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Improvement of Resolution, Mass Accuracy, and Reproducibility in Reflected Mode DE-MALDI-TOF Analysis of DNA Using Fast Evaporation−Overlayer Sample Preparations

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DNA analysis by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry is hindered by two processes: alkali metal adduction and fragmentation of the intact ionized molecule. The adverse effects of both processes can be reduced by adding ammonium ion salts or compounds such as fructose to the sample preparations. Matrix additives improve sensitivity and resolution of DNA analysis by MALDI. In addition, spot-to-spot reproducibility, resolution, and mass accuracy for DNA oligonucleotides (≤12 mer) can be improved by the use of overlayer sample preparations with matrixes that have low aqueous solubilities, such as α-cyano-4-hydroxycinnamic acid, ferulic acid, and 2,4,6-trihydroxyacetophenone. For example, resolution for 5−12-mer oligonucleotides is greater than 7000 using overlayer matrix preparations and mass accuracy values are well below 20 ppm. In addition to these methods, a new method for analyzing DNA in positive ion mode is reported using acidified 3-hydroxypicolinic acid. This method does not lose sensitivity for higher mass oligonucleotides as quickly as overlayer methods, and spectra retain >6000 resolution and mass accuracies of ∼20 ppm between different overlayer depositions.

Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry[1] has become a routine method for the analysis of peptides and proteins, but the analysis of DNA is still quite challenging.[2] The initial reports on MALDI of DNA focused on matrixes that were compatible with DNA analysis, but more recent studies have focused on improving the signal characteristics by decreasing fragmentation and alkali metal adducts in order to lower detection limits and increase mass accuracy. It is generally recognized that the major obstacles to DNA analysis by MALDI are related to formation of alkali metal ion adducts and that the DNA ions are formed with excess internal energy resulting in fragmentation.[3−9] Although efforts aimed at discovery of new matrixes compatible with DNA have yielded significant advances, a more successful approach has been in the area of combinations of matrix, matrix additives, sample substrate, and sample deposition methods that minimize formation of adduct ions while lowering the internal energy of the ions produced. Hillenkamp and co-workers have also shown that infrared (IR) MALDI may have distinct advantages for the analysis of DNA, especially for very large DNA strands.[10−11]

The most commonly used ultraviolet MALDI matrixes for DNA are 3-hydroxypicolinic acid,[12] picolinic acid,[13] 2,5-dihydroxybenzoic acid,[14−15] 2,3,4- or 2,4,6-trihydroxyacetophenone,[16−18] and 6-aza-2-thiothymine.[19] In addition, Hathaway[16] reported successful use of sinapinic acid and of α-cyano-4-hydroxycinnamic acid as matrixes for DNA oligonucleotides; however, it has been our experience that use of these matrixes alone leads to excessive fragmentation and alkali metal adducts. Clearly, the presence of fragment ions in the MALDI mass spectra has considerable utility, because the presence of intact protonated or deprotonated molecule ions and fragment ions can be used for molecular weight determinations and sequencing from individual spectra.[20−23] The composition and

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partial (if not complete) sequence can be determined by "in-source" fragmentation of DNA oligonucleotides; however, alkali adducts must be eliminated, so that the protonated or deprotonated DNA ion is detected with a sufficient signal-to-noise ratio to perform accurate calibrations.[24] Furthermore, fragmentation must be controlled to balance between sequence determination and signal for $(M + H)^+$ or $(M - H)^-$ ion yields.

Matrix additives have proven to be very effective for controlling the abundances of alkali adducts and fragment ions. Of the numerous matrix additives reported, ammonium salts appear to be the most useful for reducing alkali metal binding to the phosphate backbone and decreasing the amount of fragmentation.[25−26] Simmons and Limbach[27] argued that matrix additives which promote negative ion formation have a higher affinity for the phosphodiester backbone of DNA than alkali metals and higher proton affinities than the DNA bases to prevent adduct formation and fragmentation, respectively. Comparative studies of different ammonium salts indicate that diammonium hydrogencitrate and ammonium fluoride are the most successful;[25,26,28] however, other amine-containing matrix additives, including spermine, triethylamine, imidazole, and piperidine, have been successfully used.[27,29−30]

The chemical properties of the surface can also be used to enhance the DNA ion signal in MALDI. Ion yields and mass resolution from hydrophobic surfaces, e.g., Parafilm[31] and Teflon,[32] are better than those obtained from stainless steel surfaces. Improved ion yields have also been demonstrated with nitrocellulose films[33] and a perfluorosulfonate membrane, Nafion.[34,35] These hydrophobic surfaces appear to work by corralling the droplet and forcing it to concentrate as it dries. Silicon wafers with etched wells[36] or with derivatized surfaces[37] are also used to obtain high resolution and high reproducibility for repetitive DNA analysis, presumably also by a corralling mechanism.

Independent of matrix, additive, and sample support surface, the most commonly used sample preparation method is dried droplet deposition.[1] Fast evaporation methods reported by Vorm et al.[38] and variations reported by Edmondson and Russell[39] and Dai et al.[40] produce higher resolution spectra for peptides. Here, we show that a similar sample preparation greatly improves analysis of oligonucleotides. In addition to increased resolution, the subsequent increased mass accuracy for peptides[39,41−43] should also be possible for oligonucleotides. Thus far, the overlayer, fast evaporation deposition method has been used to analyze only chemically modified DNA oligonucleotides, where "charge-tagged" analytes are cocrystallized with the methyl ester of α-cyano-4-hydroxycinnamic acid.[5,9] Improvements in resolution and mass accuracy have been reported for larger DNA oligonucleotides (17−35 mer) using an overlayer method;[44] mass resolutions of up to 1300 and mass measurement errors less than 100 ppm are reported. The matrix preparation in that study is a modification of the Xiang and Beavis[45] crushed crystal method, which is another type of overlayer preparation.

In this paper, the overlayer preparation method is examined using three matrixes: α-cyano-4-hydroxycinnamic acid, ferulic acid, and 2,4,6-trihydroxyacetophenone. The effect of the matrix additives (diammonium hydrogencitrate and triammonium citrate) on crystallization is investigated by fluorescence microscopy. We also examine the effects of using fructose as an additive. Previous work has suggested that fructose effectively reduces the internal energies of the desorbed ions, reducing the abundance of fragment ions and increasing the yield of the protonated or deprotonated molecules.[46−49] In addition, a new dried droplet method using acidified 3-hydroxypicolinic acid is also reported. These new sample preparation methods are evaluated using the following criteria: sample deposit morphology, ion yield reproducibility, alkali metal adducts, fragmentation, resolution, and mass accuracy.

EXPERIMENTAL SECTION

Three DNA oligonucleotides, which were purchased from OnlyDNA (Midland, TX; http://www.onlydna.com), are used in these studies: dGGATC (mass 1502), dAACCGTT (mass 2078), and dAACAGATCGGTT (mass 3667). Another oligomer, TGGGGTT (mass 2166) was purchased from Midland Certified Reagent Co. (Midland, TX). All matrixes and matrix additives are purchased from Sigma (St. Louis, MO) and Aldrich (Milwaukee, WI). The matrixes include 2,4,6-trihydroxyacetophenone (THAP),

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Table 1. List of All Combinations of Matrix Concentrations and Matrix Additives Examined in Overlayer Sample Preparations[a]

underlayer matrix A	overlayer matrix B. mg/mL	matrix additive C
ferulic acid	6	none
(30 mg/mL)	30	10 mg/mL $(NH2)_2$hcitrate
	80	10 mg/mL $(NH2)_3$citrate
		3 mg/mL fructose + 10 mg/mL $(NH2)_2$hcitrate
α-CHCA	6	100 mg/mL $(NH2)_2$hcitrate
(30 mg/mL)	30	100 mg/mL $(NH2)_3$citrate
		10% formic acid (aqueous)
2,4,6-THAP	8	
(80 mg/mL)	16	
	40	
	80	

[a]All combinations of the matrix B and matrix additive C solutions were mixed 1:1:1 with analyte and tested with reflected mode analysis.

α-cyano-4-hydroxycinnamic acid (αCHCA), ferulic acid, 2,5-dihyroxybenzoic acid, 6-aza-2-thiothymine (ATT), and 3-hydroxypicolinic acid (3-HPA). The matrix additives are restricted to diammonium hydrogencitrate, triammonium citrate, and fructose. In some samples, formic acid is also used.

Samples are prepared by a modified overlayer method, which was previously described.[36,37] Table 1 lists all of the overlayer solutions that are examined. The matrix solution B, matrix additive C, and sample at 10 pmol/μL are mixed 1:1:1; then 0.5 μL is deposited on a bed of matrix deposited previously from concentrated matrix solution A. Solutions A and B are prepared in HPLC grade methanol. All combinations of matrix B and additive C solutions are mixed 1:1:1 with analyte and tested with reflected mode analysis. The DNA concentration is chosen so that 1.67 pmol, an amount well above the detection limit of the instrument, is deposited on the sample stage. The matrix concentrations are chosen so that matrix-to-analyte ratios of 1000:1−15 000:1 can be examined. Also, the higher concentrations of matrix are similar to those used for current dried droplet deposition, so comparisons can be made between these overlayer samples and dried droplet depositions.

Dried droplet preparations are made using the following matrix solutions: 40 mg/mL 2,5-dihydroxybenzoic acid, 6-aza-2-thiothymine, or 3-hydroxypicolinic acid in 85% aqueous diammonium hydrogencitrate (10−20 mg/mL) and 15% acetonitrile. In addition, a new dried droplet method is characterized using 3-hydroxypicolinic acid dissolved in 0.5% aqueous formic acid and mixed 1:1:1 with DNA analyte and aqueous diammonium hydrogencitrate (10 mg/mL).

All spectra are acquired using a Perseptive Biosystems Voyager Elite XL (PE Biosystems, Framingham, MA) equipped with delayed extraction and a nitrogen laser.[50] Reflected mode acquisition is used to monitor both negative and positive ions. Accelerating voltage is set at either 20 kV in negative ion mode or 25 kV in positive ion mode, the grid voltage is 69−71% of the acceleration voltage, and the guide wire is set to 0.010−0.025% of the acceleration voltage. Resolution and mass accuracy were comparable for the same matrix preparations, but signal-to-noise ratios were higher in positive ion mode, so it was used primarily. For studying resolution and mass accuracy, laser fluences were above but still near the threshold for production of DNA ions so that peaks with signal-to-noise ratios greater than 10 are observed in the spectra. For the studies of fragmentation and alkali metal adduct formation, the laser fluence was increased significantly above the threshold.

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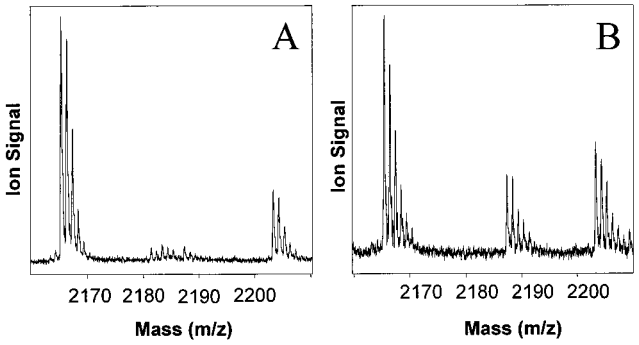

Figure 1. MALDI spectra of TGGGGTT obtained from dried droplet depositions using 3-hydroxypicolinic acid (A) and 2,5-dihydroxybenzoic acid (B).

Spectra are collected and calibrated in Perseptive Grams386 (Galactic Salem, NH). Resolution measurements are always marked at the baseline, giving true full width at half-maximum (fwhm) values. Signal-to-noise ratios are determined by selecting at least a 15 mass unit window with the peak of interest at the far left. Mass accuracies are determined after internal calibration using the monoisotopic peaks.

Fluorescence micrographs are acquired using a Nikon Eclipse E800 microscope fitted with epifluorescence with illumination from a mercury arc lamp. Images are acquired with a Photometrics Sensys 8073 CCD camera and imported with Metamorph software. Images are recorded using Nikon Plan Fluor 4X and 10× objectives creating 40× and 100× images of the sample depositions. For 3-HPA, the FITC filter set is used. The excitation band is at 470 nm, and emission is observed at 515 nm. For α-CHCA and THAP, the UV-2A filter set is used for illumination at 330−380 nm and fluorescence observation at 420 nm. Images are cropped with Paint Shop Pro 5.0 (Jasc Software, Eden Prairie, MN).

RESULTS AND DISCUSSION

Traditional dried droplet sample deposition methods using 2,5-DHB, ATT, and 3-HPA are tested with ammonium citrate matrix additives. In Figure 1, representative spectra are shown for 3-hydroxypicolinic acid (A) and 2,5-dihydroxybenzoic acid (B) dried droplet depositions. All of the dried droplet sample deposits are similar in morphology, but 3-HPA crystals appear to be more uniform in shape and size. For example, the 2,5-DHB and ATT deposits often do not thoroughly cocrystallize with the matrix additive, because two distinct crystal morphologies are observed by microscopy.

The mass spectra obtained from each dried droplet preparation varies considerably in relative abundances of alkali metal ion adducts. The general trend observed for relative abundance of alkali metal ion adducts is 2,5-DHB > 3-HPA > ATT. Alkali metal

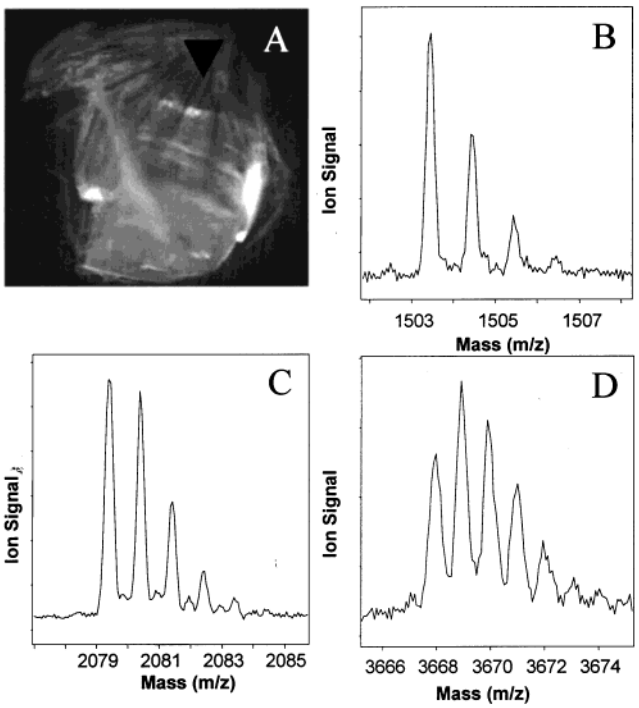

Figure 2. Fluorescence micrograph (A) and positive ion MALDI spectra of three oligonucleotides, GGATC (B), AACCGTT (C), and AACAGATCGGTT (D) obtained from acidified 3-hydroxypicolinic acid dried droplets on Parafilm.

adducts produce approximately 46.8, 19.5, and 10.5% of the signal from the ionized molecule from 2,5-DHB, 3-HPA, and ATT, respectively. Fragment ions are observed in greater abundance in spectra obtained using 2,5-DHB as compared to the other matrixes we have examined using dried droplet preparations.

The resolutions observed for 5- and 12-mer oligonucleotides in traditional dried droplet samples are in the range 4000−7500 and 6000−9500, respectively. The resolution in ATT spectra is the lowest of the dried droplet preparations: 4000−5500 on the 5 mer, dGGATC. The ion yields for 3-HPA deposits are generally more reproducible and the resolution (>6000 for the 5 mer) and mass accuracy is better than the other two matrixes. Flight time reproducibility in dried droplet preparations is ∼20 ppm within the same deposition and 50 ppm between different depositions. With internal calibration, dried droplet methods yield average mass accuracies between 8 and 18 ppm.

We also compared dried droplet sample preparations using the concentrating effect of Parafilm[31] in combination with acidified 3-HPA with results obtained using conventional dried droplet deposits. A representative dried droplet deposition is shown in Figure 2A; the best spectra are obtained from the area marked with the white triangle, where the crystals are shaped like long blades radiating out from one center. The matrix crystals do not begin to form until after the solution is evaporated to about half its original volume. The formation of the crystals then occurs slowly as the remaining solvent evaporates. This method of sample preparation seems to work very well, presumably because the slow evaporation of solvent and rate of crystallization yields a more even distribution of the DNA analytes in the crystal lattice.

The most intense signals in spectra acquired from Parafilm, acidified 3-HPA deposits correspond to the singly and doubly protonated molecule, and very little fragmentation is observed. For example, the only fragmentation that is seen with any regularity is loss of guanine. Furthermore, this method yields minimal amounts of alkali adduct ions, and in most cases this is less than 5% of the total ion yield.

In positive ion mass spectra obtained from acidified 3-HPA dried droplet depositions on Parafilm, resolution values range from 6950 to 7350 for the 5 mer and from 8500 to 10 500 for the 12 mer. Representative spectra for the 5, 7, and 12 mer are shown in Figure 2B−D, respectively. Although mass resolution is improved with this sample preparation method, the mass accuracy using external calibration is about the same as that for dried droplet sample preparation methods; i.e., spot-to-spot reproducibility is poor (20−50 ppm flight time variations). On the other hand, the mass accuracy values for internal calibration are better than the traditional dried droplet methods tested, most likely because S/N ratios are higher due to better ion yields.

The overlayer sample preparation method appears to give the best mass spectral data; e.g., the ion yields are higher and more reproducible both in terms of laser shot-to-shot and from sample spot-to-spot when compared to dried droplet preparations. Overlayer sample depositions appear to be more homogeneous when prepared from solutions with lower matrix concentrations. In fact, overlayer crystals prepared from solutions containing higher matrix concentrations are very similar in appearance to those prepared as dried droplet samples, and these deposits do not appear to cocrystallize with matrix additives. From these solutions, we observe rapid deposition of a large portion of the matrix prior to crystallization of the matrix additive. A similar observation is made for dried droplet deposits prepared from highly concentrated THAP overlayer depositions (40−80 mg/mL matrix). However, the matrix and analyte rapidly coprecipitate when a drop of solution is placed on the sample stage, and matrix additive crystals are not observed with microscopy after the solvent is completely evaporated (Figure 3).

Fluorescence micrographs for two overlayer deposits of THAP at 16 (A) and 80 mg/mL (B) are shown in Figure 3. The deposit shown in Figure 3A contains weblike fine crystals, whereas crystals contained in Figure 3B more closely resemble dried droplet deposits in physical appearance, spectral resolution, and signal reproducibility. Mass spectra acquired at points C and D are shown in Figure 3 to illustrate the difference in resolution (almost 2-fold improvement) for the deprotonated molecule of dAACCGTT. Similar results are observed in positive ion mode as well.

In general, significant reductions in alkali metal adduct ions from αCHCA and ferulic acid matrixes are only observed at high additive concentrations, and under these conditions, the matrix bed dissolves upon addition of the overlayer sample. The sample deposit no longer appears homogeneous (Figure 4), and it more closely resembles deposits prepared by dried droplet methods in appearance, resolution, and S/N ratios. The overlayer deposits shown in Figure 4 were obtained by (A) mixing aqueous 10 mM DNA, 10 mg/mL aqueous diammonium hydrogencitrate, and 6 mg/mL matrix in methanol and (B) the same as (A) but 100 mg/mL aqueous matrix additive in an effort to reduce the amount of alkali adduct ions. The first deposit (Figure 4A) appears to be more homogeneous, but spectra obtained from these spots contain abundant alkali metal adduct ions. Mass spectra obtained from

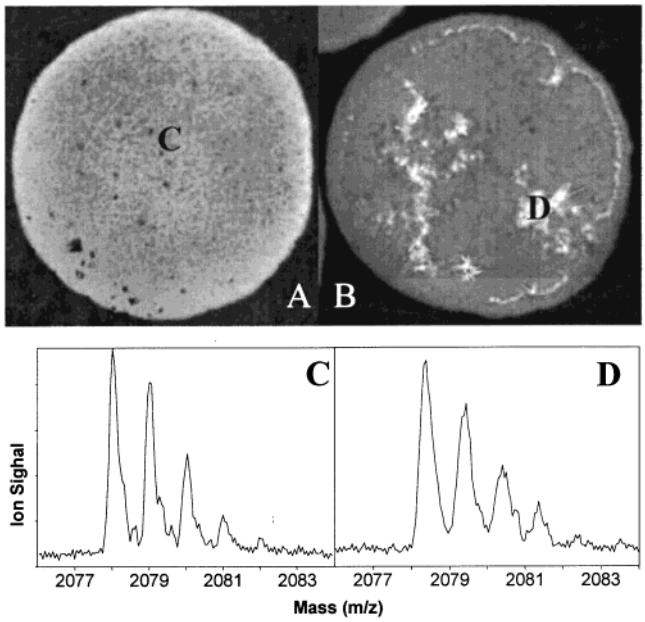

Figure 3. Fluorescence micrographs of two overlayer preparations made with 2,4,6-THAP with mass spectra acquired from the indicated locations. (A) 16 mg/mL THAP in the overlayer deposit and (B) 80 mg/mL THAP in the overlayer deposit.

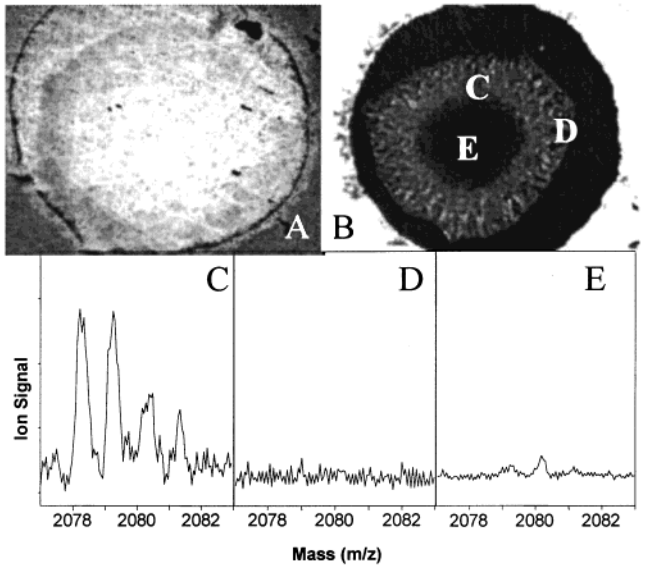

Figure 4. Fluorescence micrographs of two overlayer preparations made with α-cyano-4-hydroxycinnamic acid with mass spectra acquired from the indicated locations. (A) 10 mg/mL ammonium matrix additive and (B) 100 mg/mL ammonium matrix additive.

the second deposit (Figure 4B) contain fewer alkali adduct peaks. Note that the matrix bed has been redissolved, and the mass resolution of spectra acquired from these preparations is degraded. As can be seen in the spectra (Figure 4C−E), signals are only obtained from the interface region (C) between the bed of matrix (E) and the elongated ammonium matrix additive crystals (D). The maximum concentrations of matrix additive that can be tolerated without redissolving the underlayer are between 10 and 20 mg/mL in the overlayer solution.

Ferulic acid and αCHCA both exhibit a rapid decrease in ion yield as the length of the oligonucleotide strand increases. THAP spectra also show decreasing S/N ratios with increasing strand

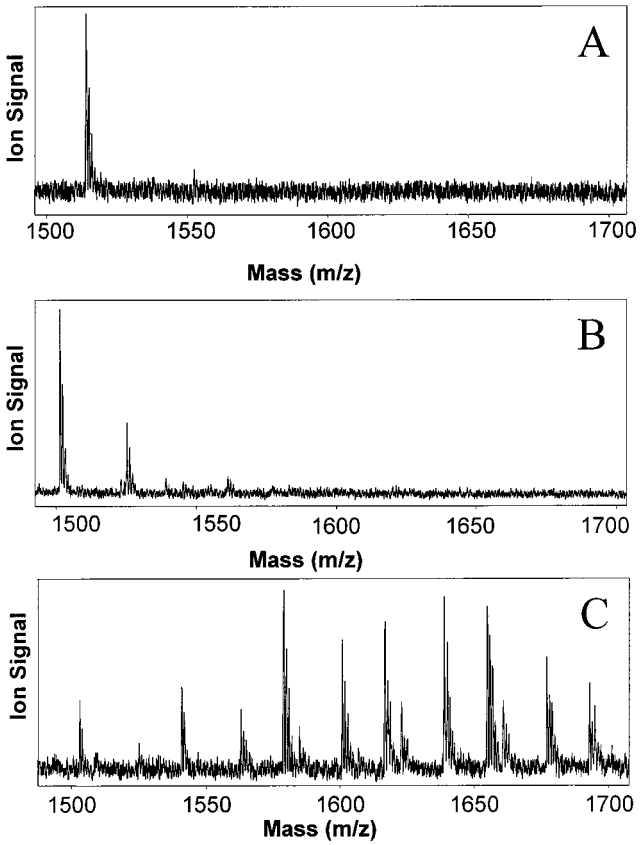

Figure 5. Alkali adduct formation observed in positive ion mass spectra of 5-mer DNA from 2,4,6-THAP (A), αCHCA (B), and ferulic acid (C) samples with 10 mg/mL diammonium hydrogencitrate additive.

length, but not to the extent that is observed for the other two matrixes. Signals for the protonated or deprotonated 12-mer ion could not be obtained at S/N ratios greater than 10 from αCHCA or ferulic acid, whereas the S/N ratios for the 5 mer are frequently in excess of 100.

The two factors that seem to limit the utility of MALDI for DNA analysis are formation of alkali metal adduct ions and fragmentation. The abundance of adduct ions increases in the following order, THAP < αCHCA < FA, whereas fragmentation increases as follows: THAP < FA < αCHCA. The abundance of alkali metal adduct ions of dGGATC obtained using these three matrixes can be seen in Figure 5 A−C, respectively. In these spectra, some adducts are observed in αCHCA spectra, while the signal for the protonated molecule in ferulic acid is small compared to the adduct ion signals. The abundance of fragment ions also depends on the amount of matrix used. As the matrix-to-analyte (M/A) ratio is varied from 1000:1 to 15000:1 for the two cinnamic acid derivatives, the protonated or deprotonated molecule signal decreases significantly.

Fragmentation of DNA analytes is a major problem for both αCHCA and ferulic acid when compared to THAP. Both αCHCA and ferulic acid produce more prompt and metastable fragments than THAP. Samples made with ferulic acid (Figure 6A) and THAP (B) both show significant loss from the signal of the protonated molecule due to fragmentation. The fragment ion yield increases with increased matrix concentration in the overlayer, and ferulic acid induced more fragmentation than THAP. Spectra acquired

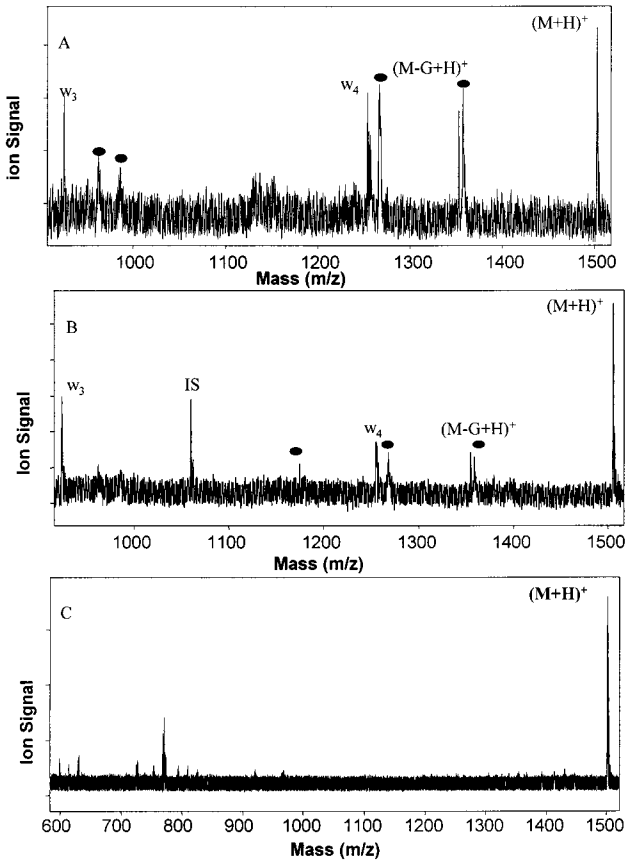

Figure 6. Positive ion mass spectra acquired using ferulic acid (a) and 2,4,6-THAP (B) matrixes. The black circles indicate metastable ions, while the identities of the prompt fragments are indicated. The matrix/additive combination in (C) includes ferulic acid and fructose to demonstrate the reduced fragmentation.

from αCHCA overlayer samples show even higher fragment ion yields and decreased signals for the $(M + H)^+$ of the DNA when compared to the other two matrixes.

Addition of fructose to the matrix significantly decreases the amount of fragmentation observed for DNA samples; however, the observed effect is greatest for ferulic acid and THAP samples and almost no decrease in fragmentation is observed for αCHCA. A positive ion spectrum (Figure 6C) of dGGATC clearly illustrates the effect fructose has on ferulic acid samples. Note the decreased fragmentation obtained from an overlayer deposit of ferulic acid with diammonium hydrogencitrate and fructose compared to Figure 6A and B. When added at low concentrations (up to ∼6 mg/mL), fructose does not appear to influence the crystallization process or degrade resolution or mass accuracy. Wilkins and coworkers previously used fructose to reduce the amount of fragmentation for peptides ionized by MALDI and mass analyzed by Fourier transform ion cyclotron resonance (FTICR) mass spectrometry.[47,48] They explained the stabilization of protein ions in terms of gas-phase collision processes involving laser desorption/degradation of the fructose; however, the fact that fragmentation is observed with some matrixes and not with others is not totally consistent with their explanation. More detailed studies on the mechanism by which fructose and other additives alter the internal energies of MALDI formed ions are underway.

In general, spectral quality examined in terms of signal-to-noise ratios, resolution, and mass measurement accuracy is improved

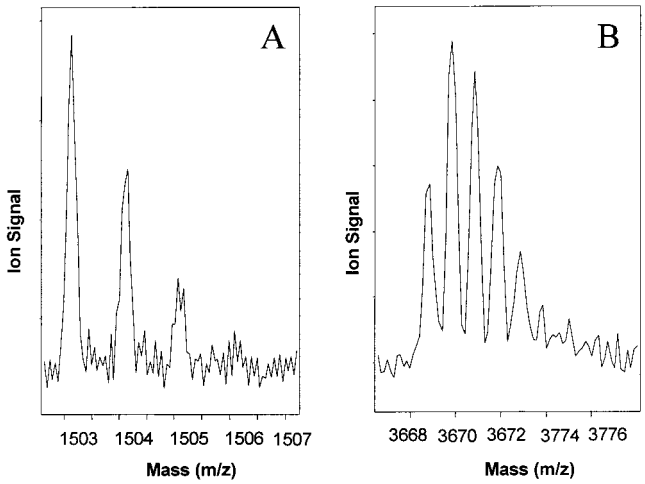

Figure 7. MALDI spectra of (A) 5 mer, dGGATC, and (B) 12 mer, dAACAGATCCGTT, DNA obtained from 2,4,6-THAP overlayer deposits.

and more persistent in overlayer samples when compared to dried droplets. Ion signal appears to be more evenly distributed in the overlayer preparations, and its quality is significantly more reproducible within the same sample depositions and between multiple depositions. We routinely see resolution increase by a factor of 1.5 and in some cases by as much as a factor of 3. These results are consistent over the entire surface of the overlayer deposition and spot-to-spot reproducibility is high as well. The resolution values calculated for overlayer samples of 5 mer are 7000−8700 and those for the 12 mer are 6600−14000; two spectra of DNA oligomers, dGGATC (Figure 7A) and dAACAGATCGGTT (B), show resolution, and S/N ratios characteristic of overlayer THAP preparations. The resolution on the 5 mer is 8700 at S/N 27.2 and the resolution on the 12 mer is 9532 at S/N 31.5. The best resolution values are obtained in spectra with S/N ratios between 20 and 40. The lowest resolution values for the 12-mer oligonucleotide are obtained from noise-broadened signals in αCHCA and ferulic acid.

In addition to increased resolution, mass measurement accuracy is also improved for overlayer deposits when compared to dried droplet deposition. The flight time reproducibility from within the same overlayer deposition is very high, usually ∼5 ppm, and spot-to-spot reproducibility ranges between 10 and 25 ppm in most cases. The advantage of overlayer deposits is only realized when the matrix bed is not redissolved by the overlayer solution. Spectra from THAP matrix overlayer sample depositions (Figure 8A) have much better resolution and higher reproducibility in flight time and intensity than dried droplet samples (B). On the basis of these data, the overlayer method can be used for significantly better external calibrations. With internally calibrated overlayer spectra, the average mass accuracy values lie between 3 and 8 ppm. So, the overlayer methods also improve data obtained using internal calibration. In both cases, the highest mass accuracy values are obtained from spectra that have S/N ratios higher than 10.0 for all peaks and similar intensities for the two calibrant peaks.

CONCLUSIONS

MALDI mass spectra of DNA samples prepared by fast evaporation−overlayer matrix deposition can be acquired at

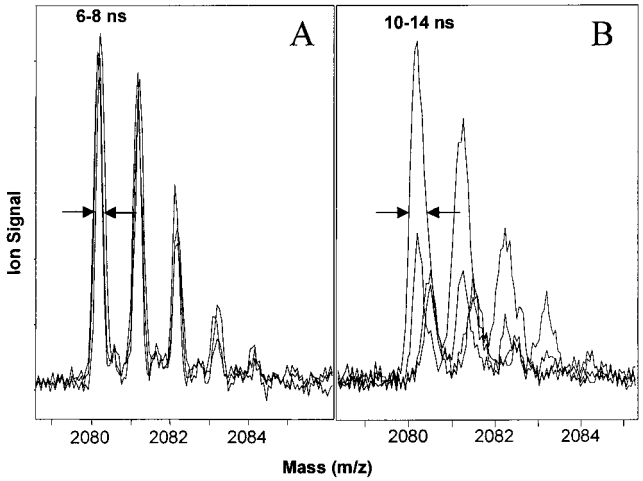

Figure 8. Comparison of resolution and flight time reproducibility in overlayer (A) and dried droplet (B) methods using 2,4,6-THAP matrix.

sufficiently high resolution to facilitate accurate mass measurement. These mass spectra also contain fragment ions that can be used to assign composition and sequence to DNA oligomers. Although 2,4,6-trihydroxyacetophenone is shown to be the best matrix for such studies, ferulic acid (6 mg/mL overlayer concentration) can also be applied to DNA analysis. The best results with ferulic acid are obtained with fructose added to reduce fragmentation.

The data can be as good from dried droplet samples, but the laser spot-to-laser spot variations in signal and resolution are much higher. However, the mass accuracy is not as good as overlayer methods, because of the lack of homogeneity of the surface. The spot-to-spot signal reproducibility and analyte ion flight times observed by fast evaporation−overlayer methods are higher than that of dried droplet methods. Also, in individual sample depositions, the fast evaporation, overlayer method produces much more homogeneous crystallization and more even incorporation of the ssDNA analyte.

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