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A comparison of enzymatic digestion for the quantitation of an oligonucleotide by liquid chromatography–isotope dilution mass spectrometry

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Abstract

DNA is a universal analyte found in almost every organism. It is the code that dictates our genetic make-up and it provides a vast library of information. DNA sequences can indicate genetic modification of foodstuffs, how we may metabolise pharmaceuticals and the likelihood of suffering particular diseases. The basis for many of these genetic tests would benefit greatly from procedures that can accurately quantitate DNA in an absolute manner. This would then provide a sound and universally consistent foundation for regulatory and diagnostic decision making. This work compares two different enzymatic digestion systems as precursor steps to high accuracy isotope dilution mass spectrometry (IDMS) quantitation of a 20mer oligonucleotide. In the first approach, snake venom phosphodiesterase (SVP) digests the oligonucleotide to its constituent deoxynucleotides (dNMPs), followed by liquid chromatography–IDMS (LC–IDMS) quantitation. The second enzyme digestion approach used a combination of snake venom phosphodiesterase and shrimp alkaline phosphatase (SAP) which reduces the oligonucleotide to its constituent deoxynucleosides (dNs). This was then followed by an alternative LC separation and equivalent IDMS measurements. Total phosphorous content of the 20mer oligonucleotide was measured by inductively coupled plasma optical emission spectroscopy (ICP-OES). This provided independent data for comparison with the two enzyme digestion–IDMS based procedures. The most appropriate method of quantitation was found to be the combined SVP and SAP digestion. This approach negates the need to consider and/or account for the lack of a 5′ terminal phosphate residue. It also enables the use of positive ion mass spectrometry which simplifies the chromatographic requirements. Based on the exact matched IDMS of the adenine deoxynucleoside, the concentration of the original 20mer oligonucleotide was found to be $110 \pm 9\,\mu g\,g^{-1}$. This showed good agreement with the ICP-OES data based on the measurement of phosphorus which gave an equivalent value for the original 20mer oligonucleotide of $108 \pm 5\,\mu g\,g^{-1}$ (uncertainties at the 95% confidence interval). It is intended that this high accuracy methodology should be used to produce high calibre reference standards. These, in turn, could then be used to underpin the quality and consistency of routine measurements involving a variety of more commonly encountered methodologies. It should be noted that the IDMS procedures are equally applicable to both sequenced and non-sequenced oligonucleotide materials.

Keywords: Oligonucleotide; Quantitation; Liquid chromatography–isotope dilution mass spectrometry; Digestion; Enzyme

1. Introduction

The field of molecular biology has undergone vast expansion since the discovery of the polymerase chain reaction (PCR) [1] 19 years ago. The ability to amplify DNA has fostered a wealth of techniques and more recently, advances in the technology have enabled more sensitive, automated, reduced scale, high throughput analyses. Applications of DNA-based analytical experiments include the determination of genetically modified organisms in foodstuffs, calculation of viral load in clinical prognosis, scene of crime genotyping, adulteration and bacterial contamination of food. The consequences of these genetic tests im-

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pinge on people and on the environment. Naturally, there is currently a drive towards accurate, traceable methods that are suitable for the characterisation of certified reference materials and standards for use in this field. Such materials would then provide the basis for consistency between a wide range of users. Traditionally, the concentration of the DNA analyte has been determined by ultra-violet (UV) absorbance at a wavelength of 260 nm [2–5]. However, more recently DNA quantitation methods have involved real-time fluorescent amplification combined with standard curve interpolation [6,7]. Whilst both these approaches give a good indication of the concentration of DNA present, they do not take into account, extraction contaminants or matrix effects which may contribute to the result and presently, there is a lack of reliable, traceable reference standards for DNA measurement.

Liquid chromatography–isotope dilution mass spectrometry (LC–IDMS) [8] has already been demonstrated, in principle, to be an accurate, primary method of oligonucleotide quantitation [9,10]. This technique counters the lack of pure oligonucleotide standards, in suitable quantities, by using deoxynucleotides (dNMPs) as the calibrants. Deoxynucleotides are the products of phosphodiesterase I digestion of DNA and there are four different sub-units which differ in mass and base composition (adenine, guanine, cytidine, thymine). Oligonucleotides are a good model for DNA quantitation since they are short, synthetic single-stranded fragments. However, water, salts and the by-products of synthesis such as shortened or incorrectly synthesised oligonucleotides may contaminate them. In addition, as a consequence of the synthetic process, the 20mer oligonucleotide actually consists of 19 dNMPs and one deoxynucleoside where a 5′ terminal hydroxyl group replaces the phosphate. Therefore, the latter residue is undetected by the earlier method [9] and must be accounted for in the molar calculations.

Hence, we propose a different enzymatic digestion approach which counters the lack of a phosphate group on the 5′ terminal residue. The technique involves an initial phosphodiesterase I digestion of the oligonucleotide to produce dNMPs, followed by dNMP digestion by shrimp alkaline phosphatase (SAP) to produce deoxynucleosides (dNs). The labelled and natural dNMP standards obtained for the original SVP digestion method are also digested by the SAP and produce labelled and natural deoxynucleoside standards, respectively, to ensure accurate quantitation by IDMS.

In addition, inductively coupled plasma optical emission spectroscopy (ICP-OES) has been employed to determine the phosphorous content of the intact oligonucleotide so as to provide independent data for comparison with the LC–IDMS results. This technique provides an alternative to the digestion-based methods. The IDMS methods highlighted here are intended to be suitable for characterising reference standards that underpin other customary DNA analyses.

2. Experimental

2.1. Oligonucleotide digestion and quantitation by LC–IDMS

2.1.1. Preparation of the sample (natural intact oligonucleotide “unknown”)

The sequence of the 20mer oligonucleotide sample was 5′-CTTCCCGAGTGGGTGAGGAT-3′ (3A, 4C, 8G, 5T). Twelve individual preparations, each containing nominally 400 μg of desalted, lyophilised oligonucleotide were obtained from Sigma-Genosys (Cambridge, UK). Each sample was re-constituted with 750 μl of water (ELGA STAT; 18.2 MΩ) at room temperature and then pooled quantitatively into a single tube according to the previously published method [9]. The empty tubes were then freeze dried and difference weighings of these tubes before and after sample removal determined the mass of oligonucleotide pooled. Water was gravimetrically added to the oligonucleotide solution and the concentration calculated. Further dilution of this solution by the gravimetric addition of water was undertaken in order to obtain a nominal oligonucleotide concentration of 200 $\mu g\,g^{-1}$. Although the purity of an oligonucleotide at the 0.2 μmol synthesis scale is generally greater than 95%, in-house assessment by ESI/MS was performed. The most likely impurities, dNMPs and $n \pm 1$mers were not observed. However, it was impossible to accurately define the water content of the oligonucleotide due to the small amount of starting material. Therefore, it is particularly advantageous that the standards for this approach are dNMPs and the method is not reliant on ‘pure’ DNA standards.

An oligonucleotide purity of 95% with an associated uncertainty of 5% was assigned as published previously [9], to give a final oligonucleotide concentration that was nominally $200 \pm 5\,\mu g\,g^{-1}$. This mass fraction, however, makes no impurity allowance for moisture or inorganic salts. This solution was used as the “unknown” sample.

2.1.2. Preparation of the natural deoxynucleotides (dNMPs) standard

One gram quantities of 2′-deoxyadenosine 5′-monophosphate (dAMP), 2′-deoxycytidine 5′-monophosphate (dCMP), 2′-deoxyguanosine 5′-monophosphate (dGMP) and 2′-deoxythymidine 5′-monophosphate (dTMP) were obtained from Sigma-Aldrich Co. (Poole, Dorset, UK). A mixed standard of all four deoxynucleotides (dNMPs) was prepared gravimetrically in water as reported earlier [9]. The mixed stock standard contained $32.1 \pm 0.6\,\mu g\,g^{-1}$ of dAMP, $39.7 \pm 0.7\,\mu g\,g^{-1}$ of dCMP, $89.8 \pm 1.7\,\mu g\,g^{-1}$ of dGMP and $49.0 \pm 0.9\,\mu g\,g^{-1}$ of dTMP dissolved in water. These concentrations matched those of the dNMPs in the oligonucleotide solution assuming complete digestion of the oligonucleotide to its constituent dNMPs and assuming that the oligonucleotide concentration was $200\,\mu g\,g^{-1}$.

2.1.3. Preparation of the labelled deoxynucleotides (LdNMPs) internal standard

A mixed solution of isotopically labelled, ^{13}C and ^{15}N enriched, dNMPs was obtained from Silantes (München, Germany). The isotope purity of the LdNMPs was stated to be greater than 98%. Chemical purity was assessed using LC/UV/MS and the isotopic purity was tested by direct infusion ESI/MS [9]. No natural dNMPs were detected in the sample and only the four fully labelled dNMPs were detected.

A reverse IDMS experiment was performed as described previously [9] to accurately determine the concentration of each isotopically labelled dNMP in the new working solution. This solution was then gravimetrically diluted to yield a final concentration of 32.1 $\mu g\,g^{-1}$ of LdAMP.

2.1.4. Preparation of sample and calibration blends

Sample and calibration blends were prepared gravimetrically. For the sample blend, typically 1 g of sample was gravimetrically transferred to a 5 ml screw cap polypropylene sample tube. An appropriate amount of internal standard was added to give a molar ratio of 1:1 between dAMP and the labelled dAMP. A calibration blend was prepared in an identical fashion using exactly the same amount of internal standard and a pre-calculated amount of calibration solution; the isotopic ratio of natural dAMP to labelled dAMP in both the sample and calibration blends should be close to unity. (This approach is an iterative process and a preliminary analysis of the sample may be required in order to determine the approximate amount of dAMP in the digested sample [8].)

Five different sample blends were formulated. Each sample blend was individually digested twice and five repeat injections were performed.

2.1.5. Preparation of digestion reagents

2.1.5.1. Oligonucleotide digestion by snake venom phosphodiesterase I (SVP). The components of the digestion media were 10 μl of 25 mM magnesium chloride (Roche Applied Science, East Sussex, UK), 9 μl of 10 mM ammonium acetate (Sigma-Aldrich Co., Dorset, UK) and 1 μl of a 1 $mg\,ml^{-1}$ dilution of phosphodiesterase I (isolated from *Crotalus adamanteus* venom; 36.2 $U\,mg^{-1}$; Amersham Biosciences, Buckinghamshire, UK). In addition, 10 μl of the sample blend or calibration blend was added and the mixture was incubated for 20 min as previously detailed [9].

Three types of reaction blank were prepared by replacing the sample, enzyme and both the sample and enzyme with water, respectively.

The digest mixtures were made up to 200 μl using water (18 MΩ) prior to LC/MS analysis.

2.1.5.2. Oligonucleotide digestion by snake venom phosphodiesterase I (SVP) and shrimp alkaline phosphatase (SAP). The initial phosphodiesterase I digestion protocol differed from that outlined above, being based on a modification of the method published by Friso et al. [10] to account for single-stranded oligonucleotides rather than genomic DNA. To 10 μl of a sample or calibration blend, 4.0 μl of 1.0 M ammonium hydrogen carbonate (Fisher Scientific UK Ltd., Leics, UK) and 4 μl of phosphodiesterase I (1 $mg\,ml^{-1}$; as above) were added. The mixture was then incubated for 30 min at 37 °C in a Geneamp PCR 2400 thermocycler (Applied Biosystems, CA, USA). Following removal from the thermocycler, 0.75 μl of shrimp alkaline phosphatase (isolated from *Pandalus borealis*, 1.0 $U\,\mu l^{-1}$; Amersham Biosciences, Buckinghamshire, UK) was added together with 4.0 μl of 10× shrimp alkaline digestion buffer (Amersham Biosciences, Buckinghamshire, UK) to the tube. Further incubation at 37 °C in the thermocycler was undertaken for 1 h prior to the tubes being heated to 65 °C for 15 min in order to denature the enzymes.

Blank digests were also prepared in an analogous manner to that described in the section above. The digest mixtures were made up to 200 μl using water (18 MΩ) prior to LC/MS analysis.

2.1.6. High performance liquid chromatography (HPLC)

The HPLC system employed for the separation of the deoxynucleotides (dNMPs) consisted of an Alliance 2690 (Waters, Manchester, UK) separations module, an XTerra® (Waters, Manchester, UK) analytical column (150 mm × 2.1 mm, 3.5 μm particle diameter, 125 Å pore size) and a mobile phase of 10 mM ammonium acetate buffered to pH 8.5 with ammonium hydroxide solution (specific gravity, 0.88; BDH, Dorset, UK). The flow rate was 0.2 $ml\,min^{-1}$.

The deoxynucleosides (dNs) were separated using a Synergi™ Polar RP (250 mm × 2 mm, 4 μm particle size, 80 Å pore size) analytical column (Phenomenex, Cheshire, UK). The mobile phase consisted of 10 mM ammonium acetate (Sigma-Aldrich Co., as before) buffered to pH 7.4 with ammonium hydroxide solution (specific gravity, 0.88; BDH, Dorset, UK) and was pumped at a flow rate of 0.2 $ml\,min^{-1}$. The injection volume was 10 μl for the SVP digestion method and 20 μl for the combined SVP and SAP digestion method.

2.1.7. Mass spectrometry

A Quattro Ultima quadrupole tandem mass spectrometer (Waters, Manchester, UK) was employed for the IDMS quantitation of the oligonucleotide. It was operated at unit mass resolution (full-width half-maximum) for both digestion methods.

2.1.7.1. Analysis of deoxynucleotides (dNMPs). Following chromatographic separation, the natural and labelled dNMPs entered the mass spectrometer via an electrospray probe operated in negative ion mode. They were detected by a selective ion monitoring (SIM) mode as detailed previously [9] except that the desolvation temperature was 400 °C and the dwell time was extended to 50 ms. The ions monitored corresponded to the deprotonated molecule ions observed at *m/z*: 306 $[dCMP - H]^-$, 318 $[LdCMP - H]^-$, 321 $[dTMP - H]^-$, 333 $[LdTMP - H]^-$, 330

Table 1
Mass spectrometer conditions for nucleoside detection by selected reaction monitoring (SRM)

Electrospray (+ve ion mode)	
Spray voltage (kV)	2.5
Cone voltage (V)	14.0
Source gas ($l\,h^{-1}$)	
Desolvation gas	650
Cone gas	100
Source temperature (°C)	
Source block temperature	120
Desolvation temperature	400

$[dAMP - H]^-$, 345 $[LdAMP - H]^-$, 346 $[dGMP - H]^-$ and 361 $[LdGMP - H]^-$.

2.1.7.2. Analysis of deoxynucleosides (dNs). The deoxynucleosides were transferred from the LC system to the mass spectrometer via an electrospray probe operated in positive ion mode. Tandem MS selected reaction monitoring (SRM) analysis of the deoxynucleosides was achieved using positive ion electrospray. A collision energy of 14 eV was used and the collision gas was argon. The precursor ion ($[M + H]^+$) to product ion ($[M + H - C_5H_8O_3]^+$) transitions of the protonated molecule ion for each natural and labelled dN were monitored in a time dependent mode (dwell time of 100 ms). The mass spectrometer operating conditions are outlined in Table 1.

Although deoxyguanosine and deoxythymine co-elute, they have sufficiently different masses to be resolved by mass spectrometry without cross-talk.

The precursor to product transitions monitored were 228 > 112 $[dC + H]^+$, 240 > 119 $[LdC + H]^+$, 243 > 127 $[dT + H]^+$, 255 > 134 $[LdT + H]^+$, 252 > 136 $[dA + H]^+$, 267 > 146 $[LdA + H]^+$, 268 > 152 $[dG + H]^+$, 283 > 162 $[LdG + H]^+$.

2.2. Inductively coupled plasma–optical emission spectroscopy (ICP-OES)

2.2.1. Preparation of ICP-OES samples and standards

The 20mer oligonucleotide sample was prepared as outlined above but the initial stock solution ($903\,\mu g\,g^{-1}$) was gravimetrically diluted to $85.6\,\mu g\,g^{-1}$ and a 'blank' sample of water (ELGA STAT; 18 MΩ) was also provided. Selenium was used as the internal standard. Both the selenium and phosphorous standards were obtained as $1000\,\mu g\,g^{-1}$ certified solutions (Spex Certprep, Metuchen, NJ, USA). An internal standard of selenium was mixed with the oligonucleotide sample and with a known amount of a phosphorous standard, respectively. The solutions were gravimetrically diluted to the required concentration. The use of selenium as the internal standard coupled with a bracketing calibration procedure was in line with earlier work that used ICP-OES for high accuracy analysis [11–13].

Table 2
ICP-OES conditions for phosphorous concentration determination

Gas ($l\,min^{-1}$)	
Plasma gas	15.0
Auxiliary gas	0.5
Nebuliser gas, argon	0.8
Pump	
Speed ($ml\,min^{-1}$)	1.0
Wavelength (nm)	
Phosphorous	213.618
Selenium	196.026
Time (s)	
Integration time	0.1
Read time	15.0
RF power (W)	1300

2.2.2. Analysis of the phosphorous content of the oligonucleotide by ICP-OES

A PerkinElmer Optima 3300 RL (radial view) with manually set read times was employed. The operating conditions for which are shown in Table 2. Calibration was bracketing with a single matched standard. The amount of phosphorous in the oligonucleotide sample was determined by ratio measurements. From this value the concentration of oligonucleotide could be calculated as the sequence for the 20mer was known. In addition, a sample of the gravimetrically determined mixed standard of dNMPs was also quantified by ICP-OES.

3. Results and discussion

3.1. Oligonucleotide digestion and atomisation

Accurate quantitation by IDMS is reliant upon the complete digestion of the oligonucleotide down to its constituent dNMPs or dNs (Fig. 1). In order to establish that both enzymatic methods achieved complete digestion, aliquots of the reaction mixture were removed at various time intervals during the course of the digestion method development. These solutions were kept on ice and then analysed by mass spectrometry as discussed previously [9]. Digestion was assumed to be complete for both digestion methods as no intact 20mer or large oligonucleotide fragments could be detected at any point. ICP-OES determination of the phosphorous content of the oligonucleotide was undertaken to provide supporting data for the IDMS digestion methods. Good agreement between the LC–IDMS quantitation and that based on the ICP-OES phosphorous data would be indicative of all the phosphorous being present as dNMPs.

3.2. LC–MS of dNMPs and dNs

3.2.1. Deoxynucleotides (dNMPs)

Separation of the deoxynucleotides was initially carried out using an Alliance 2690 Separations Module (Waters,

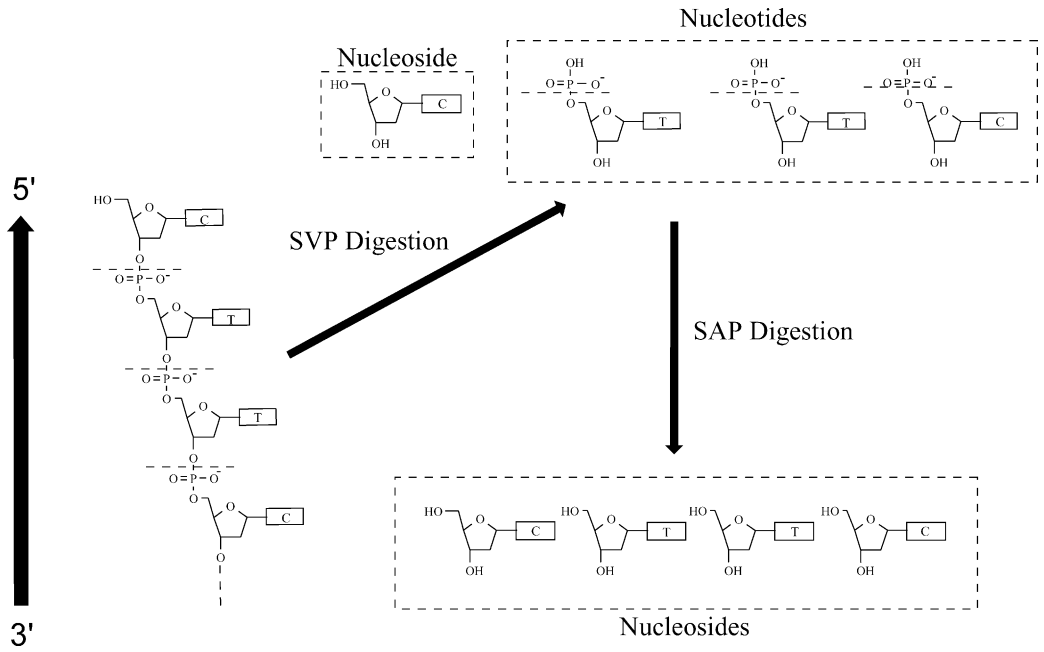

Fig. 1. Enzymatic digestion of the oligonucleotide. Snake venom phosphodiesterase digestion of the oligonucleotide produces nucleotides and one nucleoside from the 5′ terminal residue, which is not phosphorylated. Shrimp alkaline phosphatase digestion removes the phosphate group from the nucleotides. (The rectangles represent the bases [C: cytidine and T: thymine].)

Manchester, UK), an Aqua® (2 mm × 150 mm), 3 μm particle size, 125 Å pore size analytical column (Phenomenex, Cheshire, UK) and a mobile phase consisting of 10 mM ammonium acetate pH 7.5. However, an interference peak at 201 *m/z* was observed that co-eluted with dCMP (chromatogram not shown). The interference peak was found to be magnesium tetra-acetate clusters, formed from the magnesium chloride in the digestion mixture and the ammonium acetate from the mobile phase. The identity of this peak was confirmed by tandem MS which generated characteristic product ions at *m/z* 156 and 59, that corresponded to magnesium tri-acetate clusters and magnesium chloride, respectively. Removing or reducing the magnesium chloride from the digestion mix resulted in partial oligonucleotide digestion. Thus, an alternative mobile phase of 10 mM ammonium hydrogen carbonate pH 7.5 was investigated and this resulted in sharp, well separated peaks which correspond to each of the deoxynucleotides (Fig. 2). However, with this system there was a marked decrease in sensitivity after about 20 injections, presumably because of the contamination of the electrospray source with an involatile carbonate salt.

In light of this, we returned to the HPLC system previously reported [9] and although the magnesium tetra-acetate interference peak was present in this system it was separated from the ions of interest (chromatogram not presented). Using these conditions, separation of the deoxynucleotides was achieved but the signal to noise of each dNMP and LdNMP peak was poor due to competing matrix reactions by acetate ions present in the mobile phase. Unfortunately, it was not possible to reduce the ammonium acetate concentration further. A concentration of 10 mM ammonium acetate was required to maintain superior peak shape, separation and retention of the dNMPs whilst retaining adequate sensitivity, not afforded at higher concentrations, needed for accurate quantitation. Therefore, this constitutes a compromise between the demands of the separation and mass spectrometric detection.

3.2.2. Deoxynucleosides (dNs)

In order to overcome this, an additional digestion method was investigated, which removes the phosphate from the nucleotide leaving a species that readily accepts a positive charge. The signal suppression associated with the negative ion electrospray of dNMPs is effectively removed by this alternative method. Indeed, the signal to noise ratio is further enhanced by using MS/MS. Although it has been shown that SRM ratio measurements are less precise for dNMPs than SIM [9], this was not the case for the dNs due to the improvement of signal to noise levels and the lack of suppression from the mobile phase.

In addition, the alternative digestion method, adapted from Friso et al. [10] did not require magnesium chloride in the digestion mix, and therefore, potential magnesium cluster interference compounds were eliminated. However, complete chromatographic resolution of all four deoxynucleosides was not achieved. Whilst deoxyadenosine and deoxycytosine were fully resolved, both deoxyguanosine and deoxythymine eluted together (Fig. 3) but could be differentiated by their different precursor to product transitions.

Tables 3 and 4 show that the uncertainty measurements are comparable for the nucleotide and corresponding nucleoside measurements.

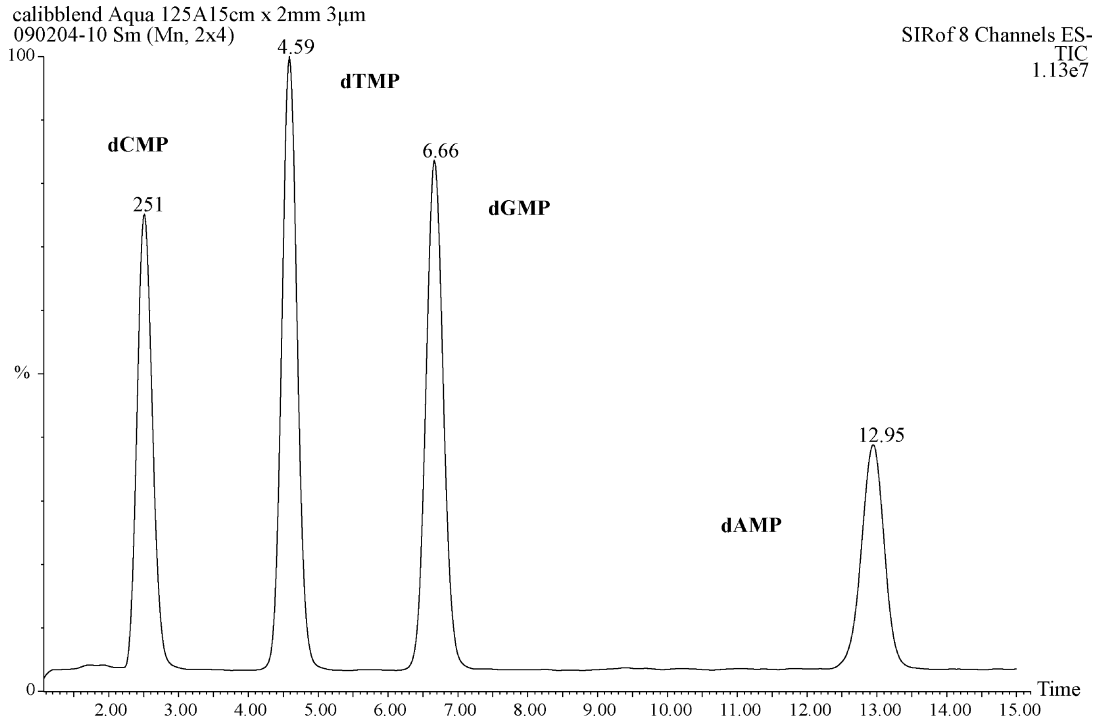

Fig. 2. Chromatographic separation of the deoxynucleotides (dNMPs) using the Aqua® 125 Å column (Phenomenex, UK) and a mobile phase of 10 mM ammonium hydrogen carbonate, pH 7.5. The retention time profile of all four deoxynucleotides (dNMPs).

3.3. Oligonucleotide quantitative results

The peak areas of the deoxynucleosides or deoxynucleotides and their labelled analogues were calculated using MassLynx (Waters; MA, USA) software. The concentration of each deoxynucleoside (dN) or deoxynucleotide (dNMP), derived from the digestion of the oligonucleotide, was determined using the double IDMS equation (Eq. (1)). An uncertainty budget was determined for each measurement undertaken using ISO guideline methodology [14] (Eq. (2)).

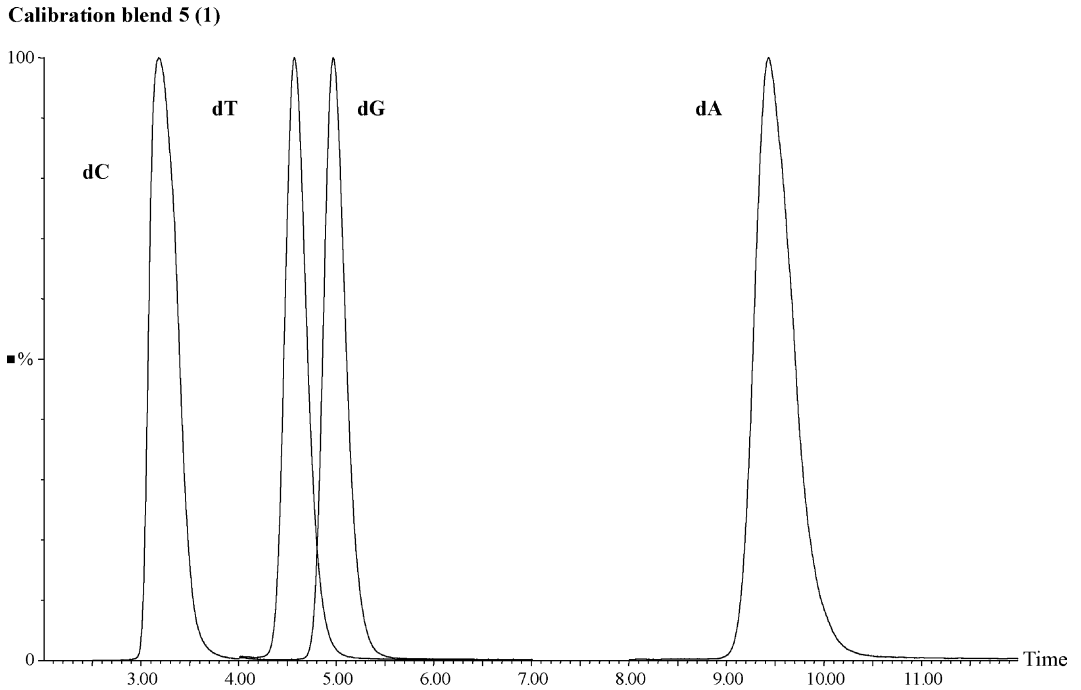

Fig. 3. Chromatographic separation of the deoxynucleosides (dNs) using the Synergi™ Polar RP analytical column.

Table 3
Calculated mass fractions ($\mu g\,g^{-1}$) of the oligonucleotide derived from the concentration of the four nucleotides (U is the expanded ($k = 2$) uncertainty of the measurement representing the 95% confidence interval)

Blend and digest	dAMP ($\mu g\,g^{-1}$)	U ($\mu g\,g^{-1}$)	dCMP ($\mu g\,g^{-1}$)	U ($\mu g\,g^{-1}$)	dGMP ($\mu g\,g^{-1}$)	U ($\mu g\,g^{-1}$)	dTMP ($\mu g\,g^{-1}$)	U ($\mu g\,g^{-1}$)
Blend 1, digest 1	98	10	93	7	110	8	90	9
Blend 1, digest 2	98	7	92	7	109	8	90	8
Blend 2, digest 1	98	8	94	11	112	8	89	7
Blend 2, digest 2	98	8	96	6	112	8	93	7
Blend 3, digest 1	97	8	94	7	111	9	88	8
Blend 3, digest 2	99	8	96	7	111	8	91	7
Blend 4, digest 1	100	7	93	8	110	8	87	7
Blend 4, digest 2	97	7	96	10	112	7	89	6
Blend 5, digest 1	98	10	99	7	113	10	87	7
Blend 5, digest 2	100	11	95	8	114	11	88	8
Mean	98	8	95	8	112	9	89	7
%R.S.D.	1.0	–	2.2	–	1.3	–	2.1	–

Optimum IDMS measurements are often conducted with the aim of having a 1:1 molar ratio between labelled and unlabelled analytes. The use of a mixed labelled standard meant that it was only possible to match one of the four analytes, which was chosen for the same reasons as those previously discussed [9].

Assuming oligonucleotide digestion was complete in both enzyme methods, then the concentration of each of the deoxynucleosides should be equal to its deoxynucleotide counterpart. In addition, following ICP-OES, the molar concentration of phosphorous atoms from the oligonucleotide break down should be equal to the molar concentration of the summed dNs or summed dNMPs.

$$W_x = W_z \cdot \frac{m_z}{m_{yc}} \cdot \frac{m_y}{m_x} \cdot \frac{R'_B}{R'_{BC}} \quad (1)$$

where W_x is the mass fraction of dNMPs in sample; W_z, the mass fraction of the natural dNMPs solution used to prepare the calibration blend; m_z, mass of the natural dNMPs standard added to the calibration blend; m_x, mass of the sample used; m_{yc}, mass of the labelled dNMPs standard added to the calibration blend; m_y, mass of the labelled dNMPs standard added to the sample blend; R'_B, measured ratio (natural/labelled) of the sample blend; R'_{BC}, average measured ratio (natural/labelled) of the calibration blend injected before and after the sample.

$$u = W_x \sqrt{\left(\frac{u_{W_z}}{W_z}\right)^2 + \left(\frac{um_x}{m_x}\right)^2 + \left(\frac{um_y}{m_y}\right)^2 + \left(\frac{um_z}{m_z}\right)^2 + \left(\frac{um_{yc}}{m_{yc}}\right)^2 + \left(\frac{uD}{D}\right)^2 + \left(\frac{uR'_B}{R'_B}\right)^2 + \left(\frac{uR'_{BC}}{R'_{BC}}\right)^2} \quad (2)$$

The uncertainty, u, associated with the final measured concentration of each analysis, was calculated by combining the relative standard uncertainties of the measured ratios with the uncertainties associated with the weighings, concentration of the natural standard solution and the uncertainty associated with the extent of digestion (D).

Fig. 4 illustrates the determined oligonucleotide mass fraction ($\mu g\,g^{-1}$) for each deoxyadenosine mono-phosphate analysis and for its corresponding deoxyadenosine analysis. The graph demonstrates that both approaches to enzymatic oligonucleotide digestion showed excellent repeatability and reproducibility. However, there is a negative bias in the mass fraction values derived from the single snake venom phosphodiesterase digestion (SVP) compared to the data for the combined SVP and SAP digestions. It was thought initially that this trend was not likely to be caused by incomplete or non-specific digestion since no small molecular weight fragments, or large oligomers, were detected by mass spectrometry during the development of the digestion processes, as mentioned previously. However, it is feasible that some low molecular weight fragments were present at low concentrations and these could not be detected.

Tables 3 and 4 feature the determined oligonucleotide mass fractions derived from the concentration of the four nucleotides and four nucleosides, respectively. The final values are derived from the amounts of each individual base unit. These individual base amounts have been multiplied up based on the known sequence of this particular 20mer. Inspection of the data in Table 3 shows that the mean concentration of the oligonucleotide, according to the SVP digestion approach, is 99 $\mu g\,g^{-1}$ with a standard deviation of 10 $\mu g\,g^{-1}$. This value agrees well with the calculated oligo mass fraction determined by summation of the individual dNMP mass fractions (97.5 $\mu g\,g^{-1}$) and requires no prior knowledge of the size or sequence of the "unknown" oligonucleotide, as illustrated in Table 5. It is also apparent in Table 3 that the oligonucleotide mass fraction derived from dGMP is far higher than that of the other dNMP derived values. The reason for this has not been resolved.

Table 4 shows that there is a good agreement between the oligonucleotide mass fractions determined by each of the nucleoside values and that the mean value was 115 $\mu g\,g^{-1}$ with a standard deviation of 3 $\mu g\,g^{-1}$. Again this agrees well with the calculated value of 113.2 $\mu g\,g^{-1}$ (Table 5) derived from the summation of the individual nucleoside mass fractions.

Table 4
Calculated mass fractions ($\mu g\,g^{-1}$) of the oligonucleotide derived from the concentration of the four nucleosides (U is the expanded ($k = 2$) uncertainty of the measurement representing the 95% confidence interval)

Blend and digest	dA ($\mu g\,g^{-1}$)	U ($\mu g\,g^{-1}$)	dC ($\mu g\,g^{-1}$)	U ($\mu g\,g^{-1}$)	dG ($\mu g\,g^{-1}$)	U ($\mu g\,g^{-1}$)	dT ($\mu g\,g^{-1}$)	U ($\mu g\,g^{-1}$)
Blend 1, digest 1	109	9	115	10	114	10	117	10
Blend 1, digest 2	111	10	117	11	116	10	117	11
Blend 2, digest 1	110	9	117	11	114	10	117	10
Blend 2, digest 2	111	9	118	11	115	11	118	10
Blend 3, digest 1	107	9	114	10	110	9	116	10
Blend 3, digest 2	109	9	115	11	114	10	118	10
Blend 4, digest 1	109	9	115	10	112	10	116	11
Blend 4, digest 2	113	9	120	11	117	10	119	11
Blend 5, digest 1	109	9	116	10	113	10	117	10
Blend 5, digest 2	109	9	117	11	114	11	117	11
Mean	110	9	117	11	114	10	117	10
%R.S.D.	1.5	–	1.6	–	1.9	–	0.9	–

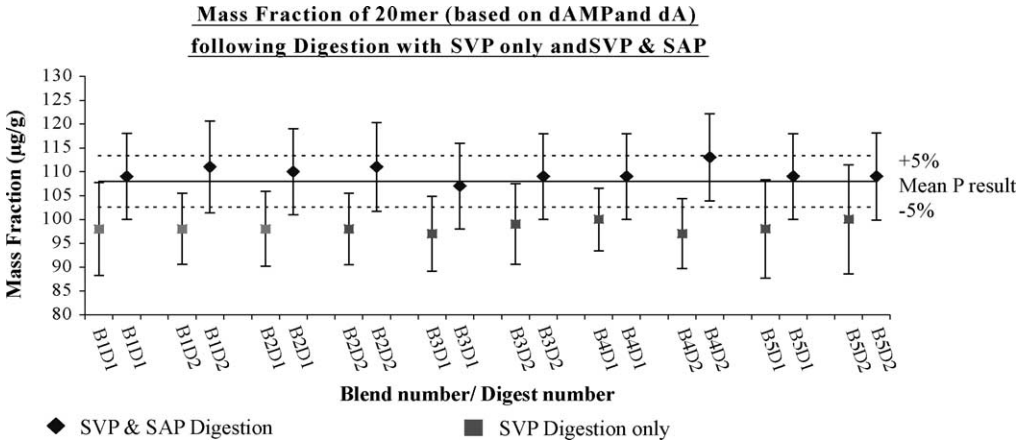

Fig. 4. Comparison of IDMS quantitation using enzymatic digestion with ICP-OES (solid horizontal line). ICP-OES uncertainty represented by the dotted horizontal line. The error bars represent the expanded ($k = 2$) individual measurement uncertainties.

Table 5
Determination of oligonucleotide mass fraction from both IDMS digestion approaches, assuming no prior knowledge of the oligonucleotide sequence or length

Base	SVP digestion		SVP and SAP digestion	
	Mass fraction of dNMP ($\mu g\,g^{-1}$)	Mass fraction of dNMP in the oligo ($\mu g\,g^{-1}$)	Mass fraction of dN ($\mu g\,g^{-1}$)	Mass fraction of dN in the oligo ($\mu g\,g^{-1}$)
A	15.8	14.9	13.4	16.7
C	14.1	13.3	17.1	21.8
G	50.0	47.4	39.3	45.9
T	23.2	21.9	22.9	28.8

Sum of the mass fractions of the dNMPs in the oligo, 97.5 $\mu g\,g^{-1}$, sum of the mass fractions of the dNs in the oligo, 113.2 $\mu g\,g^{-1}$.

Both digestion methods are repeatable with relative standard deviations ranging from 0.9 to 2.2%. The relative standard uncertainty on the oligonucleotide concentrations is comparable between the different methods. Additionally, in this instance, the oligonucleotide is known to contain three adenines, four cytosine, eight guanines and five thymines. Table 6 shows that there is a closer agreement of the dN ratios than the dNMP ratios when compared to the theoretical base ratio values. This suggests that the SVP and SAP digestion followed by IDMS quantitation is a

Table 6
The theoretical base ratio of the oligonucleotide compared to the base ratio determined by the two IDMS digestion approaches

Base	Theoretical ratio	dNMP	dN
A	1.0	1.1	1.0
C	1.3	1.0	1.3
G	2.7	3.5	2.9
T	1.7	1.6	1.7

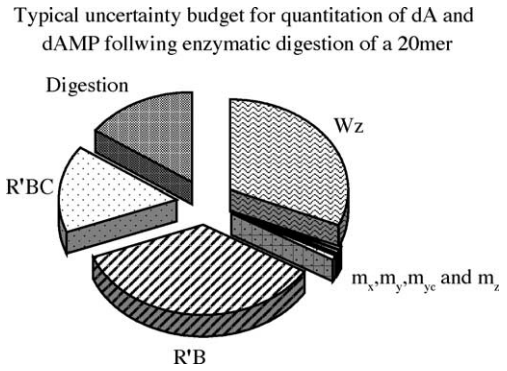

Fig. 5. A pie chart illustrating the sources of uncertainty for the IDMS quantitation of deoxyadenosine and deoxyadenosine mono-phosphate residues from the 20mer oligonucleotide. (See Eq. (1) for definitions of the factors.)

more reliable method for small oligonucleotides of unknown sequence.

The sources of uncertainty and their relative proportions are shown in Fig. 5 for dA and dAMP. Whilst the pie chart illustrates the uncertainty on the dA and dAMP measurements, the proportions are similar for every dN and dNMP in that the largest components are from the blend ratio measurements, the uncertainty associated with total digestion and the calibration blend concentration, which is a direct result of the purity of the starting material. The relative proportion of the ratio measurements can vary dependent on instrument performance.

The ICP-OES approach successfully confirmed the mass fraction of the initial calibration material. The oligonucleotide quantitation results from the ICP-OES experiments were found to closely agree with the combined digestion approach data. This provides further independent evidence to support the value of the LC–IDMS approach. However, whilst both the IDMS and the ICP-OES methods produced similar quantitative results overall, none of the methods concur with the gravimetrically determined concentration of 200 $\mu g\,g^{-1}$ of oligonucleotide. The large disparity is probably due to significant quantities of water in the oligonucleotide preparations. This in turn highlights the value of the above approach to provide reliable data for primary standard materials in the field of DNA studies.

4. Conclusions

Our previous paper described a method of oligonucleotide quantitation by LC–IDMS [9] and proved the value of such an approach but it was hindered by the possible bias in quantitation that results from the lack of a terminal phosphate group on the oligonucleotide. This paper introduced an alternative, extended digestion, with different chromatography, different mass spectrometric monitoring and is unaffected by the presence of terminal phosphate groups. This improved methodology was compared with the original published method [9]. Unlike before, the measurements in this study were compared to an independent ICP-OES technique, further supporting the complete digestion assumption, as well as to a gravimetric value. The agreement with the gravimetric value in this case was poor, and therefore, further highlights the requirement for well-characterised oligonucleotide standards for accurate quantitation of oligonucleotides and ultimately DNA.

The purpose of this research was to compare different approaches for oligonucleotide quantitation. The digestion of an oligonucleotide to its component deoxynucleosides marks a significant step in the development of an accurate, primary IDMS quantitation method for oligonucleotides.

A major benefit of the combined digestion technique (SVP and SAP) is that the mass spectrometer can be operated in positive ion mode to monitor the nucleosides without competition from acetate ions for charge and without reduction in signal intensity.

Basing the final measurements on the measurement of nucleosides also means that terminal groups that lack a phosphate moiety will also be included.

All the quantitative approaches are advantageous, as they preclude the need for a sequence specific standard. Additionally, it has been demonstrated that knowledge of the oligonucleotide sequence and length is not necessary to determine the amount of DNA in the sample.

The combined data from the LC–MS and ICP-OES has shown the value of the IDMS approach for characterising DNA standard materials. In this case the 20mer oligonucleotide as supplied had a nominal mass fraction of 200 $\mu g\,g^{-1}$. The improved IDMS methodology, supported by ICP-OES data gives an assigned value of 113 $\mu g\,g^{-1}$. For critical DNA quantitative studies it is vital that such differences are rationalised.

The IDMS techniques are recommended as a means of producing reference standards to underpin other DNA measurement techniques and to anchor DNA analyses in the laboratory.

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