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QUANTITATIVE MALDI-TOF MS OF OLIGONUCLEOTIDES AND A NUCLEASE ASSAY

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Abstract: DNA oligomers 8 and 12 nucleotides long have been detected quantitatively by MALDI-TOF mass spectrometry using longer oligonucleotides as internal standards. Employing this method, the kinetics of the nuclease degradation of the octamer were analyzed. Reactions performed in the presence of the ssDNA-binding peptide KWK yielded a footprint based on fragments from both the 3'- and the 5'-terminus of the oligonucleotide.

Oligonucleotides can be quantitatively detected by their UV absorption or, after labeling, by autoradiography or fluorescence. Analysis of nucleic acid mixtures usually requires a separation step (e.g. gel electrophoresis or HPLC). Autoradiography is most common and very sensitive, particularly when combined with the polymerase chain reaction.[1] Radioisotopes are dangerous and expensive, however, and enzymatic labeling reactions can fail with modified nucleic acids. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS)[2,3] allows for the detection of unlabeled DNA and RNA oligomers and structure assignment.[4] For short DNA and RNA oligomers, the mild desorption conditions lead to molecular ions without significant fragmentation and with high sensitivity. Thus, individual components of mixtures can be detected simultaneously, making the separation by chromatographic techniques unnecessary.[5] Uncharged nucleic acid analogs, which are intractable by classical methods, can also be analyzed by MALDI-TOF MS.[6]

One critical drawback of MALDI-TOF MS as a detection method for DNA and RNA in biochemical assays is the difficulty in obtaining quantitative results. While molecular weight determination by MALDI-TOF MS has become more and more accurate, analyte concentration cannot be determined without special techniques. Heterogeneity of the semicrystalline matrix, laser fluence fluctuations, and the formation of gas-phase adducts usually lead to very poor reproducibility of signals. Addition of a *known* quantity of a compound that is chemically similar to the analyte as an internal standard should overcome this problem. The internal standard co-localizes and co-desorbs with the analyte and its signal can serve as a reference for quantifying the unknown. It has been shown by Chen and collaborators that this approach can be successful for very short oligonucleotides (DNA dimer, trimer, and tetramer).[7] In their study, a laser and detection system was used that is not found in commercially available instrumentation. Also, it was unclear whether the method would be useful for longer oligomers as the MALDI signal of oligonucleotides often steeply declines with the length of the oligomer.[8]

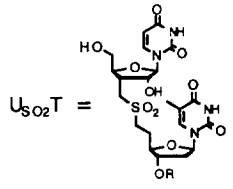
As part of a study on peptides that bind to single-stranded DNA, we became interested in monitoring the enzymatic degradation of oligonucleotides and oligonucleotide-peptide complexes. We decided to explore

quantitative MALDI-TOF MS for oligonucleotides that are long enough to hybridize to complementary strands. Such oligonucleotides have recently attracted much interest as antisense inhibitors of gene expression.[9] Here we report a facile method that allows for the quantitative detection of such oligomers in water, in buffered solutions, and in nuclease reaction mixtures. An example for a footprinting assay involving a single-stranded DNA binding peptide is reported.

As the first step towards quantitative monitoring of DNA in reactions, methods for the detection of DNA in water were studied with oligomers 8 and 12 nucleotides in length. The mixed sequence DNA octamer **1** (Table 1) was arbitrarily chosen as the first test compound. Decamer **2**, which bears two additional nucleotides, was used as internal standard.

Table 1. Oligonucleotides employed

1	5'-CATTGACA-3'
2	5'-CATGTAGACA-3'
3	5'-U_{SO_2}TTTTTTTTT-3'
4	5'-ACGTCAGTTAGC-3'
5	5'-ACGTCAGTTAGCTT-3'



It was critical to acquire spectra in a concentration range associated with linear response of the instrument as discussed in detail by Hercules and collaborators[10] and others[11] for different analytes. We used a Bruker BIFLEX® spectrometer in linear, negative mode (1.6 m drift tube, MCP detector, 1 GHz digitizer). Good linearity was observed in calibration plots when trihydroxyacetophenone was employed as matrix and diammonium hydrogen citrate as comatrix.[12] Picolinic acid and 3-hydroxypicolinic acid[13] led to less reproducible peak ratios because the intensity of sodium and potassium adducts accompanying the pseudomolecular ions varied strongly from spot to spot. Similar observations were made for 6-aza-2-thiothymine,[14] a matrix, that otherwise gave well resolved and strong signals. It should be noted that the DNA in these experiments was not pretreated with ion exchange beads to remove alkali metal ions. Metal adduct formation was suppressed by adding ammonium salt comatrices only. This approach was chosen as many enzymatic reactions (e.g. those involving nuclease S1) require the addition of a metal cofactor and are best performed in buffered solutions. Exhaustively exchanging ions with beads can be slow, making it difficult to stop enzymatic reactions accurately.

Increasing the ammonium ion concentration in the matrix mixture reduced the formation of Na^+ and K^+ adducts but also reduced the signal intensity. Easily interpretable, but not adduct-free spectra, were obtained with 27% of a 0.1 M diammonium hydrogen citrate solution in the matrix/comatrix/analyte mixture. The fluence of the laser was critical for reproducible internal standard/analyte peak ratios. If the fluence was considerably higher than the threshold for ion production, the ratio between analyte and internal standard peaks was well reproducible. The increased laser irradiance induced a noticeable but acceptable peak broadening (Figure 1a). To improve reproducibility, four spectra of 100 laser shots each were averaged. Best results were obtained when the MALDI target was pretreated with a very thin layer of silicone oil to prevent spreading of the ethanol-rich matrix mixture and to improve reproducibility of the crystallization. In addition, the target was wiped with an ammonium-hydroxide-soaked towel to remove residual alkali metal ions. Under these conditions, the matrix

crystallized in a compact, circular shape. The best signal was usually obtained at the edges of the matrix cake. Linear calibration plots with small standard deviations were obtained under these conditions (Figure 1b).[15]

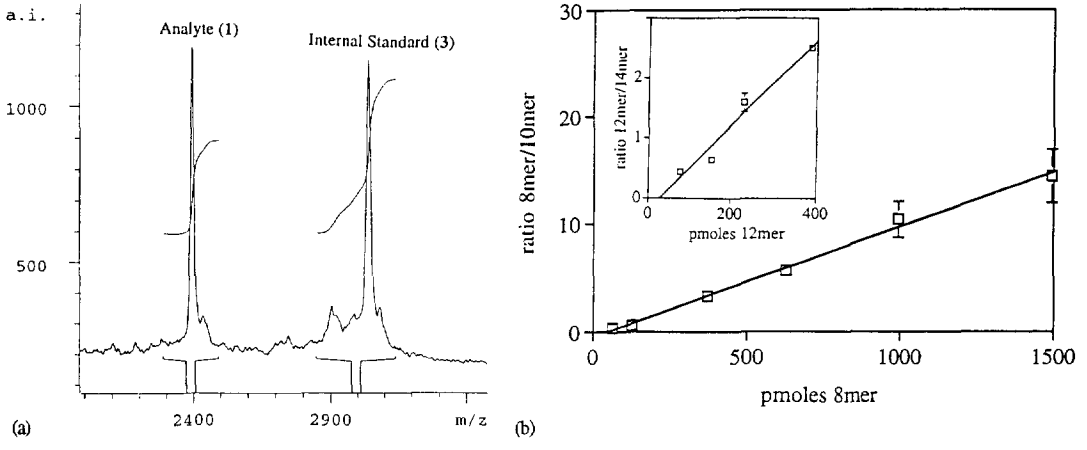


Figure 1: Quantitative MALDI-TOF of **1** using **2** as internal standard. (a) Molecular ion region of typical spectrum, (b) Calibration plot; ratio of peak integrals from four spectra, mean ± one standard deviation; inset: calibration plot for **4/5**.

The best correlation between signal ratios and analyte concentration was obtained when peaks were integrated, in contrast to results obtained by others for proteins.[16] This is probably due to the fact that a mass range that includes salt adducts and depurination products (200 mass units) was integrated. The extent of salt adduct formation and low-level fragmentation of analyte and internal standard was often different with some spot-to-spot variability. The wide integration range averages over all of these ion species. The calibration plot (Figure 1b) reveals an increase in relative standard deviation with increasing concentration of the analyte. A similar trend has been observed for proteins,[16] oligosaccharides[17] and very short oligonucleotides.[7]

Conditions established for **1** and **2** could be directly applied to the quantitative detection of 12mer **4** (Table 1) by using the 14mer **5** as internal standard. Analyte and internal standard were again chosen to differ by two nucleotide units in length. This gives a mass range between the internal standard and the analyte peaks where fragments/hydrolysis products of the 14mer can be detected. With an internal standard just one residue longer than the analyte, peaks of potential degradation products may overlap with the analyte signal. The calibration curve for the pair **4/5** is shown in the inset of Figure 1b.

Nuclease degradation of **1** was first studied with calf spleen phosphodiesterase (CSP, EC 3.1.16.1) under conditions similar to those established by Pieles and collaborators for the generation of sequencing ladders.[12] The all-pyrimidine oligomer **3** (Table 1) bearing a non-natural dimethylenesulfone linkage[18] as a 5'-cap was initially used as internal standard. This modified oligomer cannot be degraded by CSP. Calibration plots for the analyte/internal standard pair **1/3** were similar to those for **1/2**, except that the correlation factor was lower (R^2 0.966 versus 0.996). This indicates that sequence similarity between analyte and internal standard is not mandatory for analyte quantitation. As with the nonquantitative protocol,[12] the nuclease did not produce additional signals in the DNA spectra. A plot of the kinetics of the hydrolysis of **1** by CSP is shown in Figure 2a. When the same reaction was monitored with **2** as internal standard, a very similar curve was obtained,

indicating that the 10mer was not degraded with our procedure. This was probably due to the fact that the internal standard was added to the matrix preparation rather than to the reaction mixture. The matrix mixture contains 67% ethanol and was cooled to 0 °C, conditions, that can be expected to inactivate the phosphodiesterase. This was also found to be the case for nuclease S1 (EC 3.1.30.1). Since **2** has the advantage of being available by standard solid-phase DNA synthesis, all subsequent experiments were performed with this internal standard.

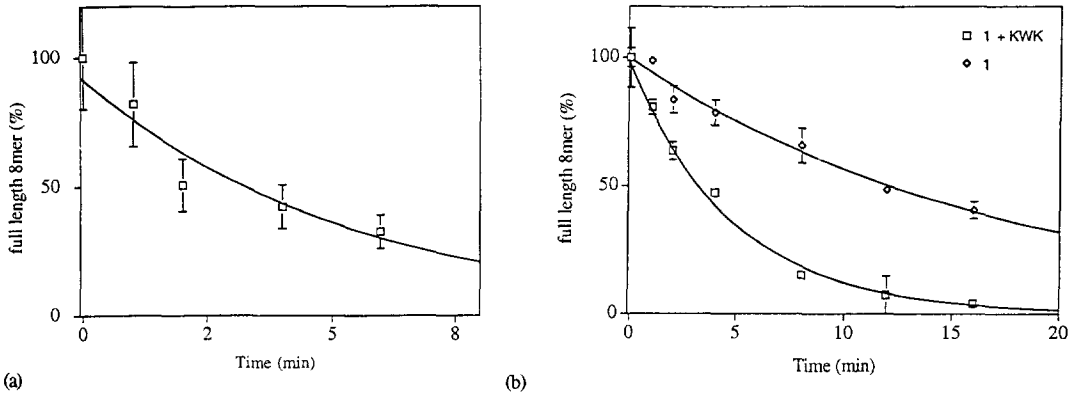


Figure 2: Kinetics of the degradation of **1** with nucleases. (a) CSP, **3** as internal standard; (b) Nuclease S1, **2** as internal standard. Monoexponential fits to normalized peak integrals (mean ± SD).

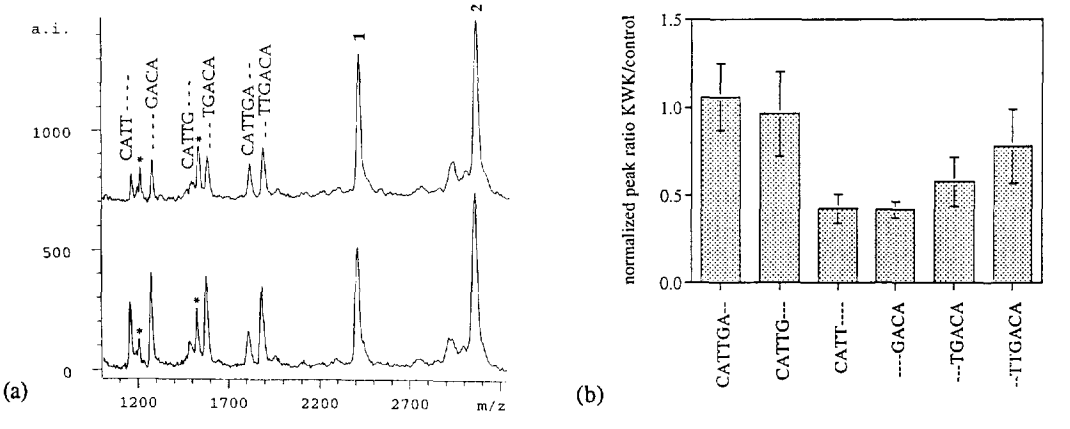


Figure 3: Footprint analysis of MALDI-TOF-monitored degradation of **1** with nuclease S1; (a) typical spectrum in the presence of KWK (upper panel), and control (lower panel), asterisks indicate M^{2-} peaks; (b) 5'-, 3'-Footprint (ratios of normalized peaks ± SD).

The kinetics of the hydrolysis of **1** with nuclease S1, monitored by quantitative MALDI, are shown in Figure 2b. When the reaction was performed in the presence of 10 mM Lys-Trp-Lys (KWK), the MALDI spectra did not show peaks corresponding to a complex between peptide and DNA. The presence of KWK did enhance the signal intensity of **1**, however, indicating, that an interaction between the two was occurring. In fact, the calibration curve for **1/2** in the presence of KWK, while linear, was steeper by a factor of 1.6 than with the peptide-free mixture. This effect was less pronounced with tryptophan-free short cationic peptides. An

improved resolution of spectra of oligopyrimidines in the presence of basic dipeptides has been reported[19] but this effect was observed in the positive mode and, most importantly, led to concurrent peaks of both uncomplexed DNA and peptide-DNA complexes. The seemingly undisturbed DNA spectra found under our conditions (negative mode, acetophenone matrix, laser power above threshold) allow for unambiguous, quantitative detection of the DNA part in peptide-DNA mixtures.

The kinetics of the nuclease S1 degradation of **1**/KWK[20] confirmed the expected[21] formation of a complex (Figure 2b). The oligonucleotide in the DNA-peptide mixture is cleaved at a slower rate than the DNA in the control sample. The half-life time of **1** is four times longer in the presence of the peptide. Structural details of the nuclease protection exerted by KWK could be revealed by analyzing the abundance of 5'- and 3'-fragments in KWK- and control-run. Figure 3 shows this analysis after 2 min reaction time at 0.13 u/μL nuclease S1, 7.2 mM of KWK, and 83 μM **1**. The "footprint" is located at the central GT step, indicating that this is where KWK binds preferentially.

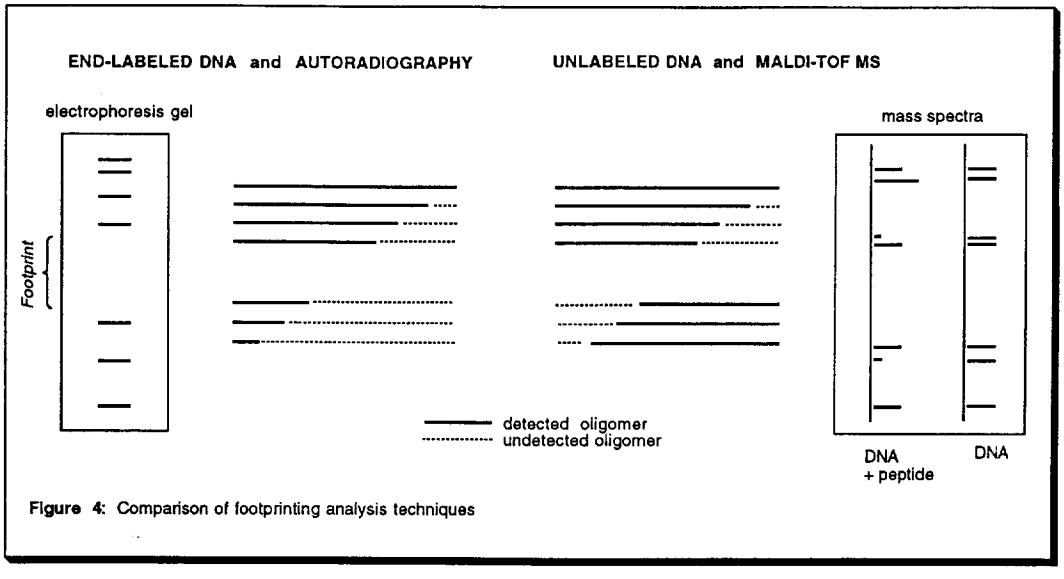


Figure 4: Comparison of footprinting analysis techniques

Our footprinting approach is different from the traditionally employed assay (Figure 4). Whereas in the case of end-labeled DNA and gel electrophoresis/autoradiography, fragments from one terminus of the DNA are detected, our MALDI-based footprinting assay uses the peak intensity information of fragments from both termini. For longer oligonucleotides, sets of matching fragments may be analyzed.

In conclusion, we have applied a quantitative MALDI-TOF detection method to DNA oligomers. This method employs commercially available instrumentation and chemicals, and is rapid and sufficiently accurate to monitor enzymatic degradation reactions in detail. Using this technique, we have performed a MALDI-TOF-based footprinting experiment for a peptide-DNA complex. It is expected that the same method can be employed for the quantitative detection of RNA oligomers and their complexes as ribonucleotides have been shown to be more stable under MALDI desorption conditions than their DNA counterparts.[22]

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15. For calibration curves, aliquots (0.5-12 μL) of a solution of **1** in deionized water (10 OD_{260}, 125 μM) were dried by lyophilization, followed by addition of **2** (3 μL, 10 OD_{260}, 93 μM), and diammonium hydrogen citrate (10 μL, 0.1 M). After vortexing and centrifugation, 2,4,6-trihydroxyacetophenone (25 μL, 0.3 M in ethanol) was added, followed by mixing. The MALDI target was coated with silicone oil (heat bath quality, Fisher), excess silicone was removed with methanol, followed by polishing of the surface with an ammonium hydroxide-soaked low-lint towel. 1 μL of the matrix mixture was applied and was left to evaporate to dryness at room temperature, resulting in a ring-shaped semicrystalline cake. Spectra were accumulated at $< 9 \times 10^{-7}$ Torr (drift tube) from 100 laser shots at 2 Hz laser frequency and 20 mV preamplifier voltage in linear, negative mode on a Bruker, BIFLEX® spectrometer at 17.5 kV extraction voltage. Matrix ions were suppressed by 2 kV deflection up to m/z 600. Five spectra were accumulated per target with best signals usually from the outer edge of the matrix. Spectra with less than 500 ion counts for the internal standard were deleted. The laser attenuation was set to produce ca. 64-70 μJ pulses (manufacturers specifications). The threshold for detectable signal under these conditions was ca. 45 μJ/pulse. Spectra were 3-point filtered to improve s/n, and a mass range of 250 mass units covering the main peak and potential adduct peaks was integrated for both analyte and standard peaks. Ratios between analyte and internal standard peak areas were plotted (mean ± SD, Figure 1b).
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20. For nuclease S1 assays, solutions of **1** (16 μL, 125 μM) and Lys-Trp-Lys (4 μL, 25mg/mL), or ammonium acetate (4 μL, 10 mM) (control) were mixed, annealed at 37 °C for 2 min and cooled to 0 °C. Nuclease S1 (4 μL, 0.8 u/μL, pH 5.6) in 0.5 M $(NH_4)_2SO_4$, 30 mM $ZnSO_4$ was added. The solution was mixed and transferred to a waterbath at 37 °C to start the reaction. Aliquots of 2 μL were removed at selected time points and pipetted into a mixture of trihydroxyacetophenone (20 μL, 0.3 M in ethanol), diammoniumcitrate (8 μL, 0.1 M), and **2** (2 μL, 93 μM) at 0 °C, followed by immediate, thorough mixing. Spectra were acquired as described above.[15]
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