH 7.4, 0.1% Triton X-100) at 4°C. Tissue was homogenized with several strokes of a glass "B" pestle. The homogenate was filtered twice. The first filtration was through cheese cloth (held in broad plastic mesh) into a 50 mL centrifuge tube and the next through a plastic fine mesh strainer (Falcon, Becton-Dickinson, Franklin Lakes, NJ #2350) into a 50 mL centrifuge tube. Nuclei were pelleted from the filtered homogenate by centrifugation at 1500 x *g* for 15 minutes. The supernatant was carefully poured off and the nuclear pellet was resuspended in nuclear isolation buffer and spun again. This last step was repeated once.

Double-Stranded DNA Preparation

DNA was prepared from isolated nuclei by an adaption of conventional means.[23] Briefly, the nuclear pellet (e.g. 100 μL) was resuspended in four volumes (e.g. 400 μL) of "RNase buffer" (100 mM NaCl, 30 mM Tris-HCl, 10 mM EDTA pH 8.4) at room temperature. An equal volume (e.g. 500 μL) of "proteinase mix" (100 mM NaCl, 50 mM EDTA pH 8.0, 1% SDS, 300 μg/mL proteinase K) was then added. This was mixed and incubated at 37°C overnight (16 hours). The next morning, the sample was treated with 2 μL of RNase (DNase free, 500 μg/mL), mixed and incubated at 37°C for 3 hours. The sample was then extracted twice with phenol:chloroform:isoamyl alcohol (24:24:1) and then once with chloroform:isoamyl alcohol (24:1). Subsequently, 0.10X volume of 3.0 M potassium acetate (pH 5.4 with acetic acid) was added (to a final concentration of 0.3M potassium acetate), and DNA was precipitated with ethanol. DNA was collected as a fluff and rinsed twice with 70% ethanol. DNA was dried (SpeedVac, Savant, Farmingdale, N.Y.) and redissolved in 10 mM Tris-HCl, 0.1 mM EDTA pH 7.4 (TE). After three days at 4°C, the DNA

was mixed to homogeneity with mild vortexing and/or pipetting. A small aliquot of each DNA sample was quantified by fluorescence using Hoechst 33258 dye on a DyNAquant 200 (Hoefer Pharmacia Biotech, Inc., San Francisco, CA). Alternatively the DNA concentration could be estimated at this step by UV spectrophotometry (A260-A320) using TE as a blank (double stranded DNA is 50 μg/mL @ 1 O.D. @ 260nm).

DNA Purification

DNA was treated with base, dialyzed and concentrated. Approximately 200 μg or less of DNA, was used in the following procedure: The DNA sample was treated with NaOH according to the procedure of Singer *et al.*[24] with modifications. Briefly, water and 1 M NaOH (standardized solution, Mallinckrodt) were added to a DNA aliquot such that DNA was in 1 mL of 0.1 M NaOH. This was lightly vortexed and incubated at 37°C overnight.

DNA in 0.1 M NaOH was transferred to a Centricon 30 spin dialysis unit (Amicon, Beverly, MA), and spin dialyzed at least seven times. Each spin dialysis was done with 1 mL of 20 mM NaOH (made from 1 M NaOH standardized solution). For each spin dialysis, the DNA was reduced to the Centricon 30 default stopping volume of about 50 μL. Standard Amicon instructions were followed for the Centricon 30 in a fixed angle rotor and two additional steps were added. The first additional step, done between spin dialyses, was rotating of the microconcentrator 180° on its long axis. This allowed for efficient dialysis of the DNA by the next 1 mL of 20 mM NaOH. After at least seven successive washes, the DNA, in 20 mM NaOH, was spun off the membrane into the recovery cap and DNA was then transferred to a microcentrifuge tube. The second additional step is washing of the membrane. Specifically, about 50 μL of 20 mM NaOH was added to the membrane, allowed to soak at room temperature for 10 minutes and spun off the membrane into the recovery cap. This recovered material was then added to the DNA in the microcentrifuge tube. This washing and recovery step was repeated once. We have also used Centricon 100 and Microcon 30 and 100 devices (Amicon) for spin dialysis with good results (not shown).

Recovered DNA was mixed and stored at 4°C. A small aliquot was diluted in 20 mM NaOH and quantified by UV spectrophotometry (A260-A320) using 20 mM NaOH as a blank (single stranded DNA is 33 μg/mL @ 1 O.D. @ 260nm). From this the concentration of the stock DNA was determined.

An exact standard volume and concentration of DNA was chosen for the series of digests (e.g. 100 μg/100 μL). DNA solutions of exactly the same concentration were prepared from the stock solutions using 20 mM NaOH.

DNA Digestion

Instructions which follow are for DNA samples (e.g. 100 μg) each in 100 μL of 20 mM NaOH. Each DNA solution was neutralized with exactly 12 μL of 200 mM acetic acid (1.20 moles of acetic acid/mole of NaOH). Acetic acid at 200 mM was made from standardized 1.0 M acetic acid (Mallinckrodt). The DNA solution was mixed, centrifuged down, heat denatured at 95°C for 5 minutes and cooled quickly on ice.

P1 nuclease solution was prepared from solid (300 units phosphodiesterase activity per mg) by dissolving at 5U/μL (1 mg in 60 μL) in 30 mM sodium acetate (pH 5.3 with acetic acid). This can be used for at least 6 months when stored frozen (-20°C).

A hydrolyzation mixture was prepared on ice just before use. The preparation of the hydrolyzation mixture for ten neutralized and heat-denatured DNA samples (100 μg in 112 μL each) is as follows: 650 μL H_2O, 130 μL sodium acetate (300 mM pH5.3 with acetic acid) and 20 μL $ZnSO_4$ (10 mM) were mixed and then 40 μL P1 nuclease solution and 40 μL alkaline phosphatase solution (1U/μL) was added and thoroughly mixed. The alkaline phosphatase stock solution was stored at +4°C.

To each DNA sample, 88 μL of the hydrolyzation mixture was added, the solution was mixed, and spun down. The final volume of each digest was 200 μL, and the final buffer and enzyme concentrations in each digest were: 30 mM sodium acetate (pH 5.3), 0.1 mM $ZnSO_4$, P1 nuclease 100 units/mL (or 0.2 unit/μg DNA, whichever is greater) and alkaline phosphatase 20 units/mL (or 0.04 unit/μg DNA, whichever is greater).

The DNA digest was incubated at 37°C for 5 hours, vortexed, two volumes of room temperature EtOH (95 to 100%) were added, and the samples mixed and spun at room temperature at 17,000 *xg* for 15 min. A small pellet or streak of protein was formed in each sample. The supernatant containing the deoxyribonucleosides (dNs) was collected, dried in a Speed Vac and redissolved in H_2O such that the dN (formerly DNA) concentration was 1.0 μg/μL.

For example, if the digestion was started with 100 μg of DNA, the dN was redissolved in 100 μL of H_2O. This digestion procedure was adapted from Palmgren *et al*[5] and Gehrke *et al.*[3] Mixtures of dNs (digested DNA or standards) were stored frozen (-20°C).

HPLC

Samples and external standards of the same volume and concentration (with respect to dC + 5MdC) were injected in each run of a series. That is, the same amount (±10%) of dN (e.g. from 10 μg of DNA) was injected for each sample. The external standards had combined peak areas of dC and 5MdC closely matching those of the samples. The samples were injected in a small volume (usually 10 μL).

HPLC was performed as described by Wise and Hass[25] as adapted from Gehrke *et al.*[3] Briefly, the samples and standards were injected into a Hewlett-Packard 1090 HPLC, equipped with a Beckman Ultrasphere ODS, 4.5 mm x 25 cm (5 μm particle size) column held at 30°C in a temperature controlled column compartment. A pre-column filter (ChromTech, Apple Valley, MN #C-751) was also used. HPLC buffer A was 0.05 M potassium dihydrogen phosphate containing 2.5% methanol while buffer B contained 0.05 M potassium dihydrogen phosphate with 9% methanol. HPLC mobile phase was delivered to the column at 0.9 mL/min. Prior to each injection, the column was equilibrated with 100% buffer A for 5 minutes. After the injection the HPLC was programmed to pump buffer A for 22 minutes, after which time it switched to buffer B over a 5 minute linear gradient. After 10 minutes of 100% buffer B, the mobile phase was changed back to 100% buffer A in preparation for the next injection. Before the run, a single batch of buffer A was made that was sufficiently large for the entire series of samples and standards. A photodiode array detector was used and data were collected at 280 nm. Under these conditions, the retention time of the 5MdC was 15-17 minutes and dC was 7.5-8.5 minutes. The purpose of buffer B was to elute the remaining dNs and to clear the column in preparation for the next analysis. The HPLC was equipped with an autosampler which allowed automatic injection of about 25 samples in a 24-hour period.

Peak Identification

In the development of these methods we verified the identity of peaks derived from DNA digests by using both internal and external 5MdC and dC

standards. Peaks were identified by their elution positions and by their 280nm/254nm absorbance ratios relative to standards as described elsewhere.[3,26]

Molar Standards for Quantifying %5MdC

The standard mixtures used here were external, i.e. they were separated and quantified in separate chromatograms from the DNA derived samples. Most of the standard mixtures and all of the samples contained both 5MdC and dC. The relative proportions of 5MdC and dC (and later the %5MdC) were determined without an internal standard.

Standard mixtures of 5MdC and dC were made as concentrated stock solutions in water. Aliquots of these were diluted in HPLC buffer or in the solution resulting from processing a mock digestion mixture (to which no DNA had been added). Using the methods described in this paper we found no quantitative difference between standards injected in HPLC buffer or injected in a processed mock digestion mixture (not shown). Standard mixtures were stored frozen (-20°C).

Standard mixtures of 5MdC and dC at relative molar concentrations of 0.0, 1.0, 2.0, 4.0, 8.0, 16.0 and 100.0% 5MdC were injected with each sample series. Standards in the range being measured were injected at the beginning and at the end of each series. Our deoxycytidine standard contained a trace (i.e. 0.5%) of 5MdC and the percent 5MdC of dC standard (no added 5MdC) was subtracted from the percent 5MdC of the other molar standard mixtures.

Quantification of Molar %5MdC

The percent 5MdC [100 x 5MdC/(5MdC + dC)] in DNA was first calculated based on the peak areas (absorbance) at 280 nm. At least two injections of each sample were done and mean of these was taken as the percent 5MdC for that sample. The molar absorbtivities of 5MdC and dC were very similar under these conditions and little or no correction was needed to obtain percent 5MdC by moles from percent 5MdC by peak areas. When necessary the percent 5MdC by peak areas at 280 nm of molar standard mixtures of 5MdC and dC were used to convert peak area percentages for DNA to molar percentages. A calibration curve can be used for this purpose when absorbtivities of 5MdC and dC differ significantly.[26] The accuracy of quantification was monitored by including DNAs of known %5MdC, such as calf thymus or salmon sperm DNA,[1,4,26] in each series of digests and standards.

Statistical Analysis

Groups were compared for significant differences by the two tailed t-test. This and other statistical analysis was done using Quattro Pro software. Groups were considered statistically different when $p < 0.05$.

RESULTS AND DISCUSSION

The methods described here are reliable and precise in comparing animal groups for the genome wide %5MdC in their DNA. These methods avoid the introduction of variables that can reduce the precision of measurement.

DNA Preparation and Purification

We used a multistep conventional method for the preparation and purification of DNA because we have found this most reliable. In particular, the purification steps are important to remove RNA that remains after RNase treatment. Residual RNA in DNA samples is a persistent nuisance in chromatographic determination of %5MdC.[3] Chromatograms of DNAs digested prior to purification (i.e. prior to NaOH treatment and dialysis) have numerous peaks in addition to the five deoxyribonucleosides expected for most eukaryotic DNA (not shown). After base treatment, dialysis and concentration, the only significant peaks are the expected dC, 5MdC, thymidine, deoxyguanosine and deoxyadenosine. In particular, there are no other significant peaks in the region of the 5MdC peak (Figure 1).

Other methods, sometimes elaborate, have been used for DNA purification before %5MdC determination by HPLC. These include cesium chloride gradient centrifugation,[4,27] gel filtration,[5] hydroxyapatite chromatography or gel electrophoresis.[3,28] The base treatment and spin dialysis purification method we use here is highly reproducible, convenient and requires no specialized equipment.

DNA Quantification and Digestion

After purification, we carefully quantify DNA by ultraviolet spectrophotometry. This provides a measure of the DNA concentration (RNA has been removed) by a very reliable method, absorbance at 260nm, that works on single stranded DNA (base treatment denatures DNA) and is done after manipulations that could result in DNA loss are finished.

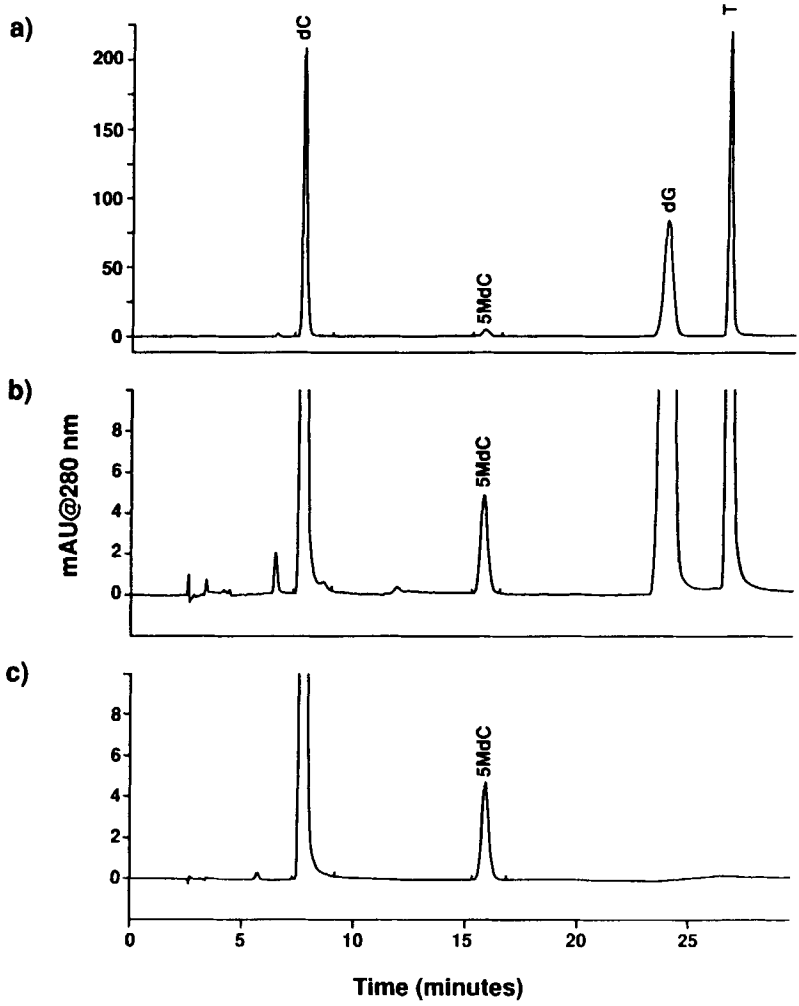

Figure 1. Chromatograms of deoxyribonucleosides from mouse liver DNA as described in the text (a, b) and of 4% 5MdC standard mixture (c). dG= deoxyguanosine, T= thymidine. Deoxyadenosine elutes after 30 minutes and is not shown. a) Full scale (from mouse liver DNA); b) Enlargement 15X to show detail around the 5MdC peak (from mouse liver DNA); c) 4% 5MdC standard mixture (shown at same scale as in b).

We used an adaption of Palmgren et al.[5] and Gehrke et al.[3] methods, in which digestion is achieved in a short, two enzyme, single buffer, single step procedure. This digestion was conducted with a digestion mix such that the same amounts and concentrations of the same enzymes and buffers were added to DNA samples, each of which are also of the same concentration and DNA amount. The removal of enzymes by ethanol precipitation at room temperature helped assure clean chromatograms as described by Palmgren et al.[5]

Both alkaline phosphatase and P1 nuclease from some commercial sources contain significant amounts of adenosine deaminase activity[3,5,29] (and our unpublished results). This activity converts deoxyadenosine from DNA to deoxyinosine. Deoxyinosine would elute just after 5MdC in our chromatograms and could interfere with quantifying 5MdC. Using the P1 nuclease and alkaline phosphatase enzymes and conditions as described above we saw no deoxyinosine peak in our chromatograms (i.e. <0.05% of dC peak area). Other combinations of commercial enzymes and digestion conditions have been described which largely avoid adenosine deaminase activity when digesting nucleic acids.[1,4,5,29] Kuo *et al.*[1] and Gehrke *et al.*[3] heated alkaline phosphatase before DNA digestion to destroy most adenosine deaminase. We did not need to heat or otherwise treat the P1 nuclease or alkaline phosphatase before DNA digestion.

HPLC

We injected the same amount (± 10%) of dN for each sample and used standards whose dC and 5MdC combined peak areas closely matched that of the samples. Thus peak sizes of dC were about the same in each chromatogram for all samples and for most standards. Peak sizes of 5MdC were also similar for samples with the same %5MdC. By digesting and injecting very similar amounts of each sample, no correction for possible variations in linearity of the overall method was necessary.

We used a single batch of HPLC buffer A that was sufficiently large for the entire series of samples and standards. This avoided small variations in pH, buffer concentration etc. that could occur between batches. Molar absorbtivities of 5MdC and dC are pH dependent,[3] and thus small pH changes could affect quantification.

Typical chromatograms from mouse liver DNA and of 4.0% 5MdC standard are shown in Figure 1. Chromatograms using this method are extremely consistent in such features as baseline and peak shape.

Because we apply these methods to vertebrate DNA, where the %5MdC varies between 2% and 10%, we can easily detect and quantify the %5MdC in 10 μg of DNA. Within these parameters the 5MdC peak at 280nm has sufficient area for precise integration. For quantification of %5MdC in DNA with less than 1% 5MdC a larger amount of DNA would need to be used (e.g. 40 μg or more) or the more sensitive ^{32}P-postlabeling method could be used.

More sensitive methods for quantification of genome wide DNA methylation include the ^{32}P-postlabeling method[30] and the methyl acceptor assay.[31,32] Both of these methods use radioisotope detection and can be used with less than 1 μg of DNA. The %5MdC can be determined when as low as 0.01% with less than 1 μg of DNA using the ^{32}P-postlabeling method.[30] This method is quantitiative, absolute and extremely sensitive but uses large amounts of ^{32}P and is laborious. It is the method of choice to determine %5MdC when only small quantities of DNA are available or when the %5MdC is very low, as in insect DNA.[30] The methyl acceptor assay gives a relative measure of genome wide DNA methylation but does not give data to determine the %5MdC. This method uses about 1 μg of DNA per determination and is probably the least laborious of the three methods. It is the method of choice when only small quantities of DNA are available and when only a relative measure of DNA methylation is required.

Percent 5MdC Quantification

Using the method described in this paper, the quantification of the %5MdC in DNA is excellent. For example, groups (four each, assayed as individuals) of B6C3F1 male mice fed NIH-31 diet had liver DNA levels of 4.13 ± 0.02 and 4.00 ± 0.03 %5MdC (± standard errors) at 8 weeks and 19 weeks of age respectively. While the difference in %5MdC at these two different ages is small (0.13), the animal groups are consistent enough, and the above described methods precise enough, that the difference is significant ($p < 0.01$). A difference in liver global DNA methylation is expected with age in mice.[17-20]

The precision of our method compares well with other methods. For example, we used deoxyribonucleosides from 10 μg of DNA for each injection in collecting the above data on 8 and 19 week mice. The mean precision for these %5MdC measurements expressed as percent relative standard deviation is 0.79%. This is similar to the percent relative standard deviation of 1.1% reported by Gehrke *et al.* 1984,[3] for the 5MdC peak when injecting deoxyribonucleosides from 7.5 μg of calf thymus DNA.

We do not measure our accuracy based on mouse liver DNA because huge variations in literature values do not provide a clear basis for comparison. Mouse liver DNA %5MdC as reported in the literature varies between 3% and 8%.[17-19, 21] As a check of accuracy we use calf thymus DNA. The %5MdC in calf thymus DNA has been extensively literature reviewed and carefully quantified by Kuo *et al.*[1] at 6.3%. We found a value of 6.44 ± 0.04% (± standard error) which is within the range of Kuo *et al.*[1] and of the literature. The accuracy of our method compares well with other extensively standardized HPLC methods.

CONCLUSION

The DNA purification and digestion methods we describe here easily and predictably avoid the introduction of variables that can reduce the precision of %5MdC measurement by HPLC. The precision of our method matches or exceeds that of other published HPLC methods for quantifying the %5MdC.[1-5] We have used these methods with numerous groups of animals with similar precision. This method has the precision needed to quantify small changes in global DNA methylation that occur in animals during aging as well as those occurring with modified diets or exposure to certain pharmaceuticals or carcinogens.

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