Comparison of Accuracy on DNA Quantitation Determined by MALDI-TOF Mass Spectrometry and UV Spectrometry

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Abstract:

Although the UV absorbance of DNA at 260 nm has been recognized as a standard method for DNA quantitation, there are limitations of using UV spectrometry to determine the purity and identity of DNA. Recently, MALDI-TOF MS has proven to be an accurate technique for qualitative DNA analysis. In this study, the accuracy of MALDI-TOF MS for determining the concentration of DNA is evaluated and compared with that of the standard UV method. The results indicated that the accuracy of quantitative MALDI-TOF MS was comparable to that of the standard UV method and that measured DNA concentrations correlated well with those determined by the standard UV method.

Article:

INTRODUCTION

Deoxyribonucleic acid (DNA) was first observed by a German biochemist named Frederich Miescher in the late 1800s.[1] The next major breakthrough in DNA research occurred when the anti-parallel helical structure of DNA was determined by James Watson and Francis Crick in 1953.[2] However, genomics research was not really feasible until the polymerase chain reaction was developed in 1984.[3] Since then, through many large-scale and individual studies, the knowledge of the structures and functions of different genomes have significantly increased.[4-7] Together with the results from many clinical studies, genomics science is expected to play a major role in the foreseeable future of medicine.[8,9] Among the various methods that have been used to analyze the genomes, the most commonly used reagent has been DNA oligonucleotides (oligos). This is because, through Watson–Crick base pairing, two complementary DNA fragments can bind to each other with relatively high specificity and

affinity. Based on this principle, DNA oligos can be used either as primers or probes for the detection of specific DNA targets. In general, there are two different ways to prepare DNA materials. In the first case, by using specific methods, cellular DNA can be isolated from living organisms.[10] In the second case, DNA can be synthesized by various chemical methods.[11]Before using the DNA materials for any specific analytical work or study, it is important to characterize the DNA materials.

Among the analytical techniques that have been used to measure DNA, UV absorption is the most widely used method.[12] For the UV spectrometry of DNA, the principle relies on the fact that the energy differences between two different molecular orbitals of all four natural nucleobases in DNA fall within the UV spectrum, with the highest absorption occurring at 260 nm. According to the Beer-Lambert Law, at low concentration of analyte (≤0.01 M), the UV absorbance is linearly proportional to the concentration of analyte. For the determination of DNA purity, a common practice in molecular biology is to measure the UV absorbance of a DNA sample at 260 nm and 280 nm. The absorbance reading at 280 nm can provide a rough estimate on the amount of protein in the sample. Thus, a DNA sample is normally considered to be pure if the ratio of its absorbance at 260 nm to that at 280 nm is close to 2.0.[13]

The development of soft ionization techniques, namely electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI), have revolutionized the way in which large biomolecules including DNA are measured by mass spectrometry (MS).[14] In the case of the UV MALDI mass spectrometry, UV laser is used to carry out the desorption/ionization of the analyte, which has been co-crystallized with an excess amount of MALDI matrix. The MALDI matrix is usually small organic molecules with a high absorptivity for the laser energy. Thus, the analyte in the MALDI sample is protected from the laser irradiation. By coupling the MALDI ion source to a time-of-flight (TOF) mass analyzer, $>10^3$ mass resolution can be routinely achieved. For MALDI-TOF mass spectrometry of DNA, it has been successfully applied to DNA sequencing, gene expression analysis, genotyping (single nucleotide polymorphisms and microsatellites), detection of DNA modifications (methylation, oxidation, and adduction), and ligand binding.[15-18]Although MALDI-TOF MS has been used for the quantitation of DNA, there was no direct comparison of the accuracy of quantitative MALDI-TOF MS to that of the standard UV spectroscopic method.[19-23]

MATERIALS AND METHODS

Preparation of DNA Solutions

HPLC purified DNA oligos were ordered from Integrated DNA Technologies (Coralville, IA). A 17 mer DNA oligo (5′-CCA TCC ACT ACA ACT AC-3′) was used as either a calibrant or a control analyte. For MALDI-TOF MS measurements, an additional 18 mer DNA oligo (5′-CCA TCC ACT ACA ACT ACA-3′) was used as an internal standard. Both oligos were reconstituted to 250 μM with deionized autoclaved water and stored at −20 °C. All subsequent dilutions of DNA oligos were carried out with deionized autoclaved water.

For the calibration of UV absorbance and MALDI-TOF MS measurements, the dilution of calibrant was carried out separately. In the case of MALDI-TOF MS calibration, a fixed amount of internal standard was added into each dilution of calibrant. The final concentration of internal standard in the mixture of DNA oligo was 2.00 µM.

For the determination of DNA concentrations that have been randomly selected (Table 1), the control analyte was used. Each sample was individually prepared from the stock solution. In the case of MALDI-TOF MS measurements, each sample was further diluted two-fold, and the same amount of internal standard (2.00 µM) was subsequently added to each sample.

TABLE 1 HAS BEEN OMITTED FROM THIS FORMATTED DOCUMENT

UV Absorbance Measurements

All UV absorbance measurements were carried out by using a microplate reader (Polarstar Optima, BMG Labtech, Durham, NC). The microplate reader was equipped with a high-energy xenon flash lamp. In the absorbance mode, the initial light passed through a 260-nm or 280-nm filter and was guided via a fiber optic to a position right above the well being measured. The absorbance was measured through the bottom fiber optic without any emission filter. Other specific settings on the microplate reader included 0.5-s positioning delay, and 20 flashes per well. Instead of using a standard cuvette, disposable 96-well microtiter plates with UV transparent bottom (Half Area 96 Well UV Microplate, Corning Inc., Corning, NY) were used. The sample size in each well was 50.0 µL. Deionized water was used as a blank. Each sample was measured five times.

Preparation of MALDI-TOF MS Samples

The MALDI matrix solution was prepared by dissolving 35.0 mg of 3-hydroxypicolinic acid (Sigma-Aldrich, St Louis, MO) and 7.10 mg of ammonium citrate dibasic (Fluka, Buchs, Switzerland) in 1.00 mL of 10% acetonitrile (HPLC-grade, Sigma-Aldrich, St Louis, MO). After vortexing the MALDI matrix solution for ~1 min, any undissolved particles were removed by filtering the solution with 0.22-µm Durapore (PVDF) syringe-driven filter units (Millipore Corp., Bedford, MA). The matrix solution was prepared monthly and stored at −20 °C. The stainless steel MALDI sample plate was cleaned by rinsing the plate with deionized water and followed by methanol washing. Of MALDI matrix solution, 0.30 µL was spotted on the MALDI sample plate and then allowed to air dry. Of DNA oligos mixture, 0.30 µL was then spotted over the dried matrix, and the mixture was also allowed to air dry.

MALDI-TOF MS Measurements

All MALDI-TOF MS measurements were carried out by using a 4700 Proteomics Analyzer from Applied Biosystems (Framingham, MA). Each sample was measured by using the linear positive ion mode (linear middle mass positive acquisition method in the 4000 Series Explorer Version 3.0 software). The Nd:YAG laser intensity was set at 6,000 arb. units (maximum intensity = 7,900 arb. units). To achieve adequate mass resolution for measuring the calibrant or control analyte ion (5,044.3 Da), the extraction of ions was delayed for 450 ns after the onset of

each laser pulse. The accelerating voltage was +20.0 kV, and grid voltage was +18.2 kV. The instrument was equipped with a 200-Hz digitizer. The sampling bin size was 4 ns, with an input bandwidth of 500 MHz and a vertical full scale of 200 mV. The linear detector voltage was +1.92 kV. The pressure inside the instrument was maintained at the level of 10^{-8} Torr. Each spectrum was automatically acquired with random edge-biased positioning of laser shots on an area where the entire MALDI sample was located. The default width for local noise window in the linear middle mass positive processing method was 250 m/z, which defined the background noise from the baseline within the specified mass window where a peak was detected in the mass spectrum.

RESULTS AND DISCUSSION

### UV Spectrometry Versus MALDI-TOF Mass Spectrometry

Although the UV absorbance measurement of DNA at 260 nm has been recognized as a standard method for DNA quantitation, UV spectrometry is unable to distinguish the absorbance between DNA and RNA, both of which co-exist in most living cells. This is because identical UV absorbing moieties (adenine, guanine, and cytosine) are present in both DNA and RNA molecules. Also, the UV absorbance measurement does not provide any information on the size of DNA fragment. For the same reason, UV spectrometry is unable to determine whether there is more than one particular DNA fragment in a sample. Traditionally, these limitations on using UV spectrometry to characterize a DNA sample have been overcome by using gel electrophoresis.[13] However, the experimental procedure for casting, running, and staining the gel has made the method of gel electrophoresis labor intensive and time consuming. Alternatively, the detection of a specific DNA fragment can be achieved by its nucleic acid hybridization with a complementary nucleic acid probe that has been labeled with a reporting molecule.[24, 25] Similar to the other analytical methods, there are drawbacks from using nucleic acid hybridization assays. These include that each nucleic acid probe can only be used for one specific DNA target; that it is difficult to perform multiplex assays; and that information on the actual size of DNA target analyte is lacking, i.e., the DNA target can be either longer or shorter than the complementary nucleic acid probe. From the continuous efforts to improve the qualitative measurements of DNA, the use of MALDI-TOF MS has been explored and shown to be better than the above methods in terms of specificity, sensitivity, and sample throughput.[14, 15] Despite the higher cost of the instrumentation and the extra procedure to prepare the MALDI samples, including the desalting of some biological samples, MALDI-TOF MS has recently become a standard method for characterizing DNA fragments with less than 50 nucleotides (nt). The advantages of combining qualitative and quantitative measurements of DNA on a single analytical platform are obvious. Surprisingly, the use of MALDI-TOF MS for DNA quantitation is less common. As part of our ongoing efforts to determine the accuracy of quantitative MALDI-TOF MS, in this study we compared the accuracy of the standard UV spectroscopic method and the MALDI-TOF MS method to determine the concentration of DNA.

Calibration of UV Spectroscopic Method

For performing absolute quantitation with any instrumental technique, it is necessary to calibrate the measurements with a series of calibrant dilutions whose concentrations are known. In this study, a HPLC-purified 17 mer DNA oligo was used as a calibrant. For carrying out the UV absorbance measurements, a microplate reader instead of a conventional cuvette-based UV spectrophotometer was chosen. This allows calibrant solution at different concentrations including their replicates to be placed and measured from one single 96-well plate. Equally important, the use of 96-well plates can minimize the consumption of any DNA material (50.0 µL per well). With the availability of disposable UV-transparent 96-well plates, it is possible to retrieve the measured samples from the wells with less concern of cross contamination. In Fig. 1A, the results show a linear calibration curve that can be achieved by using the microplate reader. The linear dynamic range for quantitation has slightly less than two orders of magnitude (0.462–40.0 µM). In comparison to the 1-cm path length in a standard cuvette, the 50.0-µL sample size in a microtiter well had a path length of <1 cm, which therefore lowers the absorbance. This resulted in a higher limit of quantitation than expected, and it limited the linear dynamic range for quantitation. Nevertheless, the linearity of the calibration curve in Fig. 1A has an R-squared value of 0.998_7.
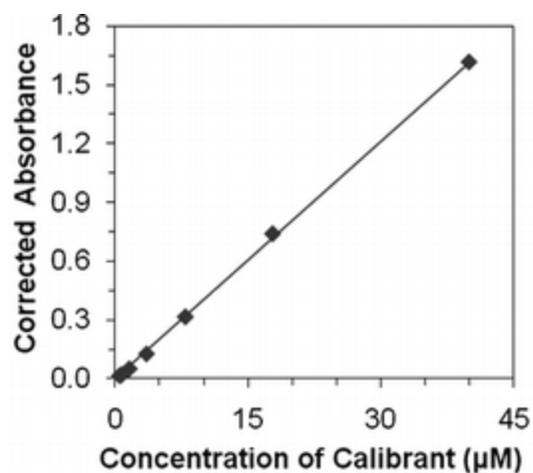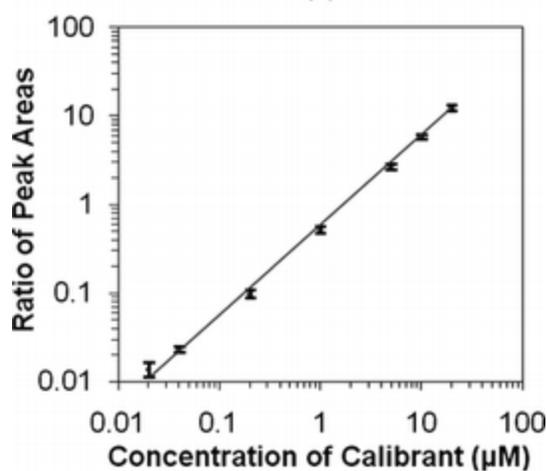
FIGURE 1 Calibration graphs: (A) calibration graph for UV absorbance measurements. The concentration of calibrant (0.462 − 40.0 µM) is plotted against corrected absorbance, which is equal to the average absorbance minus the blank (n = 5). The linear equation for regression analysis is $y = 0.0414x - 0.140$, and $R^2 = 0.998_7$. (B) Calibration graph for MALDI-TOF MS measurements. The concentration of calibrant (0.0200 − 20.0 µM) is plotted against the ratio of average peak areas of calibrant to internal standard. The equation for 2nd order polynomial regression analysis is $y = 0.0044x^2 + 0.538x - 0.142$, and $R^2 = 0.999_9$. Error bars are one standard deviation of $n \leq 12$.

Calibration of Quantitative MALDI-TOF Mass Spectroscopic Method

Due to the heterogeneity in the morphology of sample-matrix co-crystals, MALDI-TOF MS measurements have suffered from a high degree of signal variation, especially for the measurements of DNA.[19] There are several experimental approaches that can be used to improve the reproducibility of MALDI-TOF MS measurements.[26-28] To perform a calibration for quantitative MALDI-TOF MS, a common approach to overcome the variation of signal intensity is to add a fixed amount of an internal standard to each calibrant dilution and measure both calibrant and internal standard.[19-23] By plotting the concentration of calibrant against

the ratio of signals between calibrant and internal standard, a linear calibration graph can be constructed.[19-23] Unlike the protein internal standards that are labeled with stable isotope(s) in proteomics mass spectrometry, DNA internal standards that contain stable isotopes are less readily available. To ensure the internal standard has physical and chemical properties similar to those of the calibrant, a DNA oligo that had the same DNA sequence as the calibrant was used as an internal standard in this study, except an extra 2′-deoxyribonucleotide was added to the 3' end of the internal standard. In the calibration experiment of this study, the final concentration of internal standard was 2.00 µM. To further minimize the effects of signal variation and improve the accuracy, each DNA mixture was measured 12 times from different sample preparations on the same MALDI sample plate. By using the average peak areas, a calibration graph was constructed as shown in Fig. 1B. A linear dynamic range of three orders of magnitude (0.0200–20.0 µM) was achieved with an R-squared value of 0.999_9. As demonstrated by the error bar of each data point in Fig. 1B, the reproducibility of MALDI-TOF MS measurements was acceptable. In comparison of the limits of quantitation (Figs. 1A and 1B), the MALDI-TOF MS measurements were 20 times more sensitive than the UV absorbance measurements. The key to the success of the calibration of MALDI-TOF MS was in using the optimized settings in the mass spectrometer that were determined in a separated study. Although an extra DNA oligo is required as the internal standard, the convenience of combining both qualitative and quantitative measurements on the same analytical platform, the need for a much smaller sample size (~3 µL), and the lower limit of quantitation (0.0200 µM) are the apparent benefits of using the MALDI-TOF MS method. Furthermore, the same internal standard can theoretically be used in the quantitative MALDI-TOF MS measurements of other DNA fragments, whose molecular sizes are comparable to those of the internal standard. A precaution on the selection of internal standard is the fact that the signal intensity in MALDI-TOF MS measurements decreases exponentially with increasing molecular mass.

Comparison of Accuracy and Correlation Study

Following the calibration, the accuracy of MALDI-TOF MS measurements was determined by measuring multiple DNA samples whose concentrations were known and spread across the linear dynamic ranges for quantitation (Figs. 1A and1B). In this study, the concentration of each DNA sample was randomly selected. To avoid any discrepancy on the preparation of MALDI-TOF MS samples and the desorption/ionization of DNA during the MALDI process, the calibrant was also used as a control analyte in the DNA samples. To compare the accuracy of MALDI-TOF MS with that obtained by using UV spectrometry, one set of DNA samples (Table 1) was prepared and analyzed by both techniques. Owing to the difference between the linear dynamic ranges for quantitation (Figs. 1A and 1B), each sample was diluted two-fold prior to the MALDI-TOF MS measurements. In addition, the same amount of internal standard (2.00 µM) was added into each diluted sample. For the regression analysis of the experimental data in Fig. 1B, the use of 2nd-order polynomial regression analysis compared to linear regression analysis has achieved (a) a better fit between the trend line and data points and (b) a higher R-squared value. By using the average peak area (n = 12) and the 2nd-order polynomial equation in

Fig. 1B, we calculated the measured DNA concentration of each sample, as shown in Table 1. For the MALDI-TOF MS results, the majority of the percentage errors had negative values. This could be due to the fact that all DNA samples that were measured by MALDI-TOF MS were further diluted two-fold in comparison to those that were measured by UV spectrometry. In other words, that the measured DNA concentrations were lower than the expected DNA concentrations could be the result of a systematic pipetting error. Nevertheless, most of the percentage errors were attributed to the heterogeneity of MALDI sample preparation. In the case of the UV absorbance measurements, the measured DNA concentrations were calculated by using the average absorbance readings (n = 5) and the linear equation of regression analysis in Fig. 1A. The errors from using the UV method were partly attributed to the reproducibility of the absorbance mode (± 0.010 OD) of the microplate reader used in this study. By comparing the average percentage errors in Table 1, the accuracy of using MALDI-TOF MS was comparable to that obtained by using the microplate reader to measure the UV absorbance. To further evaluate the results of this study, the correlation of DNA concentrations that were determined by MALDI-TOF MS and UV spectrometry is graphically presented in Fig. 2. The correlation coefficient was calculated and was equaled to 0.998_5. This shows that the measured DNA concentrations obtained by MALDI-TOF MS correlate well with the results obtained by using the standard UV spectroscopic method.
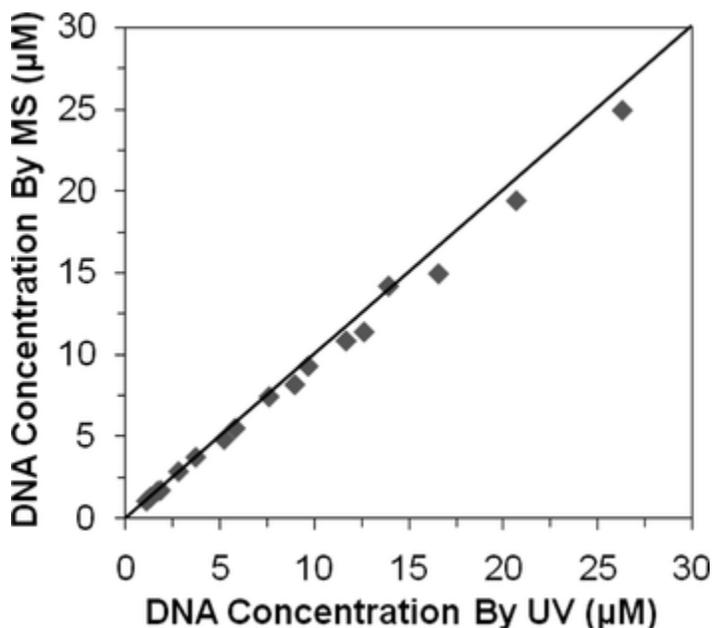

FIGURE 2 Correlation of DNA concentrations that were determined by UV spectrometry (UV) and MALDI-TOF mass spectrometry (MS). The correlation coefficient is equal to 0.998_5 (n = 17).

CONCLUSIONS

To the best of our knowledge, this study represents the first attempt to compare the accuracy of quantitative MALDI-TOF MS with the standard UV spectroscopic method. For the quantitation

of the selected DNA oligo, the results indicated that the accuracy of MALDI-TOF MS was comparable to that obtained by using the microplate reader to measure the UV absorbance. Also, the measured DNA concentrations obtained by using MALDI-TOF MS correlated well with those obtained by using the standard UV spectroscopic method. Together with the capability of determining the purity and identity of DNA, there is great potential in using MALDI-TOF MS to further improve the current analytical methods for studying genomic structures and/or functions.

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