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Analytical procedures and quality assurance criteria for the determination of major and minor deoxynucleosides in fish tissue DNA by liquid chromatography–ultraviolet spectroscopy and liquid chromatography–thermospray mass spectrometry

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ABSTRACT

Analytical procedures and quality assurance criteria have been established for enzymatic hydrolysis of fish tissue DNA to free nucleosides and their subsequent characterization by liquid chromatography–photodiode-array ultraviolet spectroscopy and liquid chromatography–thermospray mass spectrometry. Optimization of enzymatic efficiency to assure minimal loss of modified nucleosides is described. Variability in analyte capacity factors and multiwavelength response have been compared for analyte standards and hydrolysates, and results have been used to derive qualitative and quantitative quality assurance criteria. A comparison of DNA mole percent calculated using single-wavelength quantification and multiwavelength averaging quantification indicates that less variability in data may be expected using the multiwavelength technique. Finally, the composition of DNA from liver of three species of fish widely used in mutagen/carcinogen laboratory and field studies, rainbow trout (*Onchorynchus mykiss*), medaka (*Oryzias latipes*), and brown bullhead (*Ictalurus nebulosus*), has been determined. Identification of deoxyuridine in the DNA hydrolysates of each fish indicates that this analyte should be measured to accurately report DNA deoxynucleoside mole percent, especially when reporting data for the methylation of deoxycytidine.

INTRODUCTION

Chemical residues in fish have been used successfully by the U.S. EPA as an indicator of, and monitor for, environmental pollutants that readily bioaccumulate [1]. However, analytical methods must also be capable of characterizing reactive environmental pollutants, *i.e.* those chemicals that may react with important biomolecules such as DNA. Our objective was to develop standard protocols to generate reproducible data on the composition of DNA constituents which the U.S. EPA may use in both research and monitoring activities. Further, we hoped to obtain information on the composition of DNA in the liver of several fish species commonly used in toxicology research programs. We have therefore developed analytical methodology based upon liquid

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chromatography–photodiode-array ultraviolet spectroscopy (LC–UV) and liquid chromatography-thermospray mass spectrometry (LC–TSP-MS) for the characterization of major and minor deoxynucleosides in fish liver DNA, and have used the methods to characterize deoxynucleosides in three species of fish, rainbow trout, medaka, and brown bullhead. An essential part of the methodology was the development of mild DNA isolation and enzymatic digestion procedures which do not degrade modified nucleosides and are compatible with chromatographic parameters for optimal analyte resolution and thermospray ionization processes. Assessment of the variability of qualitative and quantitative parameters for the determination of quality assurance criteria was done by repetitive analysis of calibration standards and calf thymus DNA.

EXPERIMENTAL[a]

Fish care and handling

Fish were obtained from commercial sources and maintained in the fish culture facility at ERL-Duluth [2]. Trout and medaka were sexually mature, bullheads were not. Food was withheld for 24 h before using fish.

Reagents

Chemical reagents were of the highest purity commercially available and purchased from Sigma (St. Louis, MO, USA) or Aldrich (Milwaukee, WI, USA). A mixture of nucleosides used to test LC column performance was purchased from Supelco (Bellefonte, PA, USA). HPLC-grade water was purchased from EM Science (Gibbstown, PA, USA). Calf thymus and *Escherichia Coli* DNA were purchased from Sigma.

Instrumentation

UV spectrophotometry. A Beckman DU-7 multiwavelength scanning spectrophotometer (Beckman Instruments, San Ramon, CA, USA) was used to determine total microgram of nucleic acids (RNA and DNA), and purified DNA (260 nm).

HPLC–UV. A Beckman System Gold HPLC system (Beckman Instruments) consisting of a solvent delivery module (Model 126), autosampler with stream switching valves (Model 507), photodiode-array detector (Model 168), and IBM PS/2 Model 55 SX (IBM, Armonk, NY, USA) with System Gold chromatography software was used for HPLC–UV analysis. The unit was fitted with a reversed-phase column (Supelcosil LC-18, Supelco, 5 μm, 250 mm × 4.6 mm I.D.), preceded by a C_{18} guard column (Supelco LC-18S, 5 μm, 20 mm × 4.6 mm I.D.).

LC–TSP-MS. A Finnigan-MAT triple-stage quadrupole mass spectrometer (Model TSQ70B, Finnigan-MAT, San Jose, CA, USA), was coupled through a thermospray interface (TSP2, Finnigan-MAT) to a Beckman Instruments high-performance liquid chromatographic (HPLC) system (Model 340). The LC system consisted of a solvent delivery module (Model 112), gradient program controller (Model 421), UV absorbance detector (Model 160, 254 nm), and manual injector (Model 7125, Rheodyne, Cotati, CA, USA). The HPLC was fitted with a reversed-phase column (Supelcosil LC-18, 5 μm, 250 mm × 4.6 mm I.D.) preceded by a guard column (Supelco LC-18S, 5 μm, 20 mm × 4.6 mm I.D.).

Preparation of nucleoside standards

Deoxyadenosine (dAdo), adenosine (Ado), deoxyguanosine (dGuo), deoxycytidine (dCyd), thymidine (Thd), 5-methyldeoxycytidine (m^5dCyd), 5-iodocytidine (I^5Cyd), N6-methyladenosine (m^6Ado), deoxyinosine (dIno), inosine (Ino) uridine (Urd), adenosine monophosphate (AMP) and deoxyuridine (dUrd) were purchased from Sigma and stock solutions prepared (50 m*M* ammonium acetate, pH 6.2). Purity of each analyte was found to be greater than 98% except for dCyd which was contaminated with m^5dCyd. Five calibration standards were prepared, containing dAdo, dGuo, dCyd and Thd in the range 3.8–210 μg/ml, and m^5dCyd and dUrd in the range 0.43–25.6 μg/ml.

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[a] This document has been reviewed in accordance with U.S. Environmental Protection Agency policy. Mention of products or trademarks does not imply endorsement.

Sample preparation procedures

Preparation, purification, and handling of enzyme solutions. All enzymes were purchased from Sigma, and prepared and stored according to specifications. These include: Nuclease P_1 (NP_1) from Penicillium citrinum (2 U/μl in 50 mM ammonium acetate, pH 5.3), RNase A from bovine pancreas (10 mg/ml in 1 M Tris-HCl, pH 7.4), proteinase K from T. album (50 mg/ml in water), phosphodiesterase I (PDI) from Crotalux atrox venom (0.001 U/μl in water), RNase T_2 from *Aspergillus orizae* (0.5 U/μl in 60 mM Tris-HCl, pH 7.2), RNase T_1 from *Aspergillus orizae* (5000 U/ml in 2.7 M ammonium sulfate, pH 6.0) and alkaline phosphatase (AP) from *E. coli* (250 U/ml in 2.5 M ammonium sulfate). Alkaline phosphatase was washed with ammonium sulfate prior to use to remove UV-absorbing impurities. It may also be contaminated with adenine deaminases and diesterases. These contaminants were removed and alkaline phosphatase activity verified according to Crain [3]. Alkaline phosphatase may also be contaminated with cytosine deaminases not removed during the clean-up procedure described above. The action of this contaminant enzyme was limited with the addition of 5-bromocytidine (note: tetrahydrouridine may also be used).

Fish DNA isolation. Fish were anesthetized and euthanized by cervical dislocation. The liver was removed and stored in 50 mM Tris–HCl (pH 7.4). Aliquots of liver homogenates (approximately 150 mg in 500 μl of lysis buffer) were transferred to sterile 1.7-ml polypropylene vials and proteins were degraded with proteinase K (2600 U, 45 min, 37°C). Samples were then extracted with phenol (presaturated with 50 mM Tris–HCl, pH 7.8), phenol–chloroform–isoamyl alcohol (25:24:1, v/v) and chloroform–isoamyl alcohol (24:1, v/v), and nucleic acids were precipitated with the addition of 3 M LiCl and isopropyl alcohol. Nucleic acid fibers were collected by centrifuging (25 min, 10 000 g), washed with ice-cold ethanol–water (70%, v/v) and dried in a centrifuge vacuum evaporator (Savant Instruments, Minneapolis, MN, USA). The amount of nucleic acids obtained from each sample matrix (fish species) was determined spectrophotometrically (see *Analyte quantification*).

The dried nucleic acid pellet was resuspended (0.38 μg/μl) in TE buffer pH 7.4 (100 mM Tris–HCl pH 7.4–1 mM EDTA pH 8.0) and the RNA was hydrolyzed (45 min, 37°C) with RNase T_1 and RNase A, 4.4 U and 245 μg, respectively, for each 150 μg of nucleic acids. A second hydrolysis (45 min, 37°C) was done using RNase T_2 (20 U per 150 μg of nucleic acids) (45 min, 37°C). Finally, the samples were incubated with proteinase K (600 U per 150 μg of nucleic acids), and extracted with phenol–chloroform–isoamyl alcohol. DNA was precipitated, washed and dried as previously described. The total amount of DNA obtained from each sample matrix was then determined spectrophotometrically (see *Analyte quantification*).

Handling and preparation of commercially available DNA. E. coli and calf thymus DNA were dissolved in 1 mM Tris–HCl (pH 7.2), transferred to 1.7-ml polypropylene vials (50 μg), dried, and stored in the dark at room temperature until needed.

Enzymatic hydrolysis of DNA. DNA samples (50–160 μg) were hydrolyzed following a variation of the method previously reported by Crain [3]. DNA was resuspended in HPLC-grade water (0.5 μg DNA per μl), heated (3 min, 98°C) and immediately chilled (ice) to denature it. The pH was adjusted to 5.3 with ammonium acetate (100 mM). Nuclease P_1 (3 U per 25 μg DNA) was added, and the mixture incubated (2.5 h, 45°C). The pH was adjusted to 7.9 with freshly-made ammonium bicarbonate (1 M) and incubated with phosphodiesterase I (0.0025 U per 25 μg DNA) (2.5 h, 37°C). Finally, the samples were incubated with alkaline phosphatase (0.65 U per 25 μg DNA) (1.5 h, 37°C) to obtain free deoxyribonucleosides (dN). I^5Cyd and formic acid (10 μl each) were added to 80 μl of each sample in HPLC autosample vials for analysis by LC–UV and/or LC–TSP-MS. Remaining portions of sample were stored at −20°C.

HPLC conditions

Solvent composition and flow-rate program-

ming HPLC were used to separate dN for identification and quantification. Each solvent (A = 1.5% acetonitrile–water; B = 30% acetonitrile–water) was prepared to the same concentration of ammonium acetate (50 m*M*, pH 6.0) for thermospray ionization. The duration of analysis was 25 min and was followed by a 10-min column re-equilibration period. A flow-rate of 1.2 ml/min was maintained for the first 3.5 min, then increased linearly to 1.4 ml/min during the next 12 min where it was maintained until the end of the analysis. The solvent composition was maintained at 3% B for the first 3.5 min increasing linearly to 15% during the next 6 min, and finally increasing linearly to 95% during the next 6 min where it was maintained until the end of analysis. Column temperatures were maintained at 30°C.

LC–TSP-MS conditions

LC–TSP-MS was used to verify the identification of each analyte, and for the quantification of minor dN. The optimum temperatures for the capillary vaporizer and the ion source were 110 and 200°C, respectively and the analyzer pressure was $1.5 \cdot 10^{-5}$ Torr. The mass spectrometer was alternatively scanned between positive (105–650 Da) and negative (120–650 Da) ions every 0.5 s (PPINICI). When more sensitivity was required, a limited mass range positive ion only scan was used (105–305 Da, 1.0 s). Multiple ion detection (MID) scanning mode was used for the analyses of 6-methyldeoxyadenosine (m^6dAdo). The scan function for MID was: *m/z* 266 ($[M + H]^+$), 0.1 s (m^6dAdo); *m/z* 282 ($[M + H]^+$), 0.1 s (m^6Ado); *m/z* 252 ($[M + H]^+$), 0.1 s (dAdo); *m/z* 150 ($[B + 2H]^+$), 0.1 s (6-methyladenine); and *m/z* 370, 0.1 s (I^5Cyd).

Analyte quantification

Quantification of total nucleic acid (RNA and DNA) and DNA purified during sample preparation was accomplished by spectrophotometric analysis (260 nm) using the relationship of one absorbance unit for each 40 μg nucleic acid and for each 50 μg DNA [3]. Quantification of dN was done using I^5Cyd as the internal standard, and was similar to methods previously reported by Gehrke and co-workers [4,5]. The response of each analyte and the internal standard was measured at both 254 and 280 nm, and analyte peak area relative to the internal standard peak area was determined for each analyte using a five-point calibration curve generated at each wavelength. Analyte concentration was then calculated using System Gold software according to eqn. 1:

$$\text{concentration} = (\text{slope} \times \text{area}) + \text{offset} \quad (1)$$

Quality assurance/quality control

Identification of the major and minor components in DNA lysate chromatograms was based on: (1) LC capacity factors; (2) LC peak-area absorbance ratios (254 and 280 nm); (3) UV spectra; and (4) mass spectra. A sample analysis set consisted of: an instrument blank; the lowest concentration calibration standard; analyzed Supelco nucleoside test mixture; a mid-range calibration standard analyzed before and after analysis of sample set; hydrolyzed calf thymus DNA; reagent/enzyme control; five samples and an instrument blank (repeated until all samples were analyzed); and a duplicate of one of the samples. The following criteria were met before LC–UV quantification data were acceptable: (1) no analytes were to be detected in the instrument blank; (2) signal/noise (S/N) for m^5dCyd in the lowest concentration calibration standard was at least 10:1; (3) resolution of 1-methyladenosine (m^1Ado) from 2-thiocytidine (s^2Cyd) (Supelco test mixture) was at least 0.50; (4) peak symmetry of m^5Cyd (Supelco test mixture) was within 1.0–1.25; (5) quantification, capacity factor, and 254/280 nm absorbance ratio for each analyte (mid-range calibration standard) was within 5% of the expected value; (6) the difference from the mean mole percent of each dN in the duplicate sample was within ±10%; (7) mole percent of dN in calf thymus DNA sample was within ±10% of expected value; (8) identification of each analyte was confirmed (presence of B + 2H and M + H at expected capacity factor) by TSP–MS in at least 10% of all samples. Control charts were maintained for S/N of m^5dCyd in the low concentration calibration standard and for the ca-

pacity factor, 254/280 ratio, and mole percent of dAdo from calf thymus DNA.

RESULTS AND DISCUSSION

This report presents analytical methodology and quality assurance criteria developed to characterize major and minor deoxynucleosides in fish liver DNA. Methods development included the optimization of enzymatic hydrolysis, chromatographic resolution and analyte detection. A flow chart summarizing the methodology is presented in Fig. 1. Briefly, tissue was lysed with EDTA–sodium dodecyl sulfate (SDS) and centrifuged to remove cellular debris. Denatured proteinaceous material was degraded with proteinase K and removed by extraction with phenol, chloroform, and isoamyl alcohol. Resulting nucleic acids were precipitated, washed and dried. The amount of nucleic acids was determined in at least one sample of each sample type (fish species or selected tissue) so that the amount of enzyme for RNA hydrolysis could be calculated. For the first analysis of any species or tissue type, the amount of enzymes needed for RNA hydrolysis was calculated based upon the assumption that the recovered nucleic acids were all RNA. (For subsequent sample sets, the amount of enzymes required was adjusted according to the measured RNA/DNA ratio.) Samples were then treated with a mixture of RNases (A, T_1 and T_2) followed by proteinase K and repetition of the organic solvent extractions. Highly purified DNA was precipitated, washed, and dried. The amount of DNA was determined in at least one sample of each sample type so that both the amount and concentration of enzyme for DNA hydrolysis could be calculated. Samples were then treated with nuclease P_1, phosphodiesterase I, and alkaline phosphatase to release deoxynucleosides for analysis. Fig. 2 shows a typical fish liver DNA LC–UV chromatogram at 254 and 280 nm.

The effectiveness of the enzymatic procedure was determined by evaluating LC–UV chromatograms for contamination by protein, free bases, nucleosides, deoxynucleotides, and oligodeoxynucleotides. No depurinated free bases, nucleotides, or oligonucleotides (peaks less than 0.01% internal standard) were detected in any of the three fish DNA. RNA contamination was measured by the ratio of Ado/dAdo, and the detection of Urd (less than 1%, and not detected, respectively). Proteinaceous contamination was characterized by analyzing incubated samples containing enzymes but not substrate. It was found that only two enzymes, phosphodiesterase I and alkaline phosphatase, showed LC–UV peaks that could possibly interfere with dN analysis. Contaminants from alkaline phosphatase were removed by washing the enzyme with ammonium sulfate (see *Preparation, purification, and handling of enzyme solutions*). Trace amounts of contaminants in phosphodiesterase I, a lyophilized enzyme, remained, whether resuspended in HPLC-grade water, distilled water, or laboratory-distilled, deionized water. However careful control of the amounts of each enzyme resulted in final lysates which were free of proteinaceous contaminants which may possibly interfere with dN quantification. The amount of DNA per sample and the RNA/DNA ratio in trout, bullhead, and medaka from a typical analysis were 1.1 mg/g and 5.2, 0.33 mg/g and 3.0, and 0.50 mg/g and 4.2, respectively.

Nuclease P_1 was used instead of DNase I for the initial hydrolysis of DNA to 5′-mononucleotides because of its ability to hydrolyze all diester bonds, even those adjacent to modified deoxynucleosides [4,5]. Denaturing of DNA, and the optimization of enzyme concentrations and incubation times were done to insure efficient hydrolysis by NP_1. For our study, DNA was denatured at 98°C, however, subsequent experiments have shown that the maximum amount of DNA was denatured at 95°C. Further denaturing at temperatures between 85 and 100°C did not change the relative amount of m^5dCyd or dUrd. The use of phosphodiesterase I and bacterial alkaline phosphatase (also a non base-specific enzyme) completed and assured the quantitative release of dN.

The performance of the LC–UV system was evaluated before each sample set was analyzed by determining analyte resolution, sensitivity,

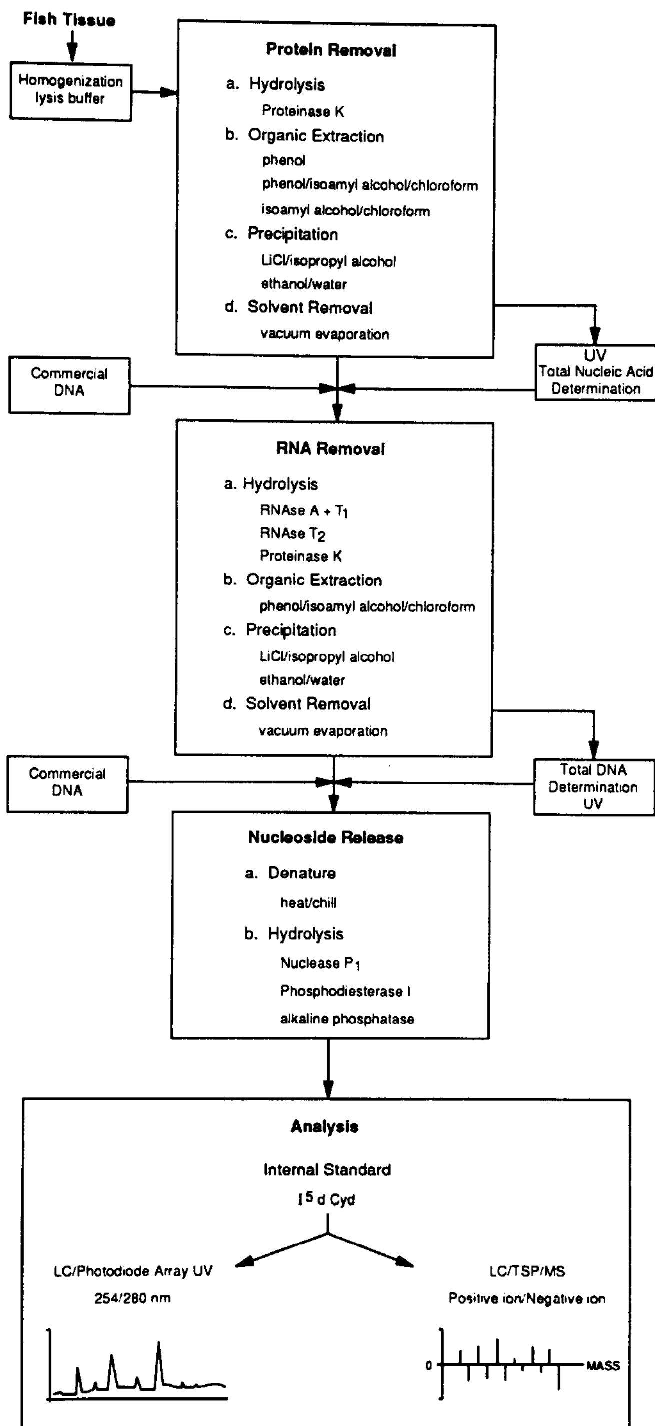

Fig. 1. Summary of analytical procedures for the characterization of DNA nucleosides.

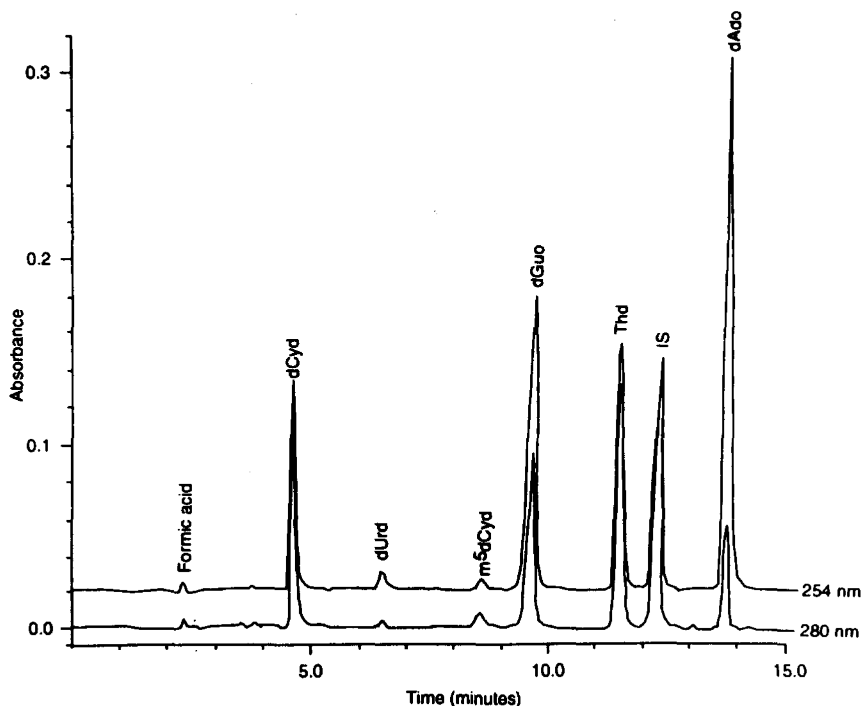

Fig. 2. LC–UV chromatogram of medaka liver DNA lysate. Upper trace 254 nm; lower trace 280 nm.

symmetry, variation of response factors from previously determined values, and the presence of residual analytes from previous analyses (peak carry-over). Resolution and symmetry were evaluated by analyzing a mixture of nucleosides (Supelco nucleoside test mixture) and calculating a resolution and symmetry coefficient. The variability of these performance parameters were determined from repetitive analysis of calibration standards and the nucleoside test mixture, and was used to set quality assurance criteria. Sensitivity was determined by analyzing the lowest concentration m^{5}dCyd standard and calculating a value for S/N. A minimum S/N was arbitrarily set at 10:1 to assure accurate measurement of small concentrations. Changes in analyte UV response from that which was determined using the original five-point calibrations were determined by measuring the concentration of dN in at least one calibration standard. The measured concentration for each analyte was compared to the expected value. The acceptable daily variability was arbitrarily set at ± 10%. The presence of residual analytes from previous analyses was evaluated by analyzing 50 m*M* ammonium acetate as an instrument blank. Samples were not analyzed until each of these performance criteria was met (see *Quality assurance*).

The capability of the LC–UV system for both qualitative and quantitative analysis was evaluated in several ways. Linearity of analyte response was verified by determining that the coefficient of determination (r^2) for each calibration curve was greater than 0.995. The accuracy of each calibration curve was verified by independent preparation of standard solutions and their quantification using the original calibration curve. Each analyte was quantified within ± 10% of the expected value. Next, five replicate injections of each of five calibration standards were analyzed and capacity factor and absorbance ratios (A_{254}/A_{280}) were determined. Capacity factor and absorbance ratio are two criteria used for the identification of analytes. The mean capacity factors and absorbance ratio (A_{254}/A_{280}) for dCyd, dUrd, m^{5}dCyd, dGuo, Thd and dAdo were 0.99

TABLE I

COMPARISON OF CONCENTRATION OF DEOXYNUCLEOSIDES IN CALIBRATION STANDARD 3 REPORTED AS SINGLE WAVELENGTH (SINGLE) AND AVERAGE OF TWO WAVELENGTHS (DUAL)

Deoxynucleoside	Concentration (mean ± S.D., n = 5) (μg/ml)		
	Single	Dual	Difference of means
dCyd	36.3 ± 0.06	36.2 ± 0.04	+0.1
m^{5}dCyd	3.33 ± 0.02	3.33 ± 0.02	0.0
dGuo	35.4 ± 0.04	35.4 ± 0.04	0.0
Thd	36.5 ± 0.03	36.5 ± 0.03	0.0
dAdo	35.5 ± 0.04	35.6 ± 0.04	−0.1

and 0.90, 1.69 and 2.52, 2.61 and 0.61, 3.01 and 1.74, 3.73 and 1.10, and 4.63 and 5.85, respectively. The relative standard deviation (R.S.D.) for the capacity factors ranged from 0.5 to 2.2%, and for the absorbance ratios from 0.4 to 2.0%. The influence of trace amounts of enzymes and organic solvents remaining in a sample upon capacity factor and absorbance ratio was evaluated by duplicate injection of five replicate preparations of calf thymus DNA. It was found that the R.S.D. varied from 0.7 to 1.9% and from 0.1 to 0.4% for capacity factor and absorbance ratio, respectively, indicating that the presence of co-extractives had little effect upon these two variables. Based upon results from these two data sets, we have set quality control criteria for the identification of analytes by LC–UV to be ±5% of the expected values, which were obtained from the calibration standards data set.

The replicate analysis of calibration standards and calf thymus DNA was also used to assess variability in analyte response, *i.e.* the ability to quantify dN. First, it was found that the percentage R.S.D. calculated from the repetitive analysis of the calibration standards was less than 2% for each analyte at each concentration, at both 254 and 280 nm, except for m^{5}dCyd which was 3.2% at 280 nm. Second, the concentration of each dN in the replicate calf thymus analysis was measured to allow us to calculate the mole percent composition of DNA. However, several different methods to determine mole percent are possible. First, concentrations at a single wavelength may be used; second, concentrations at select wavelengths for each analyte based upon absorbance maxima, *i.e.* 254 nm for purine nucleosides and 280 nm for pyrimidine nucleosides, may be used; and third, mean concentrations determined at two (254 and 280 nm) wavelengths may be used. Because our LC–UV system has the capability for dual wavelength monitoring we have chosen to compare the second and third methods. Tables I and II present a summary of the results. In each table, the mean values reported for single-wave-

TABLE II

COMPARISON OF MOLE PERCENT COMPOSITION OF CALF THYMUS DNA

Deoxynucleoside	Mole percent (mean ± S.D., n = 10)		
	Single	Dual	Difference of means
dCyd	19.78 ± 0.29	20.11 ± 0.28	−0.33
m^{5}dCyd	1.27 ± 0.07	1.28 ± 0.04	−0.01
dGuo	22.69 ± 0.08	22.66 ± 0.07	+0.03
Thd	27.36 ± 0.13	27.28 ± 0.12	+0.08
dAdo	28.90 ± 0.11	28.67 ± 0.11	+0.23

length analysis were determined at 254 nm for dGuo and dAdo, and at 280 nm for dCyd, m^{5}dCyd and Thd, while mean values for dual-wavelength analysis were determined at both 254 and 280 nm for each dN. The difference in dN concentrations measured in calibration standard 3 (Table I) and mole percent of dN in calf thymus DNA (Table II) are approximately 1 S.D. or less, indicating that the data from each method were similar within reasonable error, and neither method appeared to offer a distinct advantage. However, an advantage can be seen for dual-wavelength averaging by comparing percentage R.S.D. of each dN measured at a single wavelength to the value determined by averaging two wavelengths for the calf thymus DNA data (Table II). The percentage R.S.D. of the single-wavelength mole percent of the minor dN, m^{5}dCyd, is nearly twice as large as the value for the dual-wavelength method, 5.5% *versus* 3.1%, respectively. These data indicate that quantification errors due to interferences may be significant for minor nucleosides, and that these errors can be minimized using dual-wavelength averaging. Because of this, and because several other authors have previously chosen to report mole percent based upon the average concentration of two wavelengths [4–8], the fish liver DNA composition data reported in this paper were also calculated in this manner. Further, we have used these data to set a quality control criterium of ± 10% for the allowable variability of the expected dN concentrations in the mid-range calibration standard, in the expected dN mole percent in the calf thymus DNA control sample, and in the difference from the mean mole percent of the duplicate samples.

The dN composition of liver DNA from brown bullhead, medaka, and rainbow trout, three species of fish currently being studied in our laboratory because of their sensitivity to tumor development upon exposure to anthropogenic chemical contaminants [9–11], was determined (Table III). It was found that the mole percent decreased in the order dAdo, Thd, dGuo, dCyd, and that the relative amount of any single dN varied between species by as much as 4.5%. Our results (Table III) did not show a 1:1 mole percent correlation as expected by Watson–Crick nucleoside pairing (*i.e.* dAdo = Thd and dGuo = dCyd + m^{5}dCyd). Other authors have used relative calibration factors [4] or software adjusted LC calibration to assure a 1:1 ratio (*e.g.* salmon sperm, *E. coli* and calf thymus) [12–15]. Our chromatograms, however, did not show any free depurinated bases, nucleotides, nucleosides, oligomers or oligonucleotides that could have accounted for the error in the DNA mole percent data. Other experimental parameters were also rigorously controlled to avoid depurination or deamination. It has been suggested that the extreme accuracy of the DNA base pairing is much more exact than can be determined analytically [4]. We therefore suggest that a highly accurate compositional analysis of environmental samples might not be possible without the use of a mathe-

TABLE III

MOLE PERCENT COMPOSITIONS OF LIVER DNA FROM THREE SPECIES OF FISH FOUND TO BE SENSITIVE TO DEVELOPMENT OF TUMORS

Deoxynucleoside	Mole percent		
	Rainbow trout	Medaka	Brown bullhead
dCyd	18.69	16.76	14.49
m^{5}dCyd	1.45	1.85	1.92
dGuo	21.94	20.74	22.72
Thd	26.79	27.94	26.14
dAdo	30.29	31.22	32.50
dUrd	0.83	1.50	2.22

matical approach that may adjust the mole percent ratios of pairing ratios to a value of 1.

The mole percent of m^5dCyd varied from 1.39 to 1.93% for the three species of fish. These values are in agreement with values previously obtained for other fish species including carp (*Cyprinus carpio*) (1.41%) [16], salmon (*Oncorhynchus gorbuscha*) (2.13%) [16], and bluegill (*Lepomis macrochirus*) (1.6%) [17]. The methylation of dCyd has been shown to be an important parameter in mammalian systems influencing cell differentiation, chromosome structure stability, and DNA transcription, replication, and repair [18]. Changes in the ratio of m^5dCyd/dCyd has been suggested as a good bioindicator of exposure to carcinogens [17,18]. It is essential therefore that the m^5dCyd be carefully measured. It has been shown that m^5dCyd can be deaminated at pH < 4 to Thd [19], however, we did not observe any transformation (<0.01%) at pH values used during the sample preparation we described.

LC–TSP-MS analysis showed that the peak for m^5dCyd was free from other dN interferences and that dUrd was present in the fish DNA lysates. dUrd arises from dCyd conversion from a variety of sources including *in vivo* DNA spontaneous deamination [20,21], *in vitro* nucleotide deamination when commercial enzymes are contaminated with deaminases [22,23], or deamination by heat at neutral pH [23–25]. In this study, *in vitro* deamination of dCyd occurred to a greater extent than *in vivo* deamination as a source of dUrd in fish liver DNA lysates. Deamination studies using DNA and a cytosine deaminase inhibitor (5-bromocytidine) showed that both venom phosphodiesterase and bacterial alkaline phosphatase were contaminated with cytosine deaminases. The deaminase activity in alkaline phosphatase was found to be inversely correlated to the amount of ammonium sulfate in the preparation. Furthermore, venom phosphodiesterase contained half of the deaminase activity of phosphatase.

Methylation of dCyd can then be presented as the ratio of either m^5dCyd/dCyd or m^5dCyd/(dCyd + dUrd). Although values calculated using either method differ from each other only slightly, the difference relative to dCyd can be quite large. Further, replicate analysis ($n = 3$) of bullhead liver DNA gave dUrd mole percent data from 1.16 to 1.73%, indicating an expected variability of 1–2%. It seems, therefore, that although the presence of dUrd in fish DNA lysates might arise from either *in vivo* and/or *in vitro* sources, the quantification of the relative amount of methylation of dCyd as a bioindicator of exposure should include dUrd values. Finally, the presence of adenine deaminases in the enzyme preparations was studied using purified AMP and DNA lysates. LC–UV and LC–TSP-MS screening of these samples did not show any transformation of dAdo to dIno or of Ado to Ino under the experimental conditions used.

Although not previously identified in fish, m^6dAdo has been reported as another minor dN in prokaryotic DNA [4]. Positive ion MID-MS, a more sensitive technique, was therefore used to screen for m^6dAdo in the medaka, bullhead, and trout samples. A sample of *E. coli* DNA, known to contain m^6dAdo, was hydrolyzed to dN and spiked with m^6Ado (1% relative to dAdo). The LC capacity factors for m^6dAdo and m^6Ado were then determined using this sample. A portion of liver DNA hydrolysate from each fish was similarly spiked with m^6Ado and reanalyzed by PPINICI TSP-MS. No m^6dAdo was detected in any of the fish samples. Based upon S/N ratios of the M+H ions for m^6Ado and m^6dAdo it was estimated that the concentration of m^6dAdo in the fish samples was less than 0.01%.

CONCLUSIONS

This paper presented analytical methodology and quality assurance guidelines for both the use of enzymes and qualitative and quantitative characterization of the deoxynucleoside composition of DNA. These methods have been shown to produce accurate and reproducible results for environmental samples with minimal losses of labile modified dN. Rigorous quality assurance criteria applicable to the determination of DNA deoxynucleoside composition are essential because it has been previously shown that very subtle

changes in the composition can result in significant cellular changes. We have also shown that deamination of dCyd to dUrd can significantly change DNA mole percent composition, and can influence the presentation of information on the methylation of dCyd as a bioindicator of xenobiotic chemical exposure. Finally, this paper has been developed to serve as an analytical procedure and quality assurance guideline for future studies. Mention of trade names or commercial products does not constitute endorsement or recommendation for use by EPA.

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