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Quantitation of Oligonucleotides by Phosphodiesterase Digestion Followed by Isotope Dilution Mass Spectrometry: Proof of Concept

Gavin O'Connor,* Carol Dawson, Alison Woolford, Kenneth S. Webb, and Tim Catterick

LGC Limited, Queens Road, Teddington, Middlesex, TW11 0LY, U.K.

The importance of DNA as a regulatory analyte is well-known. Recent years have seen an increased interest in the quantitation of this analyte. Accurate quantitative measurements have been hampered by the lack of well-characterized standards and pure materials for this large-molecular-weight analyte. Outlined here is an approach for the accurate and reproducible quantitation of an oligonucleotide that is solely reliant on the availability of pure, well-characterized deoxynucleotides and not a sequence-specific pure DNA standard. The proposed procedure is intended to provide an accurate and definitive method for the quantitation of DNA for reference measurements as an improved alternative to the more conventional UV absorbance-based methods. For proof of concept, a gravimetrically prepared oligonucleotide solution was enzymatically digested to its constituent monomer-deoxynucleotide monophosphates (dNMPs), of which there are four different types. Qualitative mass spectrometry was used to confirm the 100% successful completion of the enzymatic digestion step. The dNMPs were then separated by liquid chromatography (LC) before being detected by electrospray ionization (ESI) mass spectrometry (MS). The method of quantitation was based on isotope dilution mass spectrometry (IDMS) analysis of the four different monomer units. The concentrations of the four dNMP residues were then summed to obtain the original concentration of the oligonucleotide. The concentrations determined by liquid chromatography/mass spectrometry (LC/MS) and also by liquid chromatography−tandem mass spectrometry (LC/MS/MS) differed by <2.5 and 1%, respectively, from the gravimetrically assigned value. These differences were well within the uncertainty of the gravimetrically assigned value. This highly accurate method, suitable for the definitive quantitation of oligonucleotides, should be ideal for characterizing primary calibration standards and certified reference materials that can then be used to underpin the more conventional quantitative techniques of UV and fluorescence spectroscopy.

In the past decade, we have witnessed a vast increase in the use of DNA analysis, and its associated techniques have undergone rapid developments in sensitivity, robustness, and automation. Much of this increase can be attributed to the widespread and routine use of the polymerase chain reaction (PCR) amplification of targeted DNA sequences. Large-scale genome sequencing projects, such as the human-genome-mapping project (HGMP) have created a wealth of accessible data such that many more assays can be designed and developed. At the same time, a number of important issues have prompted the need for reliable quantitation of DNA. A good example of this is highlighted by legislative requirements for genetically modified organism (GMO) analysis. The labeling of genetically modified organisms (GMOs) in foodstuffs is currently regulated by E.U. legislation (49/2000/EC, 278/97/EC)[1] that requires the compulsory labeling of foodstuffs containing GMOs at over 1% of each individual ingredient. Several real-time PCR systems for the quantitation of GMO have been reported.[2,3] These techniques reproduce well within a laboratory; however, they do not compare well in interlaboratory comparisons, in part because of a lack of accurately quantified standards.[4]

Further examples requiring DNA quantitation include clinical applications, such as the measurement of bacterial or viral loads,[5] minimal residual disease analysis, and tests for fetal DNA in maternal blood,[6] all of which could benefit from more accurate DNA quantitation techniques. In all of these cases, there is a paucity of appropriate methodology that can underpin the quantitative measurements.

For intact oligonuleotides, UV and fluorescence have been used to make quantitative estimates. In the case of UV, the adopted procedures have sometimes depended on assumed average absorbance and molecular characteristics for the individual base units making up the DNA strands, which clearly limits the accuracy of such measurements.

* Corresponding author. Telephone: 44 (0) 20 8943 7318. Fax: 44 (0) 20 8943 2767. E-mail: goc@lgc.co.uk.

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In many cases, the UV detection was linked to liquid chromatography, which was used to purify or establish the purity of the oligonucleotide,[7] rather than provide absolute quantitation. Although such assumptions are not unreasonable for many general applications, they are not deemed appropriate when trying to establish more definitive high-accuracy measurements, to underpin materials that are intended to be used as primary standards and high-caliber certified reference materials. The current method of choice for many applications is real-time PCR combined with fluorescence. However, given the possible influence of other matrix constituents to interfere with the fluorescence process, particularly by quenching, this is not deemed an ideal approach for definitive quantitation.

Quantitating DNA by measuring the individual nucleosides provides an alternative approach. Many of the early studies of the nucleosides were based around LC/UV methodologies.[8−12] However, for high accuracy, definitive quantitation, data based on UV detection can be affected by coeluting compounds and the influence of pH on the absorbance characteristics of the heterocyclic analytes.[13]

Mass spectrometry is a technique that is capable of subpicomolar detection of organic species. The development of atmospheric pressure ionization sources, especially electrospray ionization (ESI), has enabled the detection of larger nonvolatile organic species and biopolymers. It has already been noted by others that mass spectrometry can offer significant advantages over UV spectroscopy for the quantitative measurements of nucleotides.[14] The use of an isotopically labeled analogue as the internal standard provides mass spectrometry with the ideal means for compensating for potential sources of error in making quantitative measurements. This form of quantitative mass spectrometry is usually referred to as isotope dilution mass spectrometry (IDMS) and has been well-established elsewhere as a route to highly reliable quantitative trace analysis, including the ability to minimize the influence of challenging matrixes.[15,16] This has resulted in IDMS being the method of choice for the quantitation of analytes in primary standards and high-caliber certified matrix reference materials by many national measurement institutes.

Coupled LC/ESI-MS has been used for the characterization and quantitation of DNA adducts.[17,18] The method of analysis consisted of first digesting the DNA using phosphodiesterase enzymes. The modified nucleotide was then free for quantitation. Although IDMS has been used for this purpose,[19] no efforts have been made to quantify the unmodified nucleoside monophosphates.

The purpose of this study was to investigate the ability of LC/IDMS to accurately quantify a gravimetrically prepared oligonucleotide by means of quantifying its constituent nucleotides. The major benefit in achieving this is that it may aid in the realization of a quantitative reference material without the need for known-purity, sequence-specific DNA standards.

The approach described in this paper involves quantitation of an oligonucleotide without the need for a pure oligonucleotide standard. This was achieved by the quantitative digestion of the polymer to its constituent monomer units. Mass spectrometry was used to confirm the successful completion of the digestion step by confirming the absence of any other products. Each individual deoxynucleoside monophosphate (dNMP), with its labeled counterpart added as internal standards, was then quantified using LC/IDMS against pure natural standards of the dNMPs. The amount of each dNMP residue was then summed to determine the original quantity of oligonucleotide present. For the proof of principle, a gravimetrically prepared solution of an oligonucleotide was quantified, and the IDMS-derived value was compared with the gravimetrically assigned value.

EXPERIMENTAL SECTION

Reagents, Sample, and Standards. Ten individual 1-mg quantities of a 20-mer oligonucleotide, at the 0.2 μmol scale were obtained from MWG-Biotech AG, Ebersberg, Germany. The oligonucleotide was quantitatively transferred to a single 15-mL plastic tube by first dissolving it in 1 mL of water (18 MΩ). The empty vessels were then freeze-dried, and the amount of oligonucleotide transferred was calculated by difference weighings of the tubes before and after sample removal. The oligonucleotide solution was then prepared to a known concentration by the gravimetric addition of water. A gravimetric dilution of this solution to a concentration of ~200 μg/g served as the "unknown" analyte to be quantified.

One-gram quantities of 2′-deoxycytidine 5′-monophosphate (dCMP), 2′-deoxythymidine 5′-monophosphate (dTMP), 2′-deoxyadenosine 5′-monophosphate (dAMP), and 2′-deoxyguanosine 5′-monophosphate (dGMP) were obtained from Sigma-Aldrich Co., Dorset, U.K., at a purity of >98%. Mixed stock standards containing the desired concentrations of all four 2′-deoxynucleoside 5′-monophosphates (dNMPs) were prepared gravimetrically in water.

Isotopically labeled (^{13}C, ^{15}N) dNMPs were obtained in a mixed solution from Silantes GmbH, München, Germany. All carbon and nitrogen atoms of the labeled dNMPs were enriched; however, the exact concentration of each individual dNMP was not known.

Sample and calibration blend solutions were prepared by gravimetrically adding equal amounts of the mixed labeled dNMPs standard to the oligonucleotide sample and the mixed natural dNMP stock standard.

Phosphodiesterase I (*Crotalus adamanteus* venom, 36.2 U/mg) (SVPD) and phosphodiesterase II (bovine spleen, 10.8 U/mg) (CSPD) were obtained from Amersham Pharmacia, Buckinghamshire, U.K. and Sigma-Aldrich Co., Dorset, UK, respectively.

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Preparation of Standards and Samples. (a) Natural Intact Oligonucleotide "Unknown". The preparation of a simple stock solution of oligonucleotide for the purpose of validating the exact quantitation method proved more difficult than first perceived. Indeed, this fact gives extra credence to the approach described in this study, because it is not reliant on the availability of pure oligonucleotide or DNA standards, but instead, on the availability of the smaller 2′-deoxynucleoside monophosphates. To obtain the purity and amount of oligonucleotide required for this study, 10 separate freeze-dried quantities of a 20-mer oligonucleotide were purchased. The contents of each tube ranged from 600 to 1500 μg of oligonucleotide per tube, as inferred by optical density readings obtained prior to freeze-drying of the material. The material was transferred to a preweighed plastic vial by dissolving the contents of each tube in water. The final amount of oligonucleotide transferred was determined by the difference in mass between the full and empty tubes.

The purity of an oligonucleotide at the 0.2-μmol scale is generally thought to be >95%, especially if an HPLC cleanup procedure has been used. The purity of the oligonucleotide was checked by ESI-MS, and the presence of dNMPs and $N \pm 1$ products, the most likely impurities, were not detected. The determination of the water impurity was impossible because of the original small amount of oligonucleotide obtained. Therefore, the oligonucleotide was assigned a purity of 95% with an uncertainty of 5%. This, combined with the method of preparation of the stock solution, resulted in an overall expanded relative uncertainty ($K = 2$) of 7%, which is large for a gravimetrically prepared solution. The final assigned concentration of the oligonucleotide solution was 194 ± 6 μg/g.

For the direct analysis of the oligonucleotides (without an LC step) by mass spectrometry, a precleanup step was necessary. This consisted of removing the oligonucleotide (or partially digested oligonucleotide) from the digest solution by the use of a Zip-Tip (Millipore) cleanup. The oligonucleotides were eluted from the Zip-Tip in a solution consisting of 50% aqueous acetonitrile containing 1 mM piperidine and imidazole (Sigma-Aldrich, Dorset, U.K.). This solution was directly infused into the MS at a rate of 10 μL/min using a syringe pump (Harvard Apparatus Inc., MA).

(b) Natural dNMP Standards. The preparation of the stock standards for the dNMPs was relatively straightforward, since a weighed amount of stock solid easily dissolved in water. The purity of the dNMPs was stated to be greater than 95%. An uncertainty of 3% was assigned to this value. This was checked by LC/UV/MS, and no major impurities were detected. The concentration of each dNMP was corrected for purity. A mixed stock standard containing 38.8 ± 0.7 μg/g of dAMP, 53.7 ± 1.0 μg/g of dCMP, 60.7 ± 1.1 μg/g of dGMP, and 37.5 ± 0.7 μg/g of dTMP was prepared in water. These concentrations correspond to the amount of each dNMP in the prepared oligonucleotide solution (assuming total digestion of the oligonucleotide to its constituent dNMPs).

(c) Labeled Deoxynucleotide Monophosphates. The mixture of the labeled dNMPs (LdNMPs) was obtained in solution at a nominal combined concentration of 8 mg/g. To facilitate the exact matching form of IDMS[20] used in this study, an estimate of the concentration of the individual labeled dNMPs was required.

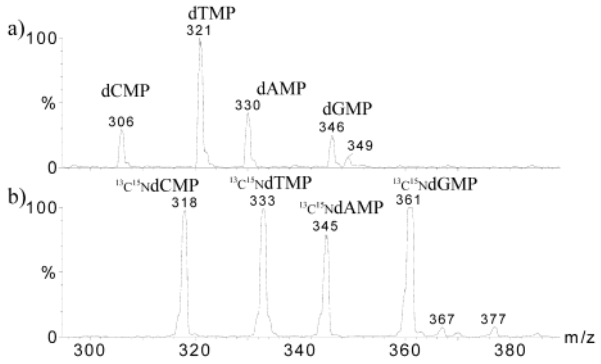

Figure 1. Electrospray mass spectrum of (a) natural deoxynucleotides and (b) isotopically enriched deoxynucleotides.

This was achieved by using the natural dNMPs as the internal standards. The concentrations of the four labeled dNMPs were determined to be 1.2, 2.5, 3.6, and 1.0 mg/g for LdAMP, LdCMP, LdGMP, and LdTMP, respectively. This solution was diluted gravimetrically to yield a final concentration of 40 μg/g of dAMP. This solution was used for the preparation of the sample and calibration blends for the IDMS experiments. The isotopic purity of the LdNMPs was stated to be >98%. The isotopic and chemical composition of the mixture was assessed using direct infusion ESI-MS and LC/UV/MS. No natural dNMPs were detected in the labeled mixture, and only the four dNMPS were detected. A mass spectrum showing the deprotonated molecule ions for the dNMPs and LdNMPs is shown in Figure 1.

(d) Digestion of the Oligonucleotide Target Sample. The digestion media consisted of 10 μL of 25 mM magnesium chloride (Applied Biosystems, CA), 9 μL of 10 mM ammonium acetate (AnalaR grade, BDH, Dorset, U.K.), and 1 μL of a 1 mg/mL dilution of the above SVPD. The sample blend (10 μL) was added to this cocktail, and the resulting mixture was incubated at 37 °C for 15 min using a GeneAmp PCR system 2400 thermocycler (Applied Biosystems, CA). This step was performed in at least triplicate for both the sample and calibration blends. Blank digests were prepared by adding water in place of the sample or calibration blends when preparing the above cocktail. Separate digests containing all the digest media apart from the oligonucleotide, SVPD, or both were also prepared. The digest mixtures were made up to 200 μL using water (18 MΩ) before being presented to the LC/MS for analysis.

(CSPD was required only for providing sequence information of the oligonucleotide. Its activity was slow in comparison to the SVPD, and it was not required for the total digestion of the 20-mer studied.)

High Performance Liquid Chromatography (HPLC). The HPLC system consisted of an Alliance 2690 quaternary pumping module, complete with integral autosampler, column oven, and vacuum degassing unit (Waters, MA). This was coupled to a Waters 2487 dual wavelength UV detector (260 nm) for preliminary experiments; however, this was removed for the bulk of the IDMS experiments. Separation of the four dNMPs was achieved using an XTerra MS C18, 3.5-μm, 2.1 × 150 mm analytical column (Waters, Hertfordshire, UK). The mobile phase consisted of 10 mM ammonium acetate buffered to pH 8.5 with ammonia and pumped at a flow rate of 0.2 mL/min. Complete resolution of the four dNMPs was achieved under isocratic conditions. Sample

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Table 1. General Mass Spectrometer Conditions

		negative ion	
electrospray	spray voltage (kV)	−2.2	
	cone voltage (V)	20−40	
source gas	desolvation gas (L/h)	650	
	cone gas (L/h)	100	
source temp	source block (°C)	100	
	desolvation (°C)	380	
		Q1	Q2
mass spectrometer	low mass resolution	15	15
	high mass resolution	15	15
	ion energy (eV)	2	2
		MS	MS/MS
collison cell	entrance	50	0
	ion energy (eV)	2	17
	exit	51	1

Table 2. Mass Spectrometer Data Acquisition Parameters

time interval (min)	SIR channels	MRM channels
1.4−2.3	306, 318	306 > 195, 318 > 200
1.7−3.7	321, 333	321 > 195, 333 > 200
2.0−4.5	346, 361	346 > 79, 361 > 79
5.0−8.0	330, 345	330 > 195, 345 > 200

aliquots of 10 μL of the above prepared sample digest and standard solutions were injected as prepared above.

Mass Spectrometry. The mass spectrometer used throughout this study was a Quattro Ultima quadrupole tandem mass spectrometer (Micromass, Wythenshawe, U.K.). The general MS and tandem MS/MS conditions used are shown in Table 1. A summary of the MS data acquisition modes used in this study is shown in Table 2.

(a) Analysis of Monomers. The monomers were introduced into the mass spectrometer via the LC system. For the MS analysis of the dNMPs, the mass spectrometer was operated in a selected ion recording (SIR) mode whereby only the deprotonated molecule ion $[M - H]^-$ of the dNMPs and labeled dNMPs were monitored. This was done in a time-dependent mode whereby only the ions for the eluting compounds were monitored for the entire peak width. The channel dwell time used was 20 ms, which resulted in the best ratio precision for the IDMS measurements. For the tandem MS/MS analysis of the dNMPs, the instrument was operated in a multiple reaction monitoring mode (MRM) whereby the precursor to product ion transitions for the dNMPs were monitored. This was also performed in a time-dependent mode with a dwell time of 200 ms.

(b) Analysis of Intact Oligonucleotides. For the study of the intact oligonucleotide, the samples were introduced by direct infusion (i.e., without any chromatographic separation) into the mass spectrometer. The mass spectrometer source settings remained the same (Table 1), but the instrument was operated in a scanning mode from 150 to 2000 m/z with a total scan time of 4 s. Data were recorded for a total of 1 min, and all the acquired scans were summed prior to deconvolution using the Max-Ent software (Micromass, Wythenshawe, U.K.).

RESULTS AND DISCUSSION

(a) Digestion of Oligonucleotide and Sequence Data. The one major assumption in the quantitation of the oligonucleotide is that total digestion of the oligonucleotide to its single nucleoside monophosphates occurs. In an effort to prove this, the oligonucleotide digest solution was analyzed at different time intervals during digestion. The mass spectrum for the undigested oligonucleotide is shown in Figure 2. This clearly shows the multiple charged series for the 20-mer being analyzed. Figure 2b shows the deconvoluted spectrum of the 20-mer, indicating the presence of the intact oligonucleotide. Digests incorporating both phosphodiesterase I and II were stopped at 5 and 60 min, respectively. The resulting deconvoluted spectra are shown in Figure 3. This shows clear evidence of digestion from the 3′ and 5′ ends of the oligonucleotide, respectively. The SVP digestion is markedly quicker than the CSP, with evidence of up to 7 bases being removed after only 5 min. A mass spectrum of a SVP digest after 15 min is shown in Figure 4. There were no oligonucleotides detected; however, evidence of the dNMPs is clearly visible (300−350 m/z). A precursor ion scan of the 79 m/z phosphate ion of a directly infused digest solution yielded no ions >350 m/z (data not shown); however, the sensitivity of this process for the detection of dimers and trimers of dNMPs was not investigated. A 15-min digestion with SVP was deemed appropriate for the total digestion of the oligonucleotide studied.

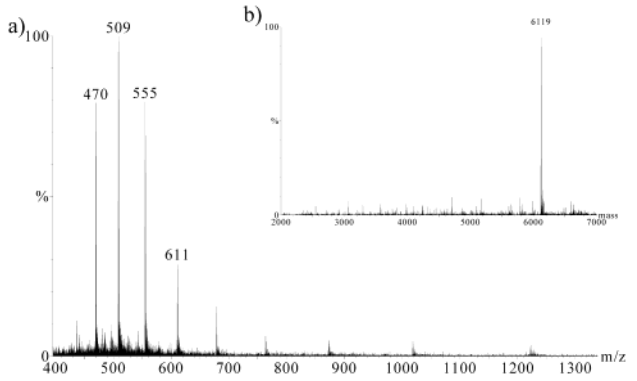

Figure 2. (a) Electrospray mass spectrum of an undigested 0.4 μM solution of a 20-mer oligonucleotide in 50:50 acetonitrile/water, 1 mM piperidine/imidazole; and (b) deconvoluted spectrum.

(b) LC Separation and IDMS Quantitation of the dNMPs in the Original Oligonucleotide. Even though the quantitation of the four dNMPs is reliant only on the signal intensities for the dNMPs and LdNMPs, it was still desirable, if not essential, to have total separation of the analytes prior to MS or tandem MS analysis. Separation of the four dNMPs was performed using a standard reversed-phase LC separation. Separation of the dNMPs was achieved only with ammonium acetate present. Because the analytes were detected in negative ion ESI, competing reactions between the acetate and the dNMPs led to reduced sensitivity. A 10-fold improvement in signal intensity was obtainable for all the dNMPs on the removal of the acetate from the mobile phase. Therefore, the minimum concentration of ammonium acetate required to separate all four dNMPs was used. An LC/UV chromatogram showing the complete separation of the four dNMPs is shown in Figure 5a. The resulting TIC for this analysis is shown in Figure 5b. The loss in chromatographic resolution observed in the TIC was due to the tubing used to couple the UV and MS in series. Consequently, the UV was taken off-line for the IDMS experiments.

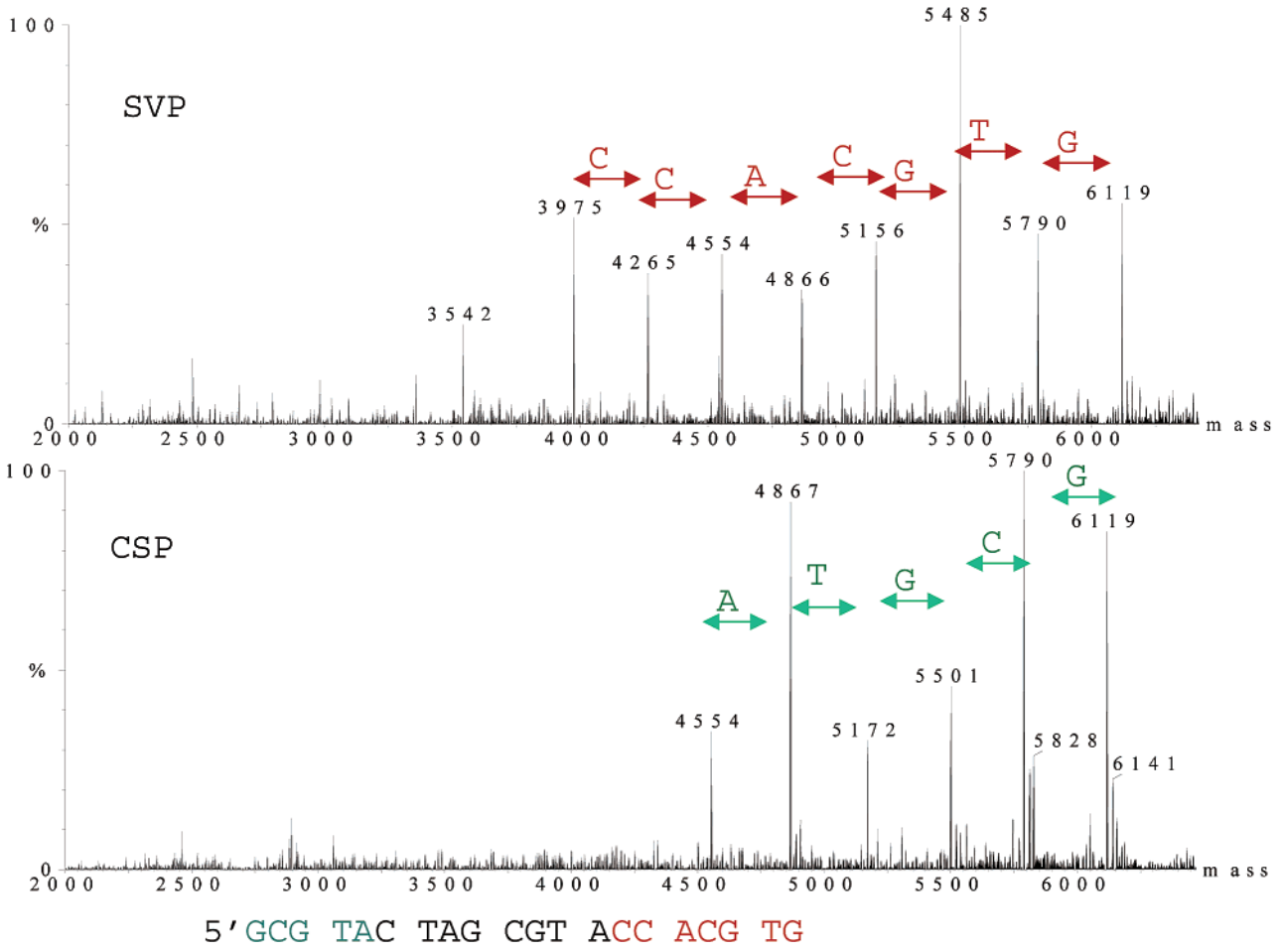

Figure 3. Deconvoluted electrospray mass spectrum of a partially digested 0.4 μM solution of a 20-mer oligonucleotide in 50:50 acetonitrile/water, 1 mM piperidine/imidazole (a) using snake venom phosphodiesterase and (b) using calf spleen phosphodiesterase.

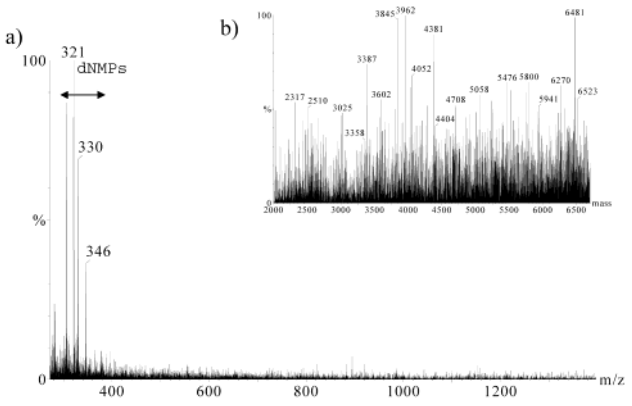

Figure 4. (a) Electrospray mass spectrum of a fully digested 0.4 μM solution of a 20-mer oligonucleotide in 50:50 acetonitrile/water and 1 mM piperidine/imidazole; (b) deconvoluted spectrum.

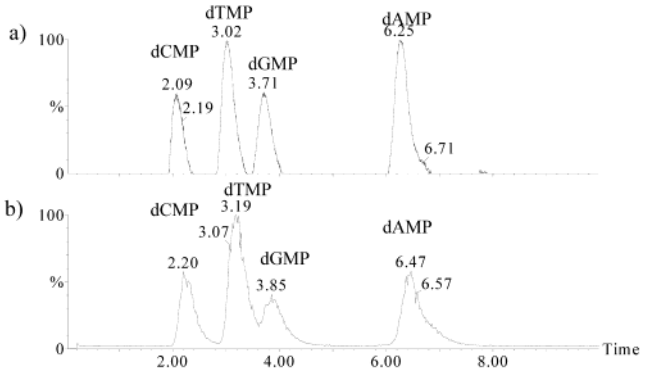

Figure 5. Chromatogram showing the separation of a mixed standard solution (~20 μg/g) of four deoxynucleotides: (a) UV trace and (b) total ion chromatogram.

A major component of the uncertainty associated with IDMS measurements can be attributed to the precision of the ratio measurements.[21] If all four dNMPs were to be analyzed at once, this would require eight SIR channels to be monitored simultaneously. The temporal separation of the dNMPs by HPLC required no more than four SIR channels to be monitored at any one time. This resulted in an improved measurement precision.

It is essential for accurate IDMS analysis that the ions of interest are free from mass spectral interferences. The formation of oxidative products and other adducts from the ESI of the dNMPs are possible causes of such interference. The SIR channels monitored for the dNMPs are shown in Figure 6. It is clear that the quantitation of dGMP would be hindered by interferences caused by the presence of dCMP and dAMP. This is indicated by the increase in the signal intensity of the 346 m/z channel during the elution of dCMP and dAMP.

The form of IDMS used throughout this study was exact matching double IDMS[21]. Ideally, the measured ratio for all four

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Table 3. Determined Concentrations (μg/g) of the Four Nucleotide Bases in Multiple Digests of Three Spiked Subsamples

blend no.	digest no.	dAMP (μg/g)	uncert, $K = 1$	DCMP (μg/g)	uncert, $K = 1$	dGMP (μg/g)	uncert, $K = 1$	dTMP (μg/g)	uncert, $K = 1$
1	1	44.96	1.89	61.23	1.82	64.95	1.60	41.81	1.63
1	2	46.27	0.77	59.03	2.16	67.20	2.68	43.52	1.63
1	3	46.08	1.42	58.58	1.57	67.00	2.15	42.18	1.02
1	4	45.20	1.78	59.32	1.95	71.16	2.45	42.48	1.59
1	5	45.77	1.13	57.13	1.22	67.45	1.53	40.90	1.56
2	1	43.52	1.68	54.47	1.99	65.19	2.62	40.71	1.72
2	2	45.32	0.87	55.16	1.23	70.36	3.63	40.31	1.63
2	3	44.08	2.50	54.35	2.29	68.92	4.64	40.86	1.28
3	1	45.93	1.53	63.47	2.04	62.15	2.79	42.37	1.90
3	2	43.78	3.55	54.97	2.73	68.66	2.99	41.34	1.40
3	3	44.54	1.78	55.85	2.57	66.65	2.25	39.51	1.64
mean		45.04	1.72	57.60	1.96	67.24	2.67	41.45	1.55
SD		0.95	0.78	3.00	0.49	2.56	0.89	1.15	0.24
gravimetric value[a]	assumes complete digestion	42.1	1.5	58.5	2.0	66.1	2.2	40.9	1.4

[a] The gravimetric value is the amount of each dNMP present in the sample solution, assuming the total conversion of the oligonucleotide to its constituent dNMPs

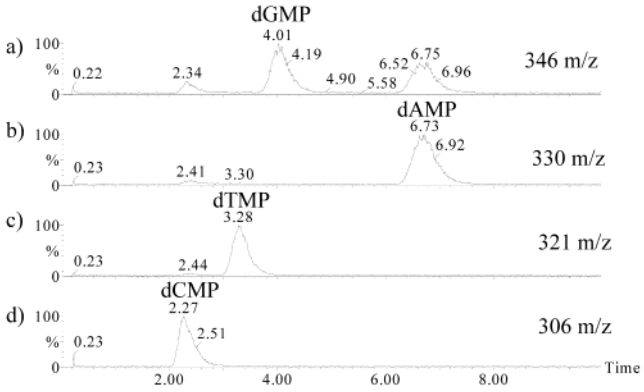

Figure 6. Selected ion chromatograms for a mixed standard of the four deoxynucleotides (a) dGMP, (b) dAMP, (c) dTMP, and (d) dCMP.

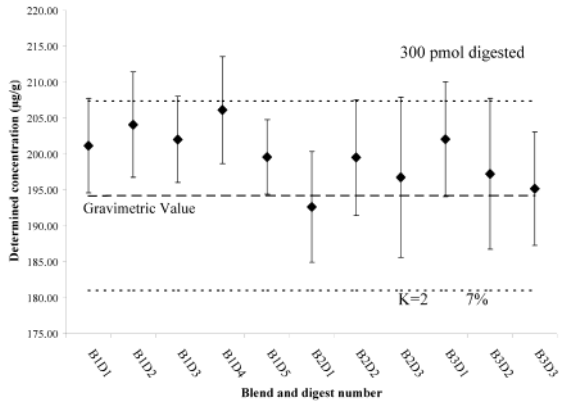

Figure 7. Determined oligonucleotide concentrations (μg/g) for the repeat subsamples and digests showing their expanded uncertainties ($K = 2$) for each individual measuremen, as compared to the gravimetrically assigned value and uncertainty.

dNMPs should be 1:1 with their labeled analogue and should match the proportions found in the spiked "unknown" digested oligonucleotide and calibration standard. However, the mixed nature of the available labeled material restricted the ideal matching to one dNMP, which was chosen to be dAMP. This has little or no bearing on the principle being demonstrated in this instance.

Quantitative Results. (a) Results as Individual dNMPs. The enzymatic digestion of the oligonucleotide involved the hydrolysis of each monomer unit to form the dNMPs. Therefore, to calculate the total concentration of oligonucleotide in solution, it was necessary to correct for this fact. The concentration of each individual dNMP was corrected to give the original concentration of the residue monomers, and these were summed to give the total concentration of oligonucleotide in the original solution.

The determined concentration of each dNMP in multiple digests of three subsamples of the oligonucleotide are shown in Table 3. The reported results were obtained from five repeat injections of each digested sample and calibration blends. An uncertainty estimate was calculated for each measurement by the method of uncertainty propagation described in the GUM guide.[22]

(22) *Guide to the Expression of Uncertainty in Measurement*; ISO: Geneva, Switzerland, 1995.

All of the determined concentrations agree within their calculated uncertainty with the theoretically calculated value for each dNMP. The error between the theoretical and mean measured value varied from 1.5% for dCMP, dGMP and dTMP to 6% for dAMP. The overall precision of the method varied from 2 to 5% RSD. The lowest RSD was associated with dAMP. This was to be expected, because the observed ratios for dAMP were close to unity. In addition, dAMP elutes by itself, and so only two SIR channels were monitored during its measurement, resulting in a more precise ratio value.

(b) Results for "Reconstructed" Oligonucleotide. The results for each individual analysis for the determination of the oligonucleotide concentration are shown in Figure 7. This plot highlights the excellent digest repeatability and the overall reproducibility of the method, because the total spread of results was less than 2%. Also plotted on this graph is the gravimetrically prepared concentration of the oligonucleotide with its expanded uncertainty of 7% relative. The overall accuracy of the method, with a mean value of 199 μg/g, resulted in an apparent difference of less than 2.5% relative. The expanded uncertainty of the determined concentration is significantly smaller than this (4% relative). This highlights one of the major advantages of exploiting

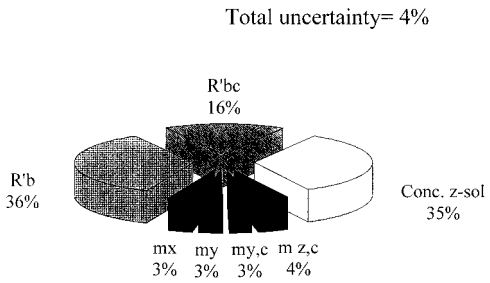

Figure 8. Pie chart representation of the uncertainty components associated with the IDMS quantitation of the dNMPs.

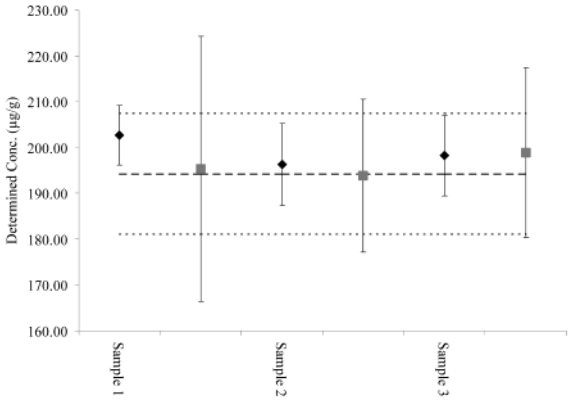

Figure 9. Comparison of isotope dilution quantitation using MS and tandem MS.

the enzymatic digestion of such a large molecule to its monomeric constituents and then applying high-accuracy IDMS measurements. The monomers can be readily obtained commercially at a purity that is superior to that of an oligonucleotide. An individual breakdown of the uncertainty components associated with the analysis of a dNMP is shown in Figure 8. This clearly shows that the largest uncertainty components are associated with the concentration of the dNMP standard solution and the measured ratio precision.

(c) Equivalent Data for Oligonucleotide Using Tandem MS. The above analysis was repeated using tandem MS detection. The combined results for the determined oligonucleotide concentration are shown in Figure 9. It was hoped that this would reduce the effects of any unknown interferences on the measured ratios. The results obtained by tandem MS analysis (195.9 μg/g) agree well with those obtained by SIR-MS and resulted in an observed difference of less than 1% from the gravimetrically assigned value. However, tandem MS analysis resulted in poorer ratio precisions and, hence, a larger uncertainty, which increased from 2.2% for LC/MS to 5.5% for LC/MS/MS.

CONCLUSIONS

A novel approach for the quantitation of an oligonucleotide using enzymatic digestion followed by LC/MS(/MS) was successfully applied for the quantification of an oligonucleotide solution to within 1%. (This was achieved by using IDMS to provide accurate quantitative measurements at the mass spectrometry stage.) However, the 1% difference quoted is well within the uncertainty associated with the gravimetrically prepared oligonucleotide solution and the purity of the dNMP standards. The digestion step on which the method hinged was shown to be reproducible, with MS data that was consistent with a successful total digestion. Interruption of the digestion stage can provide sequence data if required.

It should be noted that the digestion of the oligonucleotide was performed on a small scale, with the entire digest solution being 30 μL. This required the concentration of the original oligonucleotide solution to be rather high. However, because only 10 μL of this solution containing ~300 pmol of oligonucleotide was digested, the sensitivity of the method could be vastly improved by optimizing this step to enable the digestion of larger sample sizes.

This mass spectrometry approach, in the form of isotope dilution, offers one of the most definitive methods for obtaining high-accuracy data with small well-defined uncertainties for the individual nucleosides and, hence, the original intact oligonucleotide. As such, it will be ideally suited for characterizing primary standards and high-caliber reference materials that can be used to underpin other high throughput techniques based on UV and fluorescence detection.

Another major benefit associated with the method described is that it is applicable to any oligonucleotide sequence without the need for any standards that specifically match that same sequence. The use of IDMS for the mass spectral measurements has reduced the uncertainty in the results so that it is small when compared to the preparation of the oligonucleotide solutions themselves. The reliance on a method that relates the key measurement step to the individual dNMPS avoids the need for individually labeled analogues for each new oligonucleotide or DNA sequence. The overall approach thus offers, in principle, a sound basis for reliable and accurate quantitative measurements of DNA.

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