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A comparative study on methods of optimal sample preparation for the analysis of oligonucleotides by matrix-assisted laser desorption/ionization mass spectrometry

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Metal adducts (*e.g.*, Na^+ and K^+) significantly hinder the analysis of oligonucleotides by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Although a number of sample purification methods exist, to date no comparative study exists to determine the most efficient method for purifying oligonucleotides. The objective of this work was to perform such a study. Several different oligonucleotide samples were synthesized. Aliquots of these samples were then purposely contaminated with sodium acetate to generate representative contaminated (salted) oligonucleotide samples. A number of popular oligonucleotide purification techniques were then tested as to their effectiveness at removing Na^+ from the salted samples. The effectiveness of Na^+ removal was qualitatively assessed by comparing the MALDI mass spectra of the original sample, the salted sample, and the salted sample after purification. Micropipet tips packed with C_{18} reversed-phase packing material (*e.g.*, Zip Tips) appear to be the most effective means of purifying the oligonucleotides investigated. Minidialysis was found to be an effective alternative for purifying higher molecular weight oligonucleotides (> 10 000 u).

Introduction

Samples of oligonucleotides and nucleic acids often contain numerous metal cations and other contaminants. Alkali metal cations, such as Na^+ and K^+, are of particular concern as they may be present either in the biological system from which the sample was isolated, or as a component of a buffer solution used to prepare the sample for analysis, or they will arise as natural contaminants in the glassware and other equipment used during the handling and storage of such samples. Accurate characterization of oligonucleotides and nucleic acids by modern mass spectrometric methods requires that such impurities be reduced to as low levels as possible. In particular, matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) and electrospray ionization mass spectrometry (ESI-MS) have been shown to be extremely sensitive to the presence of metal contaminants during the analysis of oligonucleotides.[1–5]

Typically, low molecular weight oligonucleotides contaminated with metal cations exhibit adduct peaks in the mass spectrum, *e.g.*, $[M - 2H + Na]^-$, in addition to the parent molecular ion peak, $[M - H]^-$. Salt adducts for higher molecular weight oligonucleotides and nucleic acids often cannot be resolved from the unadducted parent molecular ion, resulting in a shift in the average mass of the ion to higher *m/z* values. In addition, the presence of metal cations serves to reduce the sensitivity of the mass spectral measurement as the ion current due to the analyte is divided into multiple *m/z* channels (typically one *m/z* value per adduct). As a result, the usefulness of subsequent molecular weight determinations is predicated on the ability to effectively reduce the presence of salt contaminants to levels which do not interfere with the mass spectral analysis.

A wide variety of sample desalting methods have been demonstrated for ESI- and MALDI-MS analysis of oligonucleotides. Methods common to both approaches include precipitation of the oligonucleotide as its ammonium salt from ethanol[6,7] and high performance liquid chromatography (HPLC).[8–12] With ESI-MS, ammonium acetate precipitation followed by the addition of solvent additives, such as triethylamine or imidazole, has proven to be a particularly effective approach to reducing cation adducts.[13,14] More recently, microdialysis has been shown to be useful for the removal of salt adducts from higher molecular weight oligonucleotides including those obtained *via* the polymerase chain reaction (PCR).[15–18]

Unlike ESI-MS, numerous approaches have been proposed for cation reduction in MALDI-MS. The first and still common approach has been the use of ammonium-activated cation-exchange resin beads which can be added to the sample–matrix mixture prior to spotting on the MALDI sample plate or which can be added to the spotted mixture prior to crystallization.[19] Incorporation of ammonium salts as co-matrices in MALDI for efficient alkali metal cation suppression has been reported and ammonium salts are now a standard component of oligonucleotide matrix preparations.[20,21] The addition of organic base solutions, as used previously in ESI-MS, as co-matrices has also been shown to reduce cation adduction in MALDI-MS.[22–24]

Several other approaches to sample purification have been performed prior to MALDI-MS, although most of these studies aimed to eliminate contaminants present in addition to metal cations (*e.g.*, dNTPs present from polymerase chain extension reactions). These additional approaches include spin columns[25] and molecular weight cut-off (MWCO) membranes.[26,27]

While standard practice in the MALDI-MS analysis of oligonucleotides now involves the preparation of matrix solutions with ammonium salts and the occasional use of cation-exchange resin beads for lower molecular weight oligonucleotides and ammonium acetate precipitation for higher molecular weight oligonucleotides, to our knowledge no comparative study exists which evaluates the performance of these various sample desalting methods as utilized prior to MALDI-MS analysis. Therefore, in this work we evaluated the majority of the common salt reduction approaches prior to MALDI-MS. Experiments were performed on purposefully contaminated (with sodium acetate) oligonucleotides of varying molecular weight and sequence. We found that the newly developed and

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marketed micropipet tips containing C_{18} resin (*e.g.*, ZipTips) are the most effective approach for desalting low and higher molecular weight oligonucleotides provided that the analyte can be recovered from the C_{18} resin. Higher molecular weight oligonucleotides are more problematic to characterize, although the results of our studies indicate that minidialysis, although time consuming, is an effective sample desalting approach.

Experimental

Oligonucleotides

Trityl-on oligonucleotides of dT_5 and dT_{36} were synthesized using a Perkin-Elmer/Applied Biosystems (Foster City, CA, USA) Model 394 DNA/RNA synthesizer and purified using Sep-Pak cartridges. Phosphoramidites and all synthesis reagents were obtained from ChemGenes (Waltham, MA, USA) and standard phosphoramidite chemistry was used. Two mixed-base oligonucleotides, dTACTG and dTATCGATAGGTCAGCTTCTATCGATCTAACTGCATGTC (refered to as the mixed-base 38-mer), were purchased from LSU Gene Labs (Baton Rouge, LA, USA). All samples were prepared at a final concentration of 100 μM in nanopure water. To achieve a representative 'salted' sample, a portion of each oligonucleotide was contaminated by the addition of sodium acetate to the oligonucleotide solutions. Addition of the sodium acetate salt resulted in approximate concentrations of 50 mM salt in the dT_5 sample, 40 mM salt in the dTACTG sample, 20 mM salt in the dT_{36} sample, and 25 mM salt in the mixed-base 38-mer sample. The sodium contaminated samples were then used as the baseline for judging the effectiveness of the various methods for reducing salt effects for subsequent MALDI-MS analysis.

Matrices

The matrices used in this study, 2′,4′,6′-trihydroxyacetophenone (THAP) and 3-hydroxypicolinic acid (3-HPA), were purchased from Aldrich (Milwaukee, WI, USA). The THAP matrix was prepared as a 500 mM solution in ethanol and was used in a 3 : 1 (v/v) ratio with the sample. The 3-HPA matrix was prepared as a 0.5 M solution in 50% acetonitrile and was used in a 2 : 1 (v/v) ratio with the sample.

Sample desalting approaches

Cation-exchange resin beads. AG-50W-X8 cation-exchange resin beads (Bio-Rad, Hercules, CA, USA) were activated using 10 M ammonium acetate following published procedures.[4,19] Here, the resin beads were added directly to the oligonucleotide–matrix sample mixture prior to spotting on the MALDI sample plate. Occasionally, cation-exchange resin beads would be aspirated into the micropipetter and deposited on the sample plate during sample transfer. In those circumstances where the presence of cation-exchange resin beads prohibited crystallization of the sample–matrix mixture, the sample would be re-applied at a different location on the sample plate.

Co-matrices. Ammonium acetate (Fisher Chemical, Fairlawn, NJ, USA) was prepared at three different concentrations, 1 M, 100 mM and 10 mM, in nanopure water for use as a co-matrix. This co-matrix was combined with the various matrix solutions in a 1 : 1 (v/v) ratio prior to mixing with the oligonucleotide samples. Ammonium hydrogencitrate (Aldrich) was prepared as a 100 mM solution in de-ionized water and used in a 1 : 2 (v/v) mixture with the THAP matrix only. A neat solution of triethylamine was desalted with anion and cation exchange resin beads [AG-501-X8(D), Bio-Rad] by adding 5 g of resin beads per 100 mL of solution and stirring for 1 h. After decanting, the triethylamine was diluted to 290 mM with nanopure water. A similar procedure was followed for desalting imidazole. The imidazole solution was initially prepared at 290 mM before stirring with resin beads. After 1 h, the solution was decanted from the resin beads and was ready for immediate use.

Anion-exchange HPLC. Anion-exchange HPLC separations were performed on a Beckman (Fullerton, CA, USA) System Gold HPLC instrument with the detector set to monitor at 260 nm. The column used was a Nucleogen DEAE 1000 (Nest Group, Southboro, MA, USA) anion-exchange column. Buffer A consisted of 25 mM triethylammonium bicarbonate (TEAB) and 20% acetonitrile, pH 6.4, and buffer B consisted of 1 M TEAB and 20% acetonitrile, pH 7.6. A linear gradient from 0 to 100% B at 1% min^{-1} was used. Flow rates were 1 mL min^{-1}. After HPLC purification, the samples were lyophilized and then reconstituted in nanopure water to an approximate concentration of 100 μM prior to mixing with the appropriate matrix solution.

C_{18} purification tips. Prior to C_{18} ZipTip (Millipore, Bedford, MA, USA) purification, the sample was first lyophilized and then dissolved in 0.1 or 0.5% trifluoroacetic acid (TFA). The C_{18} resin was wetted using 50% aqueous acetonitrile solution and then equilibrated by washing twice with 0.1% aqueous TFA solution. The sample was aspirated and dispensed through the purification tip approximately 5–10 times to promote binding of the oligonucleotide to the C_{18} resin in the tip. The tip was washed with 0.1% aqueous TFA several times to remove the salts from the sample. The oligonucleotide was eluted by first dispensing 2–4 μL of 50% aqueous acetonitrile solution into a clean vial using a standard tip, then aspirating and dispensing the 50% aqueous acetonitrile solution into the vial with the purification tip approximately three times. After dispensing, the analyte solution was available for immediate use.

Desalting columns. Desalting columns were obtained from Pierce (Rockford, IL, USA). Three different types of desalting columns were tested: an 1800 MWCO polyacrylamide desalting gel, a 5000 MWCO dextran plastic desalting column and a 6000 MWCO polyacrylamide plastic desalting column. The 5 mL columns were equilibrated with 25 mL of 0.2 M ammonium bicarbonate. A 100 μL volume of the sample was applied to the top of the column and allowed to permeate the gel, then 5 mL of 0.2 M ammonium bicarbonate were added and the eluate, which contained the sample, was collected. After purification using the desalting columns, the samples were lyophilized and then reconstituted in nanopure water to an approximate concentration of 100 μM prior to mixing with the appropriate matrix solution.

Minidialysis. Minidialysis cartridges (Pierce) containing either 3500 or 10 000 MWCO membranes were investigated. Volumes of 10–100 μL of the sample were injected into the cartridge, which was inserted into a flotation device in a beaker containing a buffer solution. The buffers solutions investigated were 10 mM ammonium acetate, pH 6–7, 200 mM ammonium acetate, pH 6–7, and 200 mM ammonium bicarbonate, pH 8. The sample was dialyzed for 4 h with the buffer being refreshed midway through the total dialysis time. After minidialysis, the analyte solution was available for immediate use.

Mass spectrometry

MALDI-MS analyses were carried out using a PerSeptive Biosystems (Framingham, MA, USA) Voyager linear MALDI-TOF spectrometer equipped with an N_2 laser. The laser was operated just above the threshold laser power density. Threshold laser power density is defined as the point at which the ion signals for the analytes were observed. For all of the experiments, 0.5 μL of each oligonucleotide–matrix sample mixture was spotted at two separate locations on the sample plate to gauge the reproducibility of the desalting approaches. All spectra shown here were the average of at least 50 laser shots and were obtained in the negative ion mode. External calibration was carried out using dT_5 and dT_{36} in the appropriate matrix.

Results and discussion

To characterize the effectiveness of a variety of salt reduction methods, the following methodology was employed. First, the purified oligonucleotide was analyzed to generate representative control data for these studies. The oligonucleotide was contaminated with sodium acetate at the levels described in the Experimental section. This 'salted' oligonucleotide was then re-analyzed to characterize the effects of salt on the resulting MALDI-MS data. These two sets of data were then used to provide a set of values for evaluating the sample purification methods. Aliquots of the salted sample were then purified using the approaches shown in Tables 1–3.

Three experimental criteria were used to evaluate the effectiveness of the sample purification methods at reducing cation adducts: the average mass value of the oligonucleotide, the resolution of the base peak in the mass spectrum and the signal-to-noise ratio of the base peak in the mass spectrum. The average mass value is used to determine whether the sample purification method is effective at reducing the salt adducts present in the original salted sample. As the salt adducts can be resolved using our linear instrument for dT_5 and dTACTG, the resolution for these two analytes was used to determine whether the sample purification method had any effect on the sample analysis. As the salt adducts cannot be resolved using our linear instrument for dT_{36} and the mixed-base 38-mer, the resolution for these two analytes will also be a measure of the effectiveness of the sample purification method at reducing the salt adducts. Although MALDI-MS is sensitive to sample crystallization conditions,[28] no attempts to normalize for differences in sample crystallization were made in these studies. Our rationale for this approach was to examine the utility of these different sample desalting techniques under standard experimental conditions and to evaluate each method without resorting to additional, non-standard sample spotting protocols. It should be noted that some of the following sample purification approaches may be

Table 1 Comparative results from the analysis of dT_5 and dTACTG which were purposefully contaminated with sodium acetate. The unsalted control provides a baseline with which the effectiveness of the various sample desalting approaches can be compared. For dT_5 and dTACTG, C_{18} purification tips appear to be the most effective approach at sample desalting. The reported values are the mean and standard deviations resulting from the averaging of multiple measurements from separate spots on a single sample and from analysis of multiple samples

Sample	Method	Mass[a]	Resolution[b]	Signal-to-noise ratio[c]
dT_5	Unsalted control (calc. M_r = 1458.0 Da)	1462 ± 0.3%	275 ± 8.7%	1.7 ± 5.9%
	Salted with sodium acetate	1479 ± 0.8%	248 ± 64%	1.8 ± 33%
	Cation-exchange resin beads	1470 ± 0.5%	234 ± 32%	1.6 ± 13%
	1 M ammonium acetate	1456 ± 0.8%	273 ± 9.5%	1.7 ± 12%
	100 mM ammonium acetate	1473 ± 0.6%	312 ± 23%	1.7 ± 12%
	10 mM ammonium acetate	1474 ± 0.7%	312 ± 32%	2.3 ± 30%
	Anion-exchange HPLC	1439 ± 1.5%	226 ± 24%	3.6 ± 56%
	C_{18} purification tips	1465 + 0.4%	316 ± 23%	1.7 ± 17%
d(TACTG)	Unsalted control (calc. M_r = 1477.0 Da)	1465 ± 1.1%	446 ± 34%	1.8 ± 11%
	Salted with sodium acetate	1478 ± 0.1%	291 ± 22%	1.7 ± 17%
	Cation-exchange resin beads	1492 ± 0.9%	258 ± 5.4%	2.7 ± 37%
	10 mM ammonium acetate	1489 ± 0.5%	336 ± 16%	1.7 ± 6%
	10 mM ammonium acetate	1490 ± 0.1%	293 ± 5.5%	1.5 ± 8%
	C_{18} purification tips	1480 ± 0.1%	465 ± 22%	1.6 ± 62%

[a] Reported as the average and standard deviation of multiple measurements of the most abundant ion in the mass spectrum. [b] Resolution was calculated using $R = m/\Delta m$, where m is the mass of the ion and Δm is the peak width at half-height. [c] Signal-to-noise ratio was calculated using GRAMS software included with the instrument.

Table 2 Comparative results from the analysis of dT_{36} which was purposefully contaminated with sodium acetate. The unsalted control provides a baseline with which the effectiveness of the various sample desalting approaches can be compared. C_{18} purification tips appear to be the most effective approach at sample desalting. The reported values are the mean and standard deviations resulting from the averaging of multiple measurements from separate spots on a single sample and from analysis multiple samples

Method	Mass[a]	Resolution[b]	Signal-to-noise ratio[c]
Unsalted control (calc. M_r = 10 888 Da)	10 874 ± 0.3%	323 ± 49%	18.2 ± 26%
Salted with sodium acetate	10 929 ± 0.5%	381 ± 65%	12.7 ± 29%
Cation-exchange resin beads	10 889 ± 0.5%	705 ± 77%	8.3 ± 73%
1 M ammonium acetate	10 880 ± 0.7%	252 ± 28%	9.6 ± 11%
100 mM ammonium acetate	10 940 ± 0.3%	159 ± 14%	14.1 ± 19%
10 mM ammonium acetate	10 983 ± 0.5%	143 ± 38%	15.6 ± 45%
C_{18} purification tips	10 861 ± 0.3%	251 ± 1.6%	12.6 ± 64%
1800 MWCO polyacrylamide column	10 986 ± 0.6%	125 ± 27%	20.0 ± 55%
5000 MWCO dextran column	10 913 ± 0.3%	222 ± 18%	11.4 ± 61%
6000 MWCO polyacrylamide column	10 971 ± 0.2%	159 ± 25%	18.3 ± 82%
Triethylamine co-matrix	10 963 ± 0.2%	131 ± 54%	34.0 ± 26%
Imidazole co-matrix	10 923 ± 0.2%	87 ± 1.8%	16.3 ± 1.8%

[a] See footnote *a* to Table 1. [b] See footnote *b* to Table 1. [c] See footnote *c* to Table 1.

improved if optimization of the sample crystallization is performed prior to MALDI-MS analysis.

The signal-to-noise ratios are presented as a guide to the level and reproducibility of the analyte signal strength for each of the sample purification methods tested. To gauge sample loss during the purification procedures, a fixed, constant amount of oligonucleotide was subjected to a particular desalting approach and then the volume of the resulting desalted sample was adjusted, if necessary, to generate a desalted sample solution of the same volume as that prior to the desalting step. Thus, if sample losses are severe, the resulting solution would be of a lower concentration than the solution prior to desalting, and if sample losses are minimal, the resulting solution would be of comparable concentration to that prior to desalting. In this manner, a greatly reduced signal (or signal-to-noise ratio) relative to the control salted sample would be indicative of severe sample losses.

dT_5 and dTACTG

Owing to the lower molecular weight of dT_5 and dTACTG, fewer sample purification methods are suitable for use with these two analytes. Fig. 1 shows the MALDI results from dTACTG analyzed in the THAP matrix. The unsalted control can be seen in Fig. 1(a) and the salted oligonucleotide in Fig.1(b). As is typically the case, the presence of sodium results in the generation of multiple sodium adduct peaks for the salted sample [Fig. 1(b)]. Similar results were obtained for the dT_5 sample (data not shown). THAP was found to be the most effective matrix for these lower molecular weight oligonucleotides and was the only matrix utilized for these two analytes in this study.

The following sample purification methods were tested with dT_5 and dTACTG: cation-exchange resin beads, ammonium acetate co-matrix, anion-exchange HPLC and C_{18} purification tips (Table 1). Each of these approaches was effective at reducing the cation adducts present in the salted sample. In general, no one method was found to generate reproducibly high quality mass spectral results from any location on the sample spot. Some methods, such as the cation-exchange resin beads, yielded results which were highly variable, while other methods, such as the C_{18} purification tips, yielded more reproducible results.

As an example of the variability of results found during this study, Fig. 2 shows the MALDI mass spectra obtained using cation-exchange resin beads to desalt dT_5 [Fig. 2(a)] and dTACTG [Fig. 2(b)]. The data shown here are representative of the types of mass spectral data that one obtains using cation-exchange resin beads. Fig. 2(a) is an example of a situation where the mass spectrum is of poor quality owing to the remaining presence of the cation adducts and the poor signal-to-noise ratio of the analyte. Fig. 2(b) is an example of a situation where higher quality data are obtained using this sample purification approach. Possible problems with the resin beads lie within sample preparation. Addition of too few resin beads resulted in no improvement to spectra while addition of too many resin beads led to poor sample crystal formation, which also gave poor results.

The addition of ammonium acetate as a co-matrix generally resulted in more reproducible and higher quality results than the cation-exchange resin beads (data not shown). Several different concentrations of amonium acetate co-matrix were investigated, and it was found that no dramatic difference in the quality of mass spectral data was seen with varying co-matrix concentration.

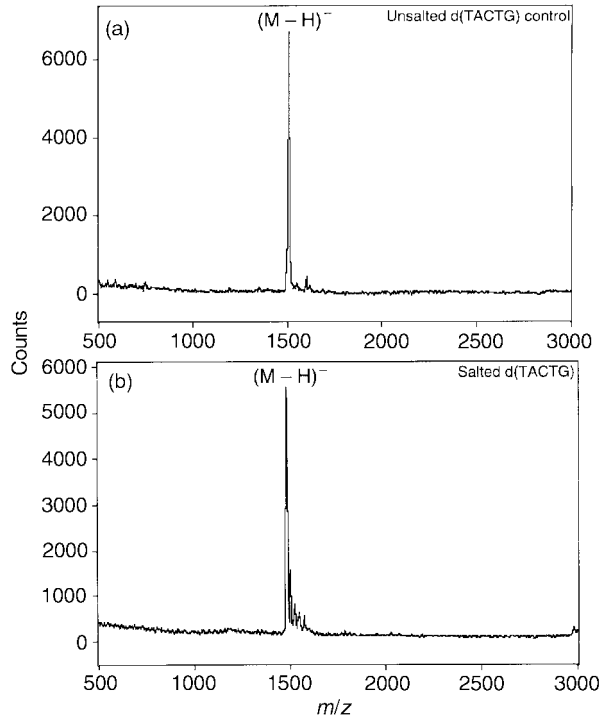

Fig. 1 MALDI mass spectra of dTACTG analysed using THAP as the matrix. (a) dTACTG analysed after synthesis and initial purification. Data acquired at this step is used to generate a baseline, unsalted control sample. (b) dTACTG analysed after addition of sodium acetate to generate a purposely contaminated oligonucleotide sample. The goal of this work was to evaluate the effectiveness of various sample desalting approaches at converting the oligonucleotide detected in (b) back to the oligonucleotide detected in (a).

Table 3 Comparative results from the analysis of a mixed-base 38-mer which was purposefully contaminated with sodium acetate. The unsalted control provides a baseline with which the effectiveness of the various sample desalting, approaches can be compared. For the mixed-base 38-mer, no method appears to be substantially more effective at sample purification than any other, although minidialysis and desalting columns generally yielded the most abundant moelcular ions at the highest resolution (see text). The reported values are the mean and standard deviations resulting from the averaging of multiple measurements from separate spots on a single sample and from analysis of multiple samples

Method	Mass[a]	Resolution[b]	Signal-to-noise ratio[c]
Unsalted control (calc. M_r = 11 618 Da)	11 764 ± 1.0%	153 ± 56%	15.6 ± 49%
Salted with sodium acetate	11 993 ± 1.1%	428 ± 89%	14.4 ± 25%
Cation-exchange resin beads	11 516 ± 0.6%	64 ± 17%	19.0 ± 32%
100 mM ammonium acetate	11 528 ± 0.5%	74 ± 20%	16.5 ± 9.1%
10 mM ammonium acetate	11 667 ± 0.6%	198 ± 75%	15.8 ± 71%
1800 MWCO polyacrylamide column	11 584 ± 0.3%	424 ± 68%	14.7 ± 74%
5000 MWCO dextran column	11 640 ± 0.5%	439 ± 78%	9.9 ± 59%
6000 MWCO polyacrylamide column	11 636 ± 0.3%	140 ± 36%	13.4 ± 90%
3500 MWCO minidialysis[d]	11 596 ± 0.1%	151 ± 56%	14.1 ± 13%
Anion-exchange HPLC	11 507 ± 0.5%	42 ± 36%	55 ± 80%

[a] See footnote *a* to Table 1. [b] See footnote *b* to Table 1. [c] See footnote *c* to Table 1. [d] 200 mM ammonium bicarbonate buffer used.

Anion-exchange HPLC, although also effective at reducing cation adducts, was significantly more time consuming than the other three approaches. The other approaches, cation-exchange resin beads, co-matrices and C_{18} purification tips, do not require additional hardware for their use, are all rapid and do not require additional sample handling after purification. In contrast, anion-exchange HPLC requires additional hardware, requires at least 1 h for purification and requires that the purified sample be re-concentrated prior to analysis. In addition, as will be discussed for the mixed-base 38-mer below, anion-exchange HPLC results in significant peak broadening during MALDI-MS analysis.

The most effective approach for desalting lower mass oligonucleotides was the use of C_{18} purification tips. An example of the variability of results found for the purification tips is shown in Fig. 3. Fig. 3(a) illustrates the application of purification tips to dT_5, and is presented as an example of poor mass spectral results using this approach. As can be seen in Fig. 3(a), a low signal response (*i.e.*, low signal-to-noise ratio) was occasionally found when using the purification tips. However, as can be seen in both Fig. 3(a) and Fig. 3(b), and C_{18} purification tips were extremely effective at removing cation adducts from the mass spectrum and also yielded molecular ion peak widths as good as or better than the original unsalted control sample. Purification tips are easy and rapid to use, and the sample can be spotted directly on the sample plate or mixed with the matrix prior to purification if desired.

dT_{36} and mixed-base 38-mer

The analytes dT_{36} and a mixed-base 38-mer were chosen to evaluate sample purification methods for higher mass oligonucleotides. The higher mass of these oligonucleotides allows for the evaluation of more sample purification methods than could be evaluated for the 5-mers, dT_5 and dTACTG. The resolution of the continuous extraction linear TOF used in these studies does not permit the salt adducts of the higher mass samples to be resolved. Broad, high mass tailing peaks with low abundance are more characteristic of a salty oligonucleotide (Fig. 4). 3-HPA was utilized as the matrix for the mixed-base 38-mer and THAP for T_{36} during these studies.

The following purification methods were tested with dT_{36} and the mixed-base 38-mer: cation-exchange resin beads, co-matrices, C_{18} purification tips, desalting columns, minidialysis cartridges and anion-exchange HPLC (Tables 2 and 3, respectively). Unlike the situation for the lower mass analytes, not all of the methods investigated were effective at reducing or removing cation adducts for the higher mass analytes. Furthermore, the broad, unresolved molecular ion and salt adducts made evaluation of the effectiveness of the various methods more problematic than for the lower mass analytes. However, based on a comparison of the results obtained from investigating the variety of sample purification methods, several approaches appear to be more effective than others at sample purification.

The least effective approach at sample desalting was the use of co-matrices. Limited reduction of salt adducts, as determined by the high average mass values detected, and broader molecular ion peaks, as determined by the resolution values obtained, were the invariable results found using any of the co-matrices tested (ammonium acetate, triethylamine or imidazole).

Mixed results were seen for the cation-exchange resin beads and the C_{18} purification tips. Although the cation-exchange resin beads and purification tips were generally effective for dT_{36}, nucleobase loss from the mixed-base analyte typically resulted in average mass values less than expected for the intact molecular ion and a broadening of the molecular ion peak for these approaches. In addition, the sample recovery was limited

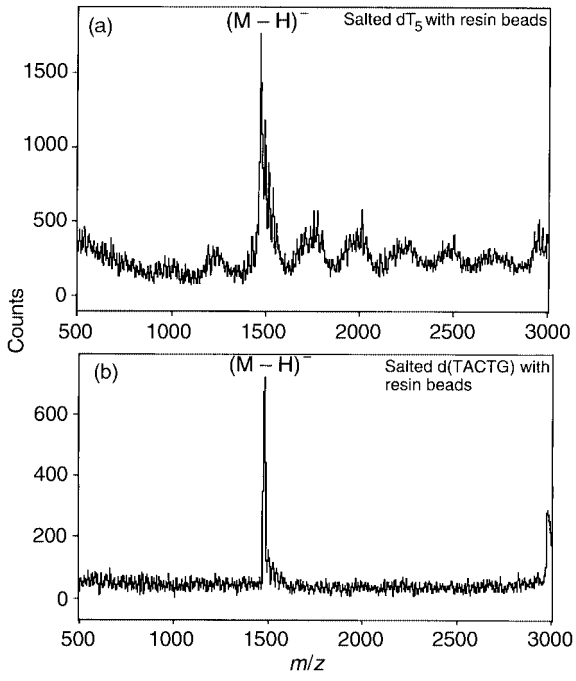

Fig. 2 MALDI mass spectra of (a) dT_5 and (b) dTACTG using THAP as the matrix. Cation-exchange resin beads were added to the salted sample in each case prior to analysis. The mass spectrum of dT_5 is representative of the poorer quality results found when using cation-exchange resin beads, while that for dTACTG represents the type of results found when cation-exchange resin beads were effective. In general, the effectiveness of cation-exchange resin beads was highly variable for the lower molecular weight oligonucleotides.

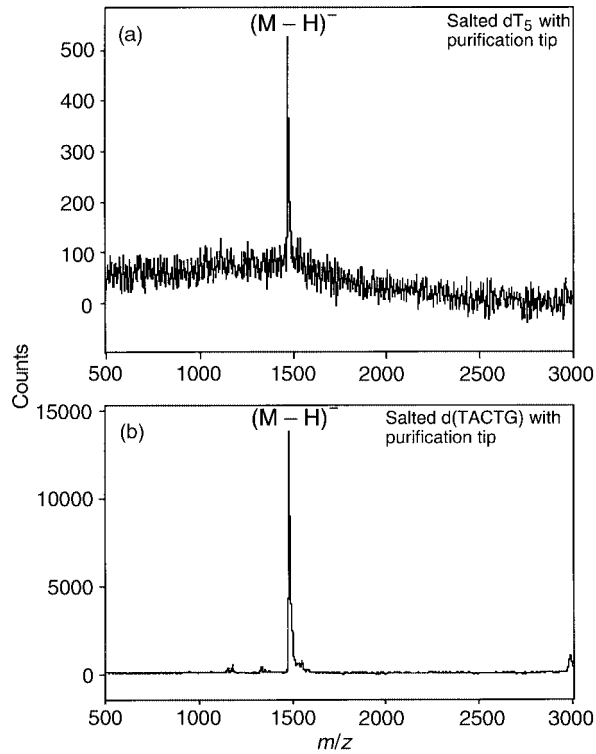

Fig. 3 MALDI mass spectra of (a) dT_5 and (b) TACTG using THAP as the matrix. The purposely contaminated analytes were purified using C_{18} purification tips in each case. Unlike the cation-exchange resin beads (Fig. 2), C_{18} purification tips yield more reproducible results for the lower mass oligonucleotides. C_{18} purification tips were found to be the most effective sample desalting method for these two analytes of the different appraoches compared in this study.

from the C_{18} purification tips, and modification to the washing–elution protocol may be necessary to ensure that these higher mass oligonucleotides do not remain bound to the C_{18} resin.

Representative results from the other three methods investigated are presented in Fig. 5. Fig. 5(a) is a representative MALDI mass spectrum arising from the analysis of the mixed-base 38-mer after sample purification using a 5000 MWCO oligonucleotide desalting column. The desalting columns yielded the highest resolutions at high signal-to-noise ratios of all of the approaches investigated for the higher mass analytes. However, as is evident in Fig. 5(a), the background contained a number of additional peaks when this sample purification method was utilized. The dextran-containing column yielded the lowest background whereas the two polyacrylamide columns both exhibited significant background ions after use. Presumably the background is due to bleeding of the desalting column material into the sample mixture. In addition, sample purification using desalting columns is significantly longer than the cation-exchange resin beads or C_{18} purification tips, requiring up to several hours for the 1800 MWCO polyacrylamide gel. However, compared with the minidialysis and HPLC, the sample purification time was not inordinately longer using the desalting columns.

Minidialysis using a variety of MWCO membranes and buffers also yielded reproducibly abundant molecular ion signals with improved resolution. The advantage of the minidialysis approach to the desalting columns is the absence of abundant background ions in the resulting mass spectrum. The disadvantage of this methodology is the longer purification time (4 h) compared with the other purification methods. It is expected that flowing microdialysis[17,18] would yield comparable results with a greatly reduced purification time.

Anion-exchange HPLC consistently yielded mass spectral data with the lowest background signal of all of the methods investigated [Fig. 5(c)]. However, the resolution obtained using HPLC is much lower than those of the other sample purification methods tested and, as mentioned earlier, HPLC is time consuming and requires additional hardware.

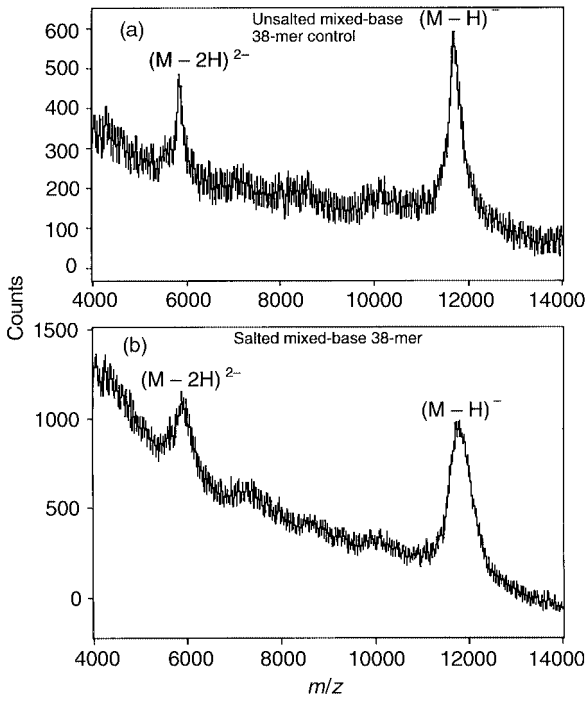

Fig. 4 (a) MALDI mass spectrum of a mixed-base 38-mer using 3-HPA as the matrix after synthesis and initial purification. Data acquired in this step were used to generate a baseline, unsalted control sample. (b) The same 38-mer as in (a) analysed after addition of sodium acetate to generate a purposely contaminated oligonucleotide sample. The goal of this work was to evaluate the effectiveness of various sample desalting approaches at converting the oligonucleotide detected in (b) back to the oligonucleotide detected in (a).

Conclusions

A variety of sample purification methods were compared to investigate which approach is the most effective at reducing or eliminating cation adducts from the MALDI mass spectral data for low and moderate molecular weight oligonucleotides. The sample purification methods investigated were cation-exchange resin beads, co-matrices, C_{18} purification tips, desalting columns, minidialysis and anion-exchange HPLC. Most of the desalting methods are effective to some degree, with some methods having greater applicability to a specific range of oligonucleotide molecular weights. When choosing an appropriate desalting method for oligonucleotides, it is important to consider several factors. Such factors include the reproducibility of the given method, its ease in application, the time restraints that the application may require, the size and stability of the oligonucleotide to be analyzed and the sample recovery after application of the desalting technique.

C_{18} purification tips appear to be the best approach to sample purification for low molecular weight oligonucleotides and also for the higher mass dT_{36} analyte studied. Poor recoveries were found when using the C_{18} purification tips and the mixed-base 38-mer, which may be attributed to the oligonucleotide remaining bound to the C_{18} resin under the elution conditions utilized in this work. If higher recoveries of mixed-base high molecular weight oligonucleotides can be achieved, *e.g.*, by use

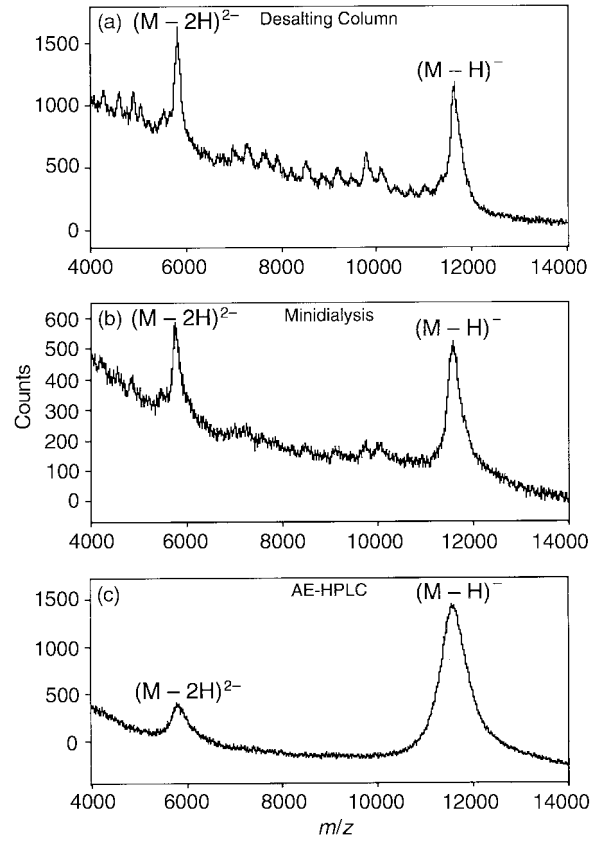

Fig. 5 MALDI mass spectra of a mixed-base 38-mer using 3-HPA as the matrix with (a) application of the 5000 MWCO dextran desalting column, (b) application of 10 000 MWCO minidialysis using 200 mM ammonium bicarbonate buffer and (c) application of anion-exchange HPLC purification. Although all three methods improve the general quality of the mass spectral data by reducing the salt adducts, the dextran desalting column gives a significantly higher background than the other two approaches. Minidialysis appears to be the best combination of salt reduction, high ion abundance, high resolution and reduced background contaminants.

of C_4 or ion-exchange resins, purification tips would appear to be the most effective sample purification approach for oligonucleotides, in general, of all those studied.

Cation-exchange resin beads remain an effective approach for desalting lower molecular weight oligonucleotides, although the results are more variable with this approach than with the C_{18} purification tips. Minidialysis, although extremely time consuming, is an effective purification approach for higher molecular weight oligonucleotides with a reduced background compared with the oligonucleotide desalting columns. In addition, a combination of sample purification approaches (*e.g.*, ethanol precipitation followed by cleanup with C_{18} purification tips or C_{18} purification tips used with co-matrices) may yield improved results for particular samples.

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