Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry Methods for Oligodeoxynucleotides: Improvements in Matrix, Detection Limits, Quantification, and Sequencing

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A comatrix of anthranilic acid and nicotinic acid is optimum for the matrix-assisted laser desorption/ionization time of flight determination of oligodeoxynucleotides that are comprised of up to 21 nucleotides. A detection limit of approximately 200 amol was obtained for an oligonucleotide 21mer. The comatrix system is also suitable for quantification of oligodeoxynucleotides provided an internal standard having one more or less nucleotide than the number in the analyte is used. Furthermore, the matrix, when used in combination with the ladder method of sequencing, allows the complete sequence of tens of picomoles of model oligodeoxynucleotides to be determined. (J Am Soc Mass Spectrom 2000, 11, 854–865) © 2000 American Society for Mass Spectrometry

Although matrix-assisted laser desorption/ionization (MALDI) mass spectrometry has rapidly developed as one of the most effective techniques for the determination of biomolecules, its application to oligonucleotides is much less routine than for peptides and proteins. One reason for the success with the latter class of biomolecules is the availability of appropriate matrices (e.g., derivatives of benzoic acid, cinnamic acid, and heterocyclic aromatic compounds). On the other hand, the development of MALDI matrices for oligonucleotides and DNA has lagged that for peptides and proteins.

There is a strong need for improved matrices that can be used for the determination of oligonucleotides, especially ones that provide lower detection limits and higher mass resolving power than those currently used. The purposes of this study are to (1) seek better detection limits by developing improved matrices for MALDI of oligonucleotides, (2) evaluate quantification by applying the improved matrices, and (3) improve sequencing of oligonucleotides by continuing the development of a ladder method involving the enzymatic digestion.

The matrix plays an important role in the desorption/ionization process of MALDI, and developing new matrices has been a focus of various research groups [1, 2]. 3-Hydroxypicolinic acid (3-HPA) [3], a 2:1 molar mixture of 2′,4′,6′-trihydroxyacetophenone (2,4,6-THAP) and 2′,3′,4′-trihydroxyacetophenone (2,3,4-THAP) [4], a mixture of 80% anthranilic acid (AA) and 20% nicotinic acid (NA) [5], and 6-aza-2-thiothymine (6-ATT) [6] are matrices that are currently used for the determination of oligonucleotides. Recently, Hunter et al. [7] reported that frozen solutions containing photoabsorbing substituted phenols are an alternative matrix system for the determination of high molecular weight oligonucleotides.

Metal-adduct formation is a major cause for low detection limit and poor mass resolving power for MALDI [1]. Even oligonucleotides that have been purified by reverse phase high-performance liquid chromatography (HPLC) may still show in the MALDI mass spectrum peaks that correspond to cationized analyte if no desalting is applied. To minimize production of metal–ion adducts in MALDI, a few beads of cation-exchange resin in the NH_4^+ form are usually added to the oligonucleotide analyte prior to the mass spectrometric analysis [1]. Another approach is to use ammonium salts as comatrix; these salts, when used in MALDI, improve significantly the detection limit and mass resolving power [1]. The basis of this approach is that ammonium salts of the oligonucleotides easily lose ammonia accompanied by proton transfer during the desorption process. Diammonium hydrogen citrate and ammonium acetate are the two most widely used comatricies. Cheng et al. [8] also reported that ammonium halides can be used as comatricies with 2-amino-5-nitro-pyridine, whereas NH_4F exhibits the greatest

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metal–cation suppression effect. More recently, Allison and co-workers [9] used spermine as a matrix additive; they reported the detection limit was 75 fmol for a 12mer when they combined spermine and 6-ATT. They also found that the spermine can be used successfully with the 80% AA/20% NA, but not with 3-HPA.

Although some improvements have come from matrix development, additional improvement is still needed. For example, 3-HPA is the common matrix for small oligonucleotides, but the sensitivity associated with its use decreases significantly when larger oligonucleotides are to be determined, especially in the analysis of oligonucleotide mixtures. The use of 2,4,6-THAP and 2,3,4-THAP for analysis of mixtures of oligonucleotides gives lower detection limit than those achieved with the other matrices mentioned above. The use of this matrix, however, requires high laser power for the desorption process, causing sometimes significant fragmentation and formation of substantial amounts of doubly charged ions. These observations are consistent with a classification of THAP as a "hot" matrix [2]. The problems of fragmentation and multiple charging provide incentive to continue the development of comatrices and to search for more suitable matrices for routine oligonucleotide screening.

Quantifying small oligonucleotides is an application of MALDI to which less attention has been paid. Oligonucleotides and modified oligonucleotides have been quantified by HPLC-electrospray ionization (ESI) MS or MS/MS approaches [10, 11]. With special techniques, quantitative measurements by MALDI are possible when appropriate measures are taken, as was illustrated for antibiotics [12] and maltooligosaccharides [13]. Sarracino and Richert [14] reported the quantitative determination of short oligonucleotides by MALDI-TOF MS at high picomole levels in 1996. Hutchens and co-workers [15] also demonstrated, by using a 36-base-pair oligonucleotide as an internal standard, the quantification of a 21-base-pair oligonucleotide. But shot-to-shot reproducibility and signal degradation are still problems associated with quantitative analysis by MALDI [16].

Therefore, another goal of the research reported here is to improve the limit of quantification by MALDI. An example of a productive approach is that of Hercules and co-workers [17, 18] who employed a matrix/comatrix to improve the signal reproducibility for the quantitative analysis of peptides. This work has relevance to oligonucleotide quantification.

Another opportunity is the use of enzymes to digest oligodeoxynucleotides as a means of improving the sequencing capabilities of MALDI for these classes of biomolecules. Tandem mass spectrometry (MS/MS) can serve this function [1, 19, 20], and we recently demonstrated the application of collisionally activated decompositions (CAD) and MALDI post-source decay (PSD) to distinguish oligodeoxynucleotides containing 4–8 bases [21–23]. There are upper-mass limits on sequencing oligonucleotides with MS/MS and PSD, however, approximately 12mers if low picomole detection limits are required, and there is a need to develop alternatives such as the exonuclease ladder method. This method may be particularly useful for modified oligodeoxynucleotides such as those that are used as models in cancer research.

To our knowledge, the ladder method in MS was first developed by Caprioli [24], who applied it to polypeptides. Sequencing oligodeoxynucleotides by exonuclease digestion and MALDI MS was later demonstrated by Schuette and co-workers [25] and further developed by Martin and co-workers [26] and by Johnston et al. [27] in 1996. Progress was recently reviewed by Limbach [20]. Smith and co-workers [28] and Owens and co-workers [29] also reviewed the subjects of oligonucleotide and peptide sequencing using both MALDI and electrospray mass spectrometry.

Three 1997 articles showed that a similar approach would work for oligoribonucleotides (RNA) that have up to nearly 50 bases [30–32]. More recently, Tolson and Nicholson [33] developed a modified method employing endonucleases and chemical methods to sequence RNA. The amount of sample consumed in these studies is in the upper picomole levels, but further improvements in detection limit would be welcome. The most recent work, to our knowledge, is that by Cadet and co-workers [34, 35], who applied MALDI-TOF-MS to investigate the mechanism of oligonucleotide cleavage by *E. Coli* repair enzymes.

Materials and Methods

Materials

The matrix components anthranilic acid and nicotinic acid were purchased from Aldrich Chemical (Milwaukee, WI). Both acids were purified by recrystallization twice from 18-MΩ water. Decolorizing charcoal was used to remove impurities during the first recrystallization. Cation-exchange beads in the NH_4^+ form were prepared from chromatography beads (AG50W-X8, 100–200 mesh; Bio-Ad, Melville, NY) in the H^+ form according to the literature [36]. All synthetic oligonucleotides were synthesized at the Nucleic Acid Chemistry Laboratory, Washington University School of Medicine, except for 5′-d(GCU^{Br}TAATTCG), which contains a 5-bromo-2′-deoxyuridine; this material was from Integrated DNA Technologies (Coralville, IA). Snake venom phosphodiesterase (SVP) was purchased from Pharmacia Biotech (Piscataway, NJ), and bovine spleen phosphodiesterase (BSP) was obtained from Sigma Chemical (St. Louis, MO).

Instrumentation

MALDI-TOF spectra were obtained with a PerSeptive Biosystems Voyager-DE RP mass spectrometer in the negative-ion mode using an accelerating voltage of 25 kV. Total length of the ion flight path was 1.2 m. The

pulse delay time was set at 100 ns, and the grid voltage was 92.0% of the accelerating voltage. Oligonucleotide molecular-weight and detection-limit determinations required 10 to 40 shots from the nitrogen laser (337 nm), whereas 60–130 laser shots were summed when the digest was analyzed. The raw data were acquired with a Tektronix 520A digitizing oscilloscope then processed with GRAMS 386 software (Galactic Industries), which was provided with the mass spectrometer.

MALDI Sample Preparation

The matrix mixture was prepared by dissolving anthranilic acid (2.74 mg, 0.02 mmol) and 1.23 mg (0.01 mmol) nicotinic acid in 50 μL of acetonitrile and 30 μL 18-MΩ water, and adding 30 μL 100-mM ammonium citrate. The final molar ratios of anthranilic acid, nicotinic acid, and ammonium citrate were 2:1:0.003. The other matrices with different ratios of anthranilic acid, and nicotinic acid were prepared similarly. Aliquots of 0.5 to 1 μL of analyte and 1 to 2 μL of matrix solution were mixed to form a spotting solution. 1 μL was spotted on the MALDI plate and air dried. Solutions of 0.2 μL of 1-μM d(T5), d(T10), d(T18), or d(T20) were used as internal standards for molecular-weight calibration.

Quantification

The analyte was diluted to 1.0; 2.0; 3.0; 4.0; 5.0 μM from a 50-μM stock solution; the 1.0-μM solution was further diluted to give 0.2; 0.4; 0.6; 0.8-μM solutions. The internal standard was kept at a constant concentration of 1.0 μM. A 2-μL volume of the analyte was premixed with 2 μL of internal standard and 6 μL of matrix solution to give a spotting solution.

For each analyte/internal standard ratio, five sample spots were prepared by loading 1 μL of the sample/matrix mixture on the MALDI plate. Mass spectra were accumulated for 50 laser shots for each sample spot. This was repeated for the other four sample spots. An 11-point Savitsky–Golay smoothing was applied to the spectra, and automatic Y offset was used to set the baseline to zero. The peak-height ratios of the analyte relative to those of the internal standard were calculated and averaged from the five measurements at each concentration. Standard deviations and relative standard deviation for average peak-height ratio from different concentrations were also determined.

Enzymatic Digestion

A 1-μL sample containing 40 pmol of the 18 to 21mers, or 10 pmol of the 10 to 14mers, was used in both snake venom phosphodiesterase (SVP) and bovine spleen phosphodiesterase (BSP) digestion experiments. For the SVP digest, 0.5-2 μL of SVP solution (10×10^{-2} units/μL) was added to 1 μL of the oligonucleotide solution, 6 μL of 100 mM ammonium citrate (pH was adjusted to 9.4 with NH_4OH), and 6 μL of H_2O. In the BSP digestion, 1–2 μL BSP (1×10^{-2} units/μL) solution was added to 1 μL of the oligonucleotide solution and 7 μL of H_2O.

The digest solution was heated to 37 °C for the SVP digestion, but kept at room temperature for the BSP digestion. A 0.6-μL aliquot of the digest solution was removed every minute until the digestion was complete. After being mixed with 1.2 μL of the matrix solution, the sample was spotted on the MALDI plate, without any additional treatment, and dried for immediate analysis.

Results and Discussion

Study Design

The principal motivation for the method development described here is a MALDI method that can used to determine damage to small oligodeoxynucleotides that serve as models in research on skin and breast cancer [21–23, 37]. Even in model studies, the reaction products (adducts) are often of low picomole to femtomole amounts. Thus, there is a need to improve the detection limits by developing improved matrices for the MALDI of small olicodeoxynucleotides.

The design of a study, from which conclusions about optimum matrix and detection limits can be drawn, is formidable because a huge variety of oligonucleotides as determined by size, composition, and sequence motif is possible. To make the research tractable, we divided the model compounds into three size classes: 8, 12–13, 18–21. Then we subdivided each size class into five additional classes based on composition: "average," "C-rich," "T-rich," "A-rich," and "G-rich" (see Table 1). By "average," we mean an oligonucleotide with approximately equal amounts of the four bases. Furthermore, we included 5′-d(TTCCCACGCCTCCAGCGC) and 5′-d(ACAGCAGGTCAAGAAGAGTAT), which have the sequences surrounding codons 13 and 61 of mouse Harvey-*ras*, and their complements in our studies. They comprise, in part, the "C," "A," "G," and "T-rich" classes, respectively.

Matrix Design

Previously, we proposed some design criteria for matrices for modified nucleobases and demonstrated that these ideas can be implemented to give a matrix that significantly enhances the sensitivity of MALDI for these materials [38]. These design criteria were also applied in the present study. The advantage of anthranilic acid is that it strongly absorbs at 337 nm (its most effective absorption is at that wavelength, λ_{max}), whereas the advantage of nicotinic acid is that it cocrystallizes well with oligodeoxynucleotides. Thus, we hypothesized that the combination of these two compounds should make an effective matrix for the MALDI of oligodeoxynucleotides. Actually, a 4:1 mixture of AA/NA was one of the first matrices to be used for

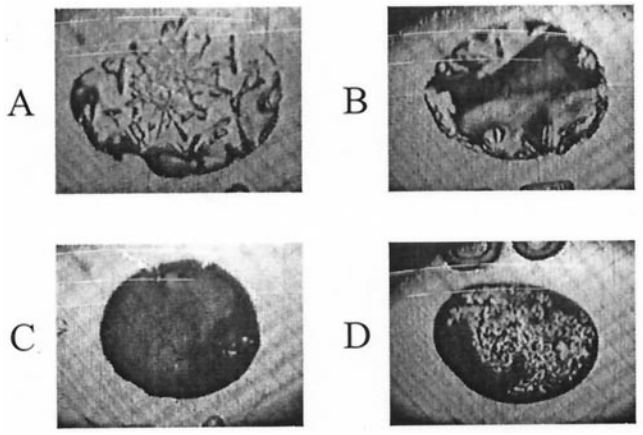

Figure 1. Images of the sample spots from the A21mer mixed with various matrices: (**A**) AA:NA = 4:1, (**B**) AA:NA = 2:1, (**C**) AA:NA = 1:1, and (**D**) AA:NA = 1:3.

nucleic acids, as reported in 1992 by Hillenkamp and co-workers [5]. This matrix, however, has not been widely used because it gives low sensitivity compared to the more commonly used matrices, 3-HPA and 6-ATT.

We anticipated that the ratio of individual matrix components plays a key role in making an effective comatrix system. Thus, we tested the combination of AA/NA at different molar ratios from 1:4 to 4:1 and used these matrices for the oligodeoxynucleotides we designed. We also increased the acetonitrile ratio in the matrix solution, because AA is less soluble in aqueous solution compare to NA, to dissolve better the matrix. We found that the AA and NA should be recrystallized twice from pure water before combining them in a matrix solution to prevent introducing metal–cation impurities into the analyte spot.

Photographs of the dried spots of a "A-rich" 21mer (A21mer) mixed with 4:1, 2:1, 1:1, or 1:3 (AA:NA) matrix (Figure 1) show that the 1:1 (Figure 1C) and 1:2 (not shown) produced the most homogeneous spots, suggesting that the analyte was best incorporated into those matrix crystals. The 2:1 (AA:NA) matrix also cocrystallized oligonucleotides well (Figure 1B), whereas the 4:1 and 1:4 (AA:NA) gave highly inhomogeneous spots for which finding a "sweet spot" is difficult (Figure 1A, D). The appearance of a 3:1 matrix was intermediate between those of 4:1 and 2:1 (AA:NA). The appearances of 1:2 and 1:3 are similar to those of 1:1 and 1:3 matrix, respectively. Preparations using the same matrix and the various oligodeoxynucleotides in Table 1 yield similarly appearing spots.

Comparative measurements of signal intensities of the oligodeoxynucleotides (Table 1) with matrices having different ratios of AA:NA allow us to choose an optimum matrix. Although all oligodeoxynucleotides (1 pmol) listed in Table 1 could be detected when either 4:1, 3:1, or 2:1 AA:NA was applied as matrix, we found the optimum ratio was AA:NA = 2:1. The signal intensities for all the oligodeoxynucleotides were consistently 2–8 times greater when we used 2:1 AA:NA matrix than when we used 4:1 or 3:1 AA:NA matrices. Some of the oligodeoxynucleotides (Table 1) at the 1-pmol level were also observed using 1:1, 1:2, 1:3, or 1:4 AA:NA matrix, but the signal intensities were significantly decreased. Furthermore, a 20% higher laser power was required to obtain the spectra compared to those obtained with 4:1, 3:1, and 2:1 AA:NA matrices. These results are consistent with the proposal that anthranilic acid is an effective absorber of energy from a nitrogen-laser pulse. The normalized signal intensities

Table 1. Oligodeoxynucleotide sequences used for matrix evaluation

Oligo name	Sequence	Base type
M8mer	5′-d(ACGTACGT)	Average
M12mer	5′-d(ACGTACGTACGT)	Average
M16mer	5′-d(ACGTACGTACGTACGT)	Average
M20mer	5′-d(ACGTACGTACGTACGTACGT)	Average
C13mer	5′-d(TTCCCACGCCTCC)	C-rich
G13mer	5′-d(GGAGGCGTGGGAA)	G-rich
A12mer	5′-d(CAAGAAGAGTAT)	A-rich
T12mer	5′-d(ATACTCTTCTTG)	T-rich
C18mer	5′-d(TTCCCACGCCTCCAGCGC)	C-rich
G18mer	5′-d(GCGCTGGAGGCGTGGGAA)	G-rich
A21mer	5′-d(ACAGCAGGTCAAGAAGAGTAT)	A-rich
T21mer	5′-d(ATACTCTTCTTGACCTGCTGT)	T-rich

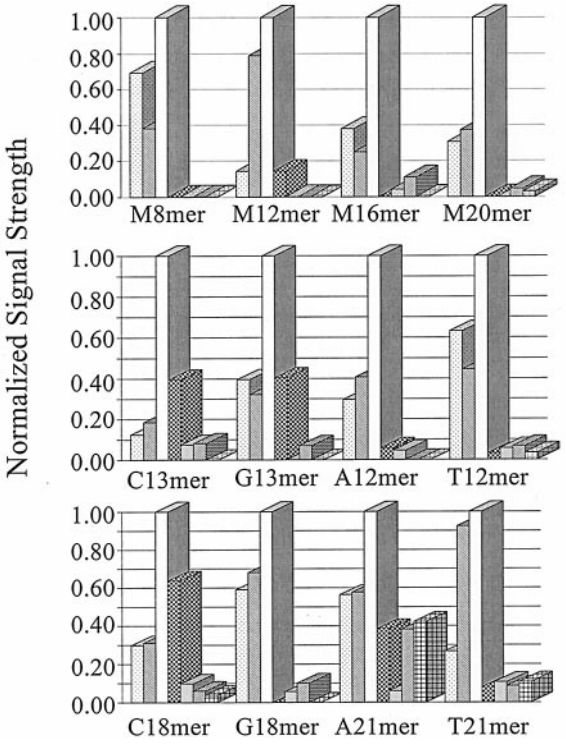

Figure 2. Normalized signal intensities obtained by using various matrices for determination of oligonucleotides listed in Table 1. (**A**) "Average" oligonucleotides, (**B**) 12–13 bases oligonucleotides, and (**C**) 18–21 bases oligonucleotides. The order of presentation for each oligo is 4:1, 3:1, 2:1, 1:1, 1.2, 1.3, 1.4 (left to right).

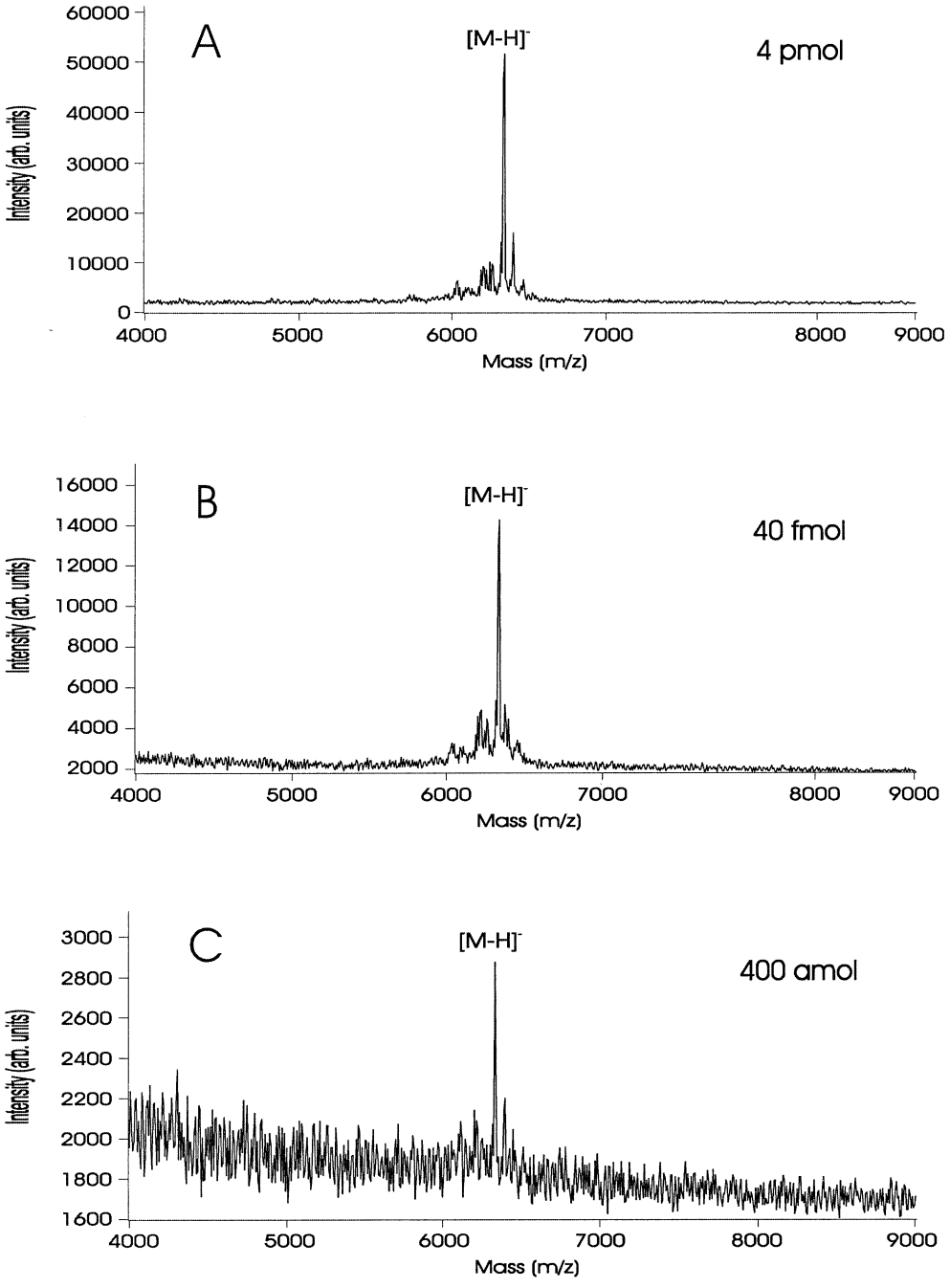

Figure 3. MALDI mass spectra of a T21mer that was loaded at various levels: (**A**) 4 pmol, (**B**) 40 fmol, and (**C**) 400 amol. The spectra were obtained by using as a matrix a 2:1:0.003 molar ratio anthranilic acid, nicotinic acid, and diammonium citrate.

obtained when using different ratios of matrix components for the oligonucleotides in Table 1 are presented in Figure 2.

The modification of the AA/NA matrix system leads to a significant improvement in both the detection limit and the mass resolving power that can be obtained in the MALDI determination of oligodeoxynucleotides. We found the detection limit of the "T-rich" 21mer to be approximately 400 amol with a signal-to-noise ratio (S/N) of 6:1 (Figure 3). This implies a detection limit of 200 amol with a S/N of 3:1, which is 20 times better than the 3.9-fmol detection limit that was previously reported for a 13mer. The authors of that report [39] used a mixture of 3-HPA, picolinic acid, and diammonium citrate as the matrix, which was applied to a Nafion-coated MALDI plate. Typically, we achieved a mass resolving power of approximately 800 (FWHM) for midsized oligodeoxynucleotides (18–31mers) when 1–4 pmol of analyte were used.

Moreover, we observed no detectable fragmentation

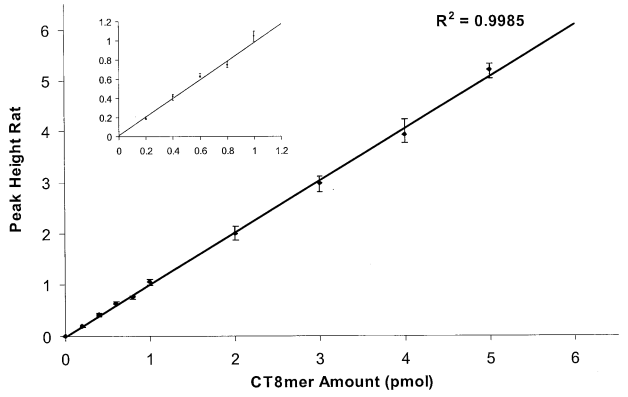

Figure 4. Calibration plot for the MALDI quantification of CT8mer using 1 pmol of CT9mer as the internal standard.

nor substantial amounts of doubly charged ions in our experiments, unlike when we used the combination of 2,4,6-THAP and 2,3,4-THAP and saw considerable amounts of doubly charged ions. In addition to the diammonium hydrogen citrate comatrix, which was chosen to suppress the cation adducts of oligonucleotides, we also used spermine as was suggested by Allison and co-workers [9]. Significant amount of spermine adducts, however, were seen upon laser desorption when AA/NA was used as the matrix.

Quantification

A second goal of this research is to use the new matrix system and MALDI-TOF mass spectrometry to quantify oligodeoxynucleotides that may be produced, for example, as the reaction products from steroid–DNA reactions. The main problem associated with MALDI-TOF quantification is poor signal reproducibility, which is often attributed to failure of the matrix, analyte, and internal standard to cocrystallize. When cocrystallization is uniform, peak ratios for standard and analyte show minimal variations from shot to shot for the laser.

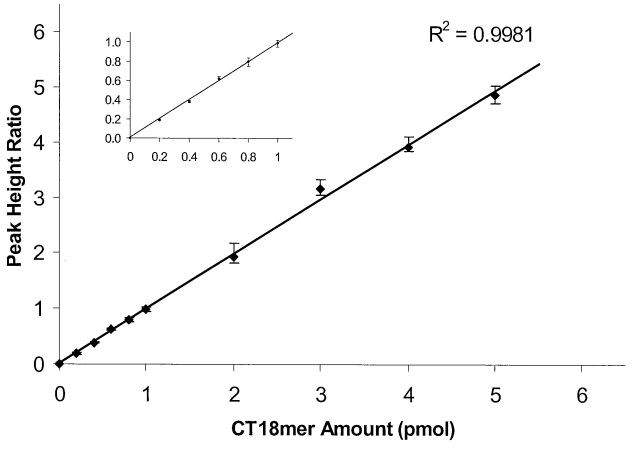

Figure 5. Calibration plot for the MALDI quantification of CT18mer using 1 pmol of CT19mer as internal standard.

Table 2. Standard deviation (SD) and relative standard deviation (RSD) for average peak–height ratio from different sample ratios of oligonucleotides

Sample ratio	Average peak–height ratio	SD	RSD (%)
CT8mer/CT9mer			
0.2	0.19	0.01	5.3
0.4	0.42	0.03	7.1
0.6	0.63	0.02	3.2
0.8	0.75	0.02	2.7
1	1.05	0.05	4.8
2	1.99	0.11	5.5
3	2.98	0.11	3.7
4	3.94	0.22	5.6
5	5.21	0.14	2.7
CT18mer/CT19mer			
0.2	0.19	0.01	5.3
0.4	0.38	0.01	2.6
0.6	0.62	0.01	1.6
0.8	0.80	0.03	3.7
1	0.99	0.03	3.0
2	1.94	0.14	7.2
3	3.17	0.11	3.5
4	3.92	0.11	2.8
5	4.87	0.14	2.9
CT9mer/CT8mer			
1	1.01	0.02	2.0
2	1.97	0.15	7.6
3	2.87	0.05	1.7
4	4.15	0.08	1.9
5	5.21	0.36	6.9

An appropriate matrix should also effectively absorb the energy from the laser beam. Unfortunately, no single matrix fulfills all requirements, and the use of comatrices offers a good prospect for improvement.

Use of an internal standard usually improves the accuracy and linearity of a calibration curve. An internal standard for MALDI quantification should have physical and chemical properties as close as possible to those of the analyte. A good choice for oligonucleotide quantification would be another oligonucleotide that differs by one nucleotide from the analyte.

We chose 5′-d(TCTTCCCTT) (CT9mer) and 5′-d(CTTTCCTCATCTTCCCTTT) (CT19mer) as internal standards to quantify 5′-d(CTTTCCTC) (CT8mer) and 5′-d(CTTTCCTCA TCTTCCCTT) (CT18mer). Our initial experiments made use of 1 μL (1 μM) of CT9mer mixed with 1 μL of CT8mer over a concentration range of 0.1 to 5 μM. When the amount of CT8mer loaded was plotted against the peak–height ratio, the calibration curve was found to be linear with a correlation coefficient (R^2) of 0.999 (Figure 4).

To test the broad applicability of this approach, we also used a 19mer, CT19mer as an internal standard to quantify an 18mer oligodeoxynucleotide (CT18mer). Similar analysis and data workup as for the shorter oligodeoxynucleotide gave a linear response, and the

Table 3. Fragments from the partial digest of A21mer with SVP

Peak	Sequence	Calculated ΔM (Da)	ΔM found (Da)	ΔΔM (Da)
1	5′-d(ACAGCAGGTCAAGAAGAGTAT)			
2	5′-d(ACAGCAGGTCAAGAAGAGTA)	304.2	304.6	0.4
3	5′-d(ACAGCAGGTCAAGAAGAGT)	313.2	313.3	0.1
4	5′-d(ACAGCAGGTCAAGAAGAG)	304.2	303.8	−0.4
5	5′-d(ACAGCAGGTCAAGAAGA)	329.2	329.7	0.5
6	5′-d(ACAGCAGGTCAAGAAG)	313.2	313.2	0.0
7	5′-d(ACAGCAGGTCAAGAA)	329.2	329.5	0.3
8	5′-d(ACAGCAGGTCAAGA)	313.2	313.1	−0.1
9	5′-d(ACAGCAGGTCAAG)	313.2	313.3	0.1
10	5′-d(ACAGCAGGTCAA)	329.2	329.3	0.1
11	5′-d(ACAGCAGGTCA)	313.2	313.6	0.4
12	5′-d(ACAGCAGGTC)	313.2	313.5	0.3
13	5′-d(ACAGCAGGT)	289.2	289.2	0.0
14	5′-d(ACAGCAGG)	304.2	304.3	0.1
15	5′-d(ACAGCAG)	329.2	329.4	0.2
16	5′-d(ACAGCA)	329.2	328.9	0.7
17	5′-d(ACAGC)	313.2	312.9	−0.3
18	5′-d(ACAG)	289.2	289.2	0.0
19	5′-d(ACA)	329.2	329.1	−0.1

correlation coefficient (R^2) for the calibration curve was found to be 0.998 (Figure 5). The concentration of the analyte was within a factor of 0.2–5 that of the internal standard, and a linear response was achieved. This outcome is adequate because often the analyte can be diluted or concentrated to fit the linear calibration range (0.1–5.0 μM).

The relative standard deviations (RSDs) for quantification of CT8mer and CT18mer are between 2.7–7.1 and 1.6–7.2 (Table 2). These results suggest the high sensitivity and mass resolving power that was achieved by using the modified matrices not only permitted the quantification of midsized oligonucleotides in the femtomole range, but also provided good reproducibility. The results of applying the smaller oligo CT8mer as internal standard to quantify the CT9mer (Table 2) show that there is no significant difference in the RSD between applying a standard that has one nucleobase less or more than in the analyte.

We also found that the internal standard should have a similar sequence to that of the analyte. Bruenner et al. [15] used a 36-base-pair oligonucleotide as an internal standard to quantify a 21-base-pair oligonucleotide. The authors showed a linear calibration over two orders of magnitude, but the errors at high concentration were relatively large (40%). These large errors compromise the ability to quantify over a large range, and they may be avoided by using a comparably sized oligonucleotide as internal standard.

Enzyme Digestion

Normal oligonucleotides. Sequencing large oligonucleotides by tandem mass spectrometry is difficult. To provide a sequencing procedure that complements the quantification method reported here, we evaluated sequencing by using exonucleases in a ladder method. We chose two enzymes, SVP and BSP, because these enzymes can remove one nucleotide at a time from the 3′ or 5′ end, respectively. Therefore, we expect the result-

Table 4. Fragments from the partial digest of A21mer with BSP

Peak	Sequence	Calculated ΔM (Da)	ΔM found (Da)	ΔΔM (Da)
1	3′-d(ACAGCAGGTCAAGAAGAGTAT)			
2	3′-d(ACAGCAGGTCAAGAAGAGTA)	313.2	313.1	−0.1
3	3′-d(ACAGCAGGTCAAGAAGAGT)	289.2	288.7	−0.5
4	3′-d(ACAGCAGGTCAAGAAGAG)	313.2	313.2	0.0
5	3′-d(ACAGCAGGTCAAGAAGA)	329.2	328.9	−0.3
6	3′-d(ACAGCAGGTCAAGAAG)	289.2	288.7	−0.5
7	3′-d(ACAGCAGGTCAAGAA)	313.2	313.2	0.0
8	3′-d(ACAGCAGGTCAAGA)	329.2	329.0	−0.2
9	3′-d(ACAGCAGGTCAAG)	329.2	329.5	0.3
10	3′-d(ACAGCAGGTCAA)	304.2	304.4	0.2
11	3′-d(ACAGCAGGTCA)	289.2	288.3	−0.9
12	3′-d(ACAGCAGGTC)	313.2	313.4	0.2

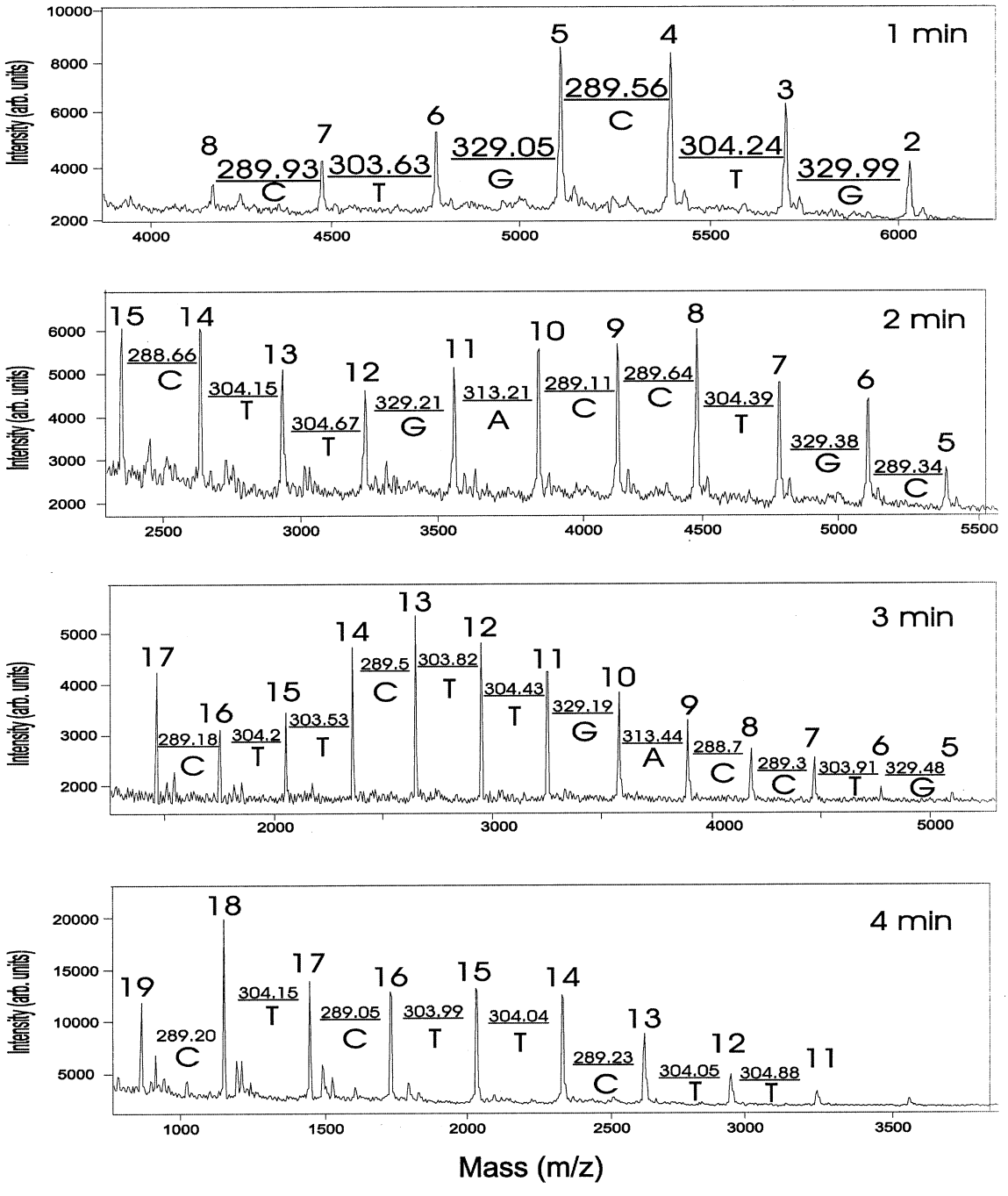

Figure 6. Time-dependent MALDI mass spectra of SVP digestion products of 5′-d(ATACTCTTCT-TGACCTGCTGT) (T21mer).

ing ladder spectra to be complementary. In a previous publication [20], the amounts of oligonucleotides consumed in the enzyme digestion are in the hundreds of picomoles. We expect that the modified procedure reported here (using a 2:1 AA/NA as matrix) can improve both the detection limit and the mass resolving power for the determination of the enzyme digestion products.

In our experiment, 40 pmol of each model oligonucleotide (C18mer, G18mer, A21mer, T21mer) was submitted to partial enzymatic digestion, and the time course of the digestion was followed by MALDI analysis. The results (see Tables 3 and 4 and Figures 6 and 7 for typical spectra) show that most sequence fragments for each model oligonucleotide can be seen. The complete sequence is obtainable by using both enzymes. The experimental masses of the fragments agree to within 1 Da of the calculated values. For the SVP digestion (Figure 6), the first eight nucleobases were revealed after 1 min. After 2 min, nucleobases at posi-

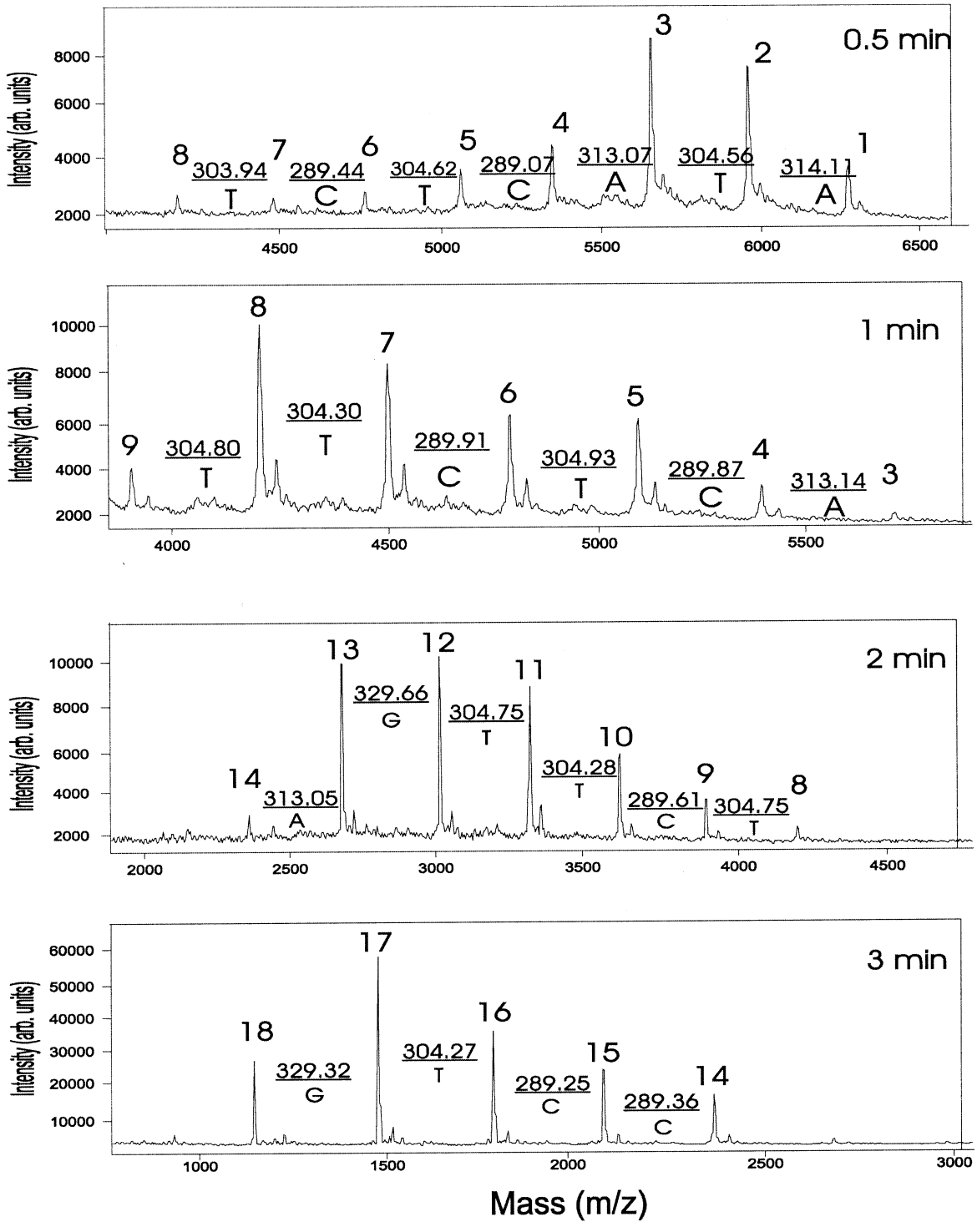

Figure 7. Time-dependent MALDI mass spectra of BSP digestion products of 5′-d(ATACTCTTCT-TGACCTGCTGT) (T21mer).

tions 5–15 were observed, and after 4 min, the sequence from nucleobases 11–19 was displayed. Time-dependent mass spectra of T21mer fragments produced by BSP digestion are shown in Figure 7. Within 3 min, the sequence of 18 nucleobases can be directly read from the 5′-end. We obtained similar results for partial digests of approximately 40 pmol of other model oligonucleotides (C18mer, G18mer, A21mer; spectra not shown here).

We found that the analyte/enzyme ratio, pH of the digestion media, and the reaction temperature are crucial for obtaining the complete oligonucleotide sequence by the ladder method. We usually used 10 mU

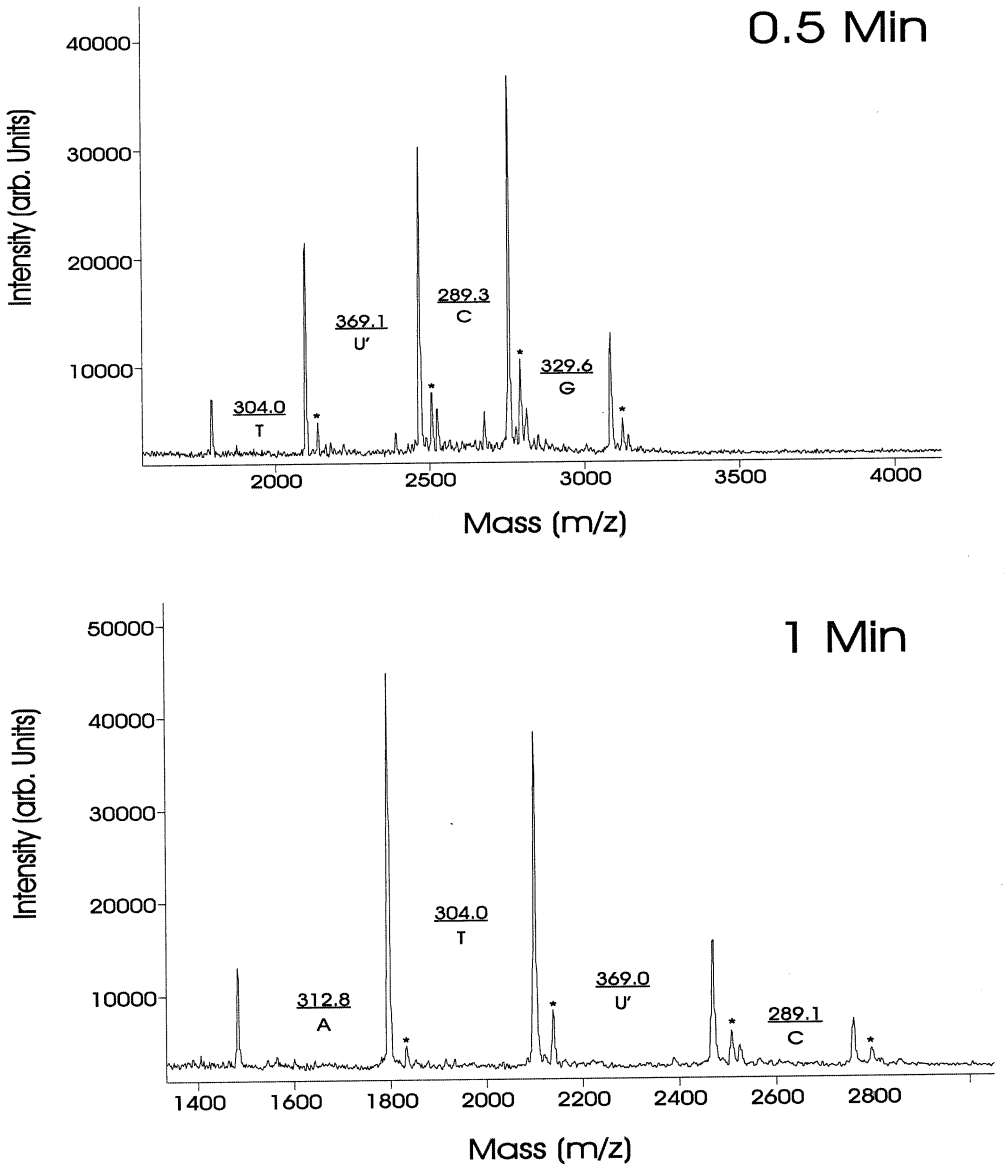

Figure 8. MALDI mass spectra of the products of BSP digestion of 5′-d(GCU′TAATTCG), where the U′ is a 5-bromo-2′-deoxyuridine.

SVP or BSP to digest 10–40 pmol of middle-sized oligos (10–21mers). Within 12 min, the digestion was complete to within the last three or four bases. The pH of the ammonium citrate buffer was adjusted to 9.4 with concentrated NH_4OH to allow for a moderate digestion rate. Without pH adjustment, only the first base was released after 10 min of digestion.

The optimum digestion conditions for BSP are pH 6.5 at 37 °C [40]. Under these conditions, however, the cleavage of the nucleobase is so fast that the peaks corresponding to ions formed by loss of the first several bases are missing or hard to read in the MALDI spectra. We performed the BSP digestion in aqueous solution without adjusting the pH, and obtained high quality spectral data (Figure 7). We insured that the buffer contained no added metal ions because metal–cation adduct ion of the oligonucleotide will cause peak broading in MALDI-MS. Oligodeoxynucleotides are more chemically stable in a salt form than in neutral form; thus, we modified the literature method [20] and performed the BSP digestion at room temperature instead of 37 °C to avoid degradation during the digestion.

Modified oligonucleotide. We anticipate that one application of this method is to probe modification of model oligonucleotides. Nucleobase modification is important because the structural integrity of DNA within cells is continuously being challenged by a variety of damaging agents, including chemicals, ionizing radiation, and UV irradiation [41]. We and others have demonstrated that mass spectrometry is a sensitive and accurate

method for characterization of modified oligonucleotides [20, 42]. Pieles et al. [43] successfully determined a 12-base oligonucleotide (5′-[GCTTXCTCGAGT], X = 2′-*O*-methyl adenosine) by MALDI and enzyme digestion, and they found that the BSP digestion is inhibited by the modification. Keough and co-workers [44] also developed a method that included failure sequence analysis, base hydrolysis, and nuclease digestion to characterize oligonucleotides containing modified phosphonate groups along their backbone.

We tested the applicability to the exonuclease ladder method to the structure characterization of a 10mer modified with 5-bromo-2′-deoxyuridine (d[GCU′TA-ATTCG]). We found that the site of modification can be determined by using either BSP or SVP digestion for several minutes (Figure 8). The calculated mass for 5-bromo-2′-deoxyuridine is 368.9, which agrees well with the mass we found (369.1). The complete sequence of this oligo was confirmed with both BSP and SVP digestion (mass spectra from the SVP digestion are not shown here). The small peaks with stars in Figure 8 correspond to the potassium adducts of the oligo fragments. Neither BSP nor SVP digestion was inhibited by the base modification.

Conclusions

The properties of anthranilic acid, which effectively absorbs the energy from a UV-laser pulse, and nicotinic acid, which cocrystallizes well with oligodeoxynucleotides, makes the combination of these two matrices with diammonium citrate (2:1:0.003) a significant improvement for the MALDI analysis of oligonucleotide and DNA mixtures at low concentration. Compared to other matrices routinely used (e.g., 3-HPA), the analyte signal intensities and mass resolving power were demonstrated to be better.

Applying this modified matrix, we also demonstrated the potential for quantitative analysis of small to middle-sized, mixed-base oligonucleotides at low picomole to femtomole level using MALDI. The choice of matrix and an internal standard that has physical and chemical properties as close as possible to those of the analyte are crucial for successful MALDI quantification of oligodeoxynucleotides.

Full sequence information is obtainable using the enzymatic degradation of normal and of one modified oligonucleotide at low levels (10–40 pmol) and MALDI analysis. This is evidence that the method will allow ladder sequencing of oligodeoxynucleotides enzymatically from in vitro or in vivo samples. We are continuing the method development for oligonucleotides that have been modified photochemically or by steroid–quinone electrophiles.

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