Dissolve-Spin: Desalting Oligonucleotides for MALDI MS Analysis

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Abstract: Desalting oligonucleotides (ONs) for matrix assisted laser desorption ionization mass spectrometry (MALDI MS) analysis was achieved using a simple dissolve-spin approach. The ON is dissolved in an organic solvent. Insoluble salts are removed by centrifugation. ONs are highly polar molecules, and are generally believed insoluble in organic solvents with moderate polarity such as acetonitrile (ACN), 1,4-dioxane, ethyl acetate and THF. However, we found that in the presence of a suitable proton source such as pyridinium chloride, a quantity of ON that is sufficient for MALDI MS analysis could be dissolved. Because inorganic salts are insoluble in such relatively non-polar solvents, the finding can be utilized for desalting ONs for MALDI MS analysis. Comparisons of MS spectra of intentionally salted ONs that underwent the new desalting procedure with those that did not undergo the procedure provided unambiguous evidence that the desalting method is highly effective.

Keywords: Desalt, DNA, MALDI MS, Oligonucleotide, RNA, Sample Preparation

Introduction

Matrix assisted laser desorption ionization mass spectrometry (MALDI MS) has been widely used for the analysis of oligonucleotides (ONs), which include synthetic DNAs and RNAs, commonly called oligodeoxyribonucleotides (ODNs) and oligoribonucleotides (ORNs), respectively. One crucial step for the success of the analysis is to desalt the ONs during sample preparation. Many methods have appeared in the literature for the purpose.[1, 2] Perhaps, the most widely used method is reversed phase filtration, which involves the use of a small deposable column filled with carbon-18 silica. The ON sample in a binding solution, which usually contains the ion-pairing agent triethylammonium acetate, is loaded onto the column. Inorganic salts are removed by washing first with an aqueous buffer that has a proton source, and then with pure water. Desalted ON is then obtained by eluding the column with an aqueous solution that has a high content of acetonitrile (ACN).[3] Other desalting methods in the literature include solid phase extraction,[4] size-exclusion chromatography,[5] fluorous affinity enrichment,[6] ion exchange chromatography,[7, 8] extraction with magnetic beads,[9] extraction with sol–gel crown ether hybrid materials,[10] attaching to MALDI substrate with an ON binding surface,[11] as well as dialysis and ethanol precipitation.[1] In this paper, we report a new method for desalting ONs for MALDI MS analysis. The method involves dissolving an ON in an organic solvent such as ethyl acetate in the

presence of a proton source such as pyridinium chloride. Inorganic salts that cause problems for MALDI MS analysis are then simply removed by centrifugation followed by separation of the supernatant from the precipitate.

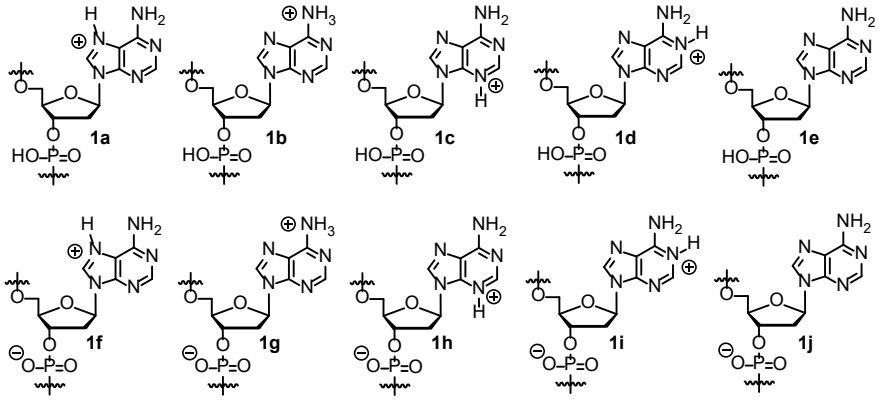
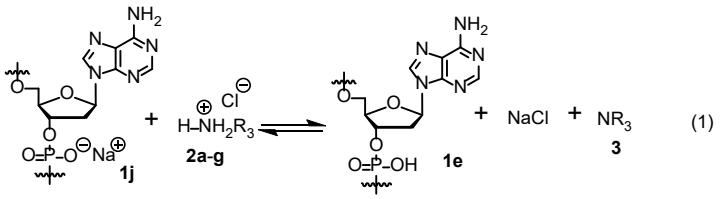


Figure 1. Possible forms of nucleotides in ON. dA is used as an example here. Similar forms for other nucleotides including those in ODN and ORN are possible.

Results and Discussion

The new ON desalting method is based on the reasoning that among various forms such as **1a-j** that can be adopted by the nucleotides in an ON molecule (Figure 1), the neutral forms such as **1e**, in which the phosphate is protonated and nucleobase is not, can exist in organic solvents with moderate polarity, and therefore some of the ON molecules containing them can become soluble in the organic solvents. Because inorganic salts including those of sodium and potassium, which are the ones that cause problems for MALDI MS analysis, are insoluble in such solvents, desalting ON can be simply achieved by dissolving in an organic solvent and remove the salts by centrifugation. Detailed explanation of the possibility of the various forms that can be adopted by nucleotides in ONs including ODNs and ORNs in aqueous and organic media is provided using dA as an example (Figure 1). Other nucleotides are similar. In aqueous media, depending on the acidity of the media, from low pH to high pH the nucleotide could adopt the positively charged forms **1a-d**, the neutral forms **1e-i**, and the negatively charged form **1j**. At moderately acidic or close to neutral pH, among the forms **1e-j**, forms **1f-i** and form **1j** with a cation such as ammonium are far more likely than form **1e** because the basic sites in the nucleobase (pKa of conjugate acid as high as 4.4)[12] and the conjugate base of ammonium (pKa 9.25) are more basic than the phosphate (pKa of conjugate acid about 1.0).[13] ONs with nucleotides adopting forms **1f-i** and **1j** are highly charged and thus are soluble in water and insoluble in organic solvents. In organic solvents, among the forms **1a-j**, the charged forms **1a-d** and **1f-j** are less stable and could be hardly soluble even if they exist. In contrast, the neutral form **1e** is more likely to exist compared with the case in aqueous media. Because neutral molecules are generally more soluble in organic solvents than charged ones, the adoption of neutral forms such as **1e** by nucleotides can increase the solubility of ONs in organic solvents. The pKa values of some compounds in the literature

support the possibility of neutral forms such as **1e**. For example, in ACN the pKa of a diphenyl hydrogen phosphate derivative was reported to be as high as 13.97.[14] The phosphate groups in ONs are connected to alkyl groups, and the pKa of their conjugate acid could possibly be even higher. The pKa of benzimidazole, which is structurally similar with the imidazole moiety of purine nucleobases, in ACN is reported to be 13.54.[15] These values indicate that it is possible for the phosphate groups of ONs to be protonated while the nucleobases are not in ACN at appropriate pH. Therefore, chances exist that nucleotides in an ON molecule adopts neutral forms such as **1e** and some ON molecules become soluble in organic solvents.



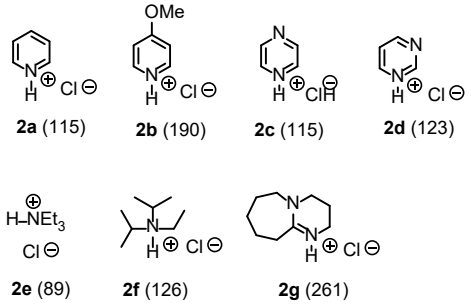


Figure 2. Proton sources screened for the ON desalting process. The numbers in the parenthesis after the compound numbers are the boiling point of the conjugate base of the acid in Celsius degrees at atmospheric pressure.

The procedure we used to drive the nucleotides in an ON to adopt the uncharged forms such as **1e** and thus to dissolve the ON in an organic solvent was to mix the dried ON with a proton source in the presence of an anhydrous organic solvent. One of the many possible scenarios is illustrated in equation (1) to serve as an example to explain the process. The nucleotide **1j** is protonated by a proton source **2** (Figure 2) at the phosphate group to give **1e**. The side product NaCl and other inorganic salts are precipitated. The neutral nitrogen-containing molecule **3** should have little effect on MALDI MS analysis when its amount is minimized. Alternatively, **3** can be removed by evaporation under vacuum if **2** is selected from candidates with a volatile conjugate base. Accordingly, using the 20-mer ODN **4a** (5'-TCATTGCTGCTTAGACCGCT-3'), we started the study by using ACN as the solvent and pyridinium chloride (**2a**) as the proton source. These materials were mixed extensively in a centrifuge tube. The insoluble materials were brought down by centrifugation. The supernatant was transferred to a clean centrifuge tube and evaporated to

dryness. The ODN was dissolved in water and the solution was mixed with the matrix solution, which is saturated 3-HPA in the solvent mixture containing equal volumes of ACN and 0.1% aqueous TFA with 10 mg/mL diammonium hydrogen citrate.[3] MALDI MS experiments were carried out under typical conditions. Details such as laser repetition rate, voltages and delay time are given in the experimental section. Gratifyingly, sweet spots, where molecular peak of ODN can be detected, could be easily located, thus providing experimental data supporting the hypothesis that chances exist for nucleotides in ONs to adopt the neutral forms such as **1e** and the ONs become soluble in organic solvents. The spectra are similar to typical spectra obtained using ONs desalted with reversed phase filtration.

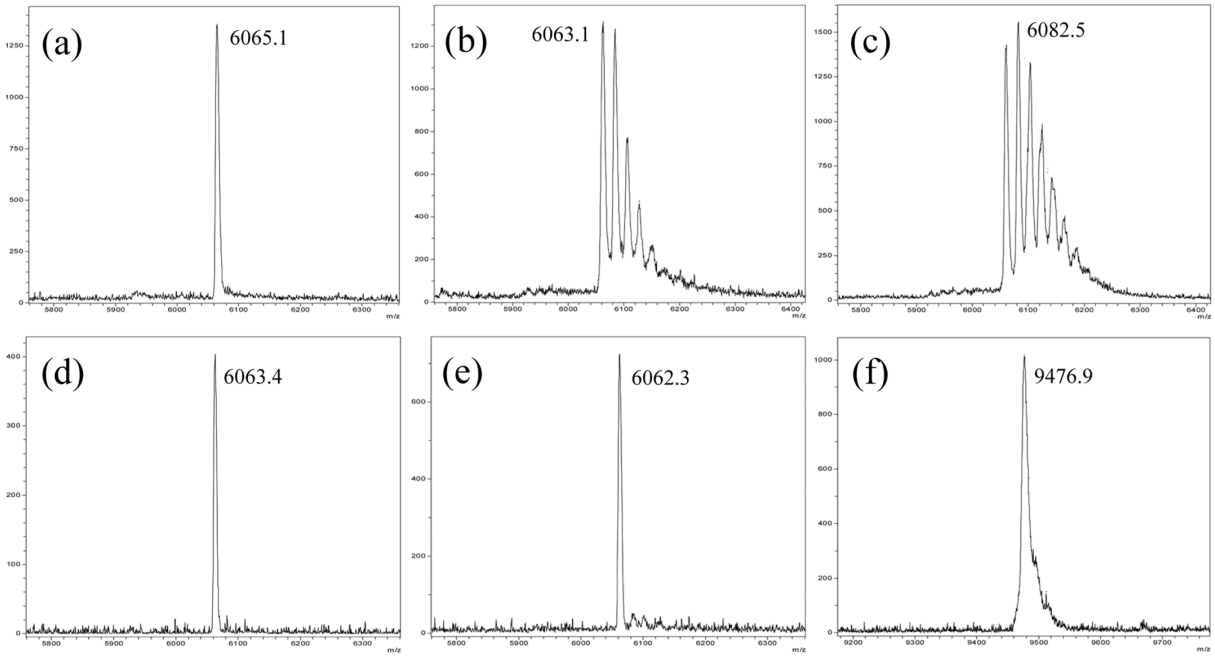


Figure 3. Selected MALDI MS spectra of the 20-mer ODN **4a** (calculated for $[M-H]^-$ 6057) and 30-mer ORN **4b** (calculated for $[M-H]^-$ 9461). Both ONs were salted with excess NaCl. (a) MS of **4a** desalted using the dissolve and spin method with ACN as solvent and **2a** as proton source (found for $[M-H]^-$ 6065). (b) MS of **4a** without desalting. Additional peaks are from adducts of ODN and sodium. (c) MS of precipitated **4a** during the dissolve and spin desalting procedure using ACN as solvent and **2a** as proton source. Additional peaks are from adducts of ODN and sodium. (d) MS of **4a** desalted using the dissolve and spin method with EtOAc as solvent and **2a** as proton source (found for $[M-H]^-$ 6063). (e) MS of **4a** desalted using the dissolve and spin method with 1,4-dioxane as solvent and **2a** as proton source (found for $[M-H]^-$ 6062). (f) MS of **4b** desalted using the dissolve and spin method with EtOAc as solvent and **2a** as proton source (found for $[M-H]^-$ 9477).

To provide clear evidence that the dissolve-spin procedure can indeed effectively remove inorganic salts from ON samples, ODN **4a** intentionally salted with 1000 equivalents of sodium chloride over ODN was used for the desalting and MALDI MS experiments. The same procedure using ACN as the organic solvent and **2a** as the proton source as described above was used for

desalting. MS spectra of the salted ODN without desalting were also obtained for comparison. Selected spectra are shown in Figure 3. As can be seen, the spectra of the sample underwent the dissolve-spin desalting procedure have no peaks of sodium-ODN adducts while the spectra of the sample without undergoing the desalting procedure have multiple peaks of sodium-ODN adducts. In addition, the precipitate was also analyzed and multiple peaks of sodium-ODN adducts were also observed. The results clearly demonstrated that the new dissolve-spin ON desalting method can indeed remove salts from ONs.

Table 1. Screening solvents for the dissolve-spin ON desalting method.[a]

Solvent (proton source used)	Note
ACN (**2a**, **2e**)	Suitable for the desalting method.[b]
Ethyl acetate (**2a**, **2e**)	Suitable for the desalting method.[b]
1,4-Dioxane (**2a**, **2e**)	Suitable for the desalting method.[b]
Toluene (**2a**)	Not suitable for the desalting method.[c]
THF (**2a**)	Not suitable for the desalting method.[d]
Chloroform (**2a**)	Not suitable for the desalting method.[d]
DCM (**2a**)	Not suitable for the desalting method.[d]
Diethyl ether (**2a**)	Not suitable for the desalting method.[d]

[a] An ON, proton source and organic solvent in a centrifuge tube were mixed. The mixture was centrifuged. The supernatant was transferred to another tube, and evaporated to dryness. The residue was dissolved in a matrix solution, loaded on a substrate, and subjected to MALDI MS analysis. See experimental section for details. [b] Sweet spots giving molecular peaks devoid of salt adducts were readily found. [c] No sweet spots could be found due to low ON solubility in the solvent. [d] The solvent evaporated too fast, and supernatant was difficult to collect. If the desalting procedure is performed at lower temperature or a larger volume of organic solvent is used, these solvents may be found suitable for the desalting method.

We next screened different solvents for the desalting process (Table 1). Using **2a** as the proton source and salted ODN **4a** as the sample, ethyl acetate and 1,4-dioxane were found as efficient as ACN for the desalting method. These two solvents are less polar that ACN. Their polarity indexes are 0.228 and 0.164, respectively, while that of ACN is 0.460.[16] Thus, they may be even more suitable for the desalting method. Selected MS spectra obtained using them for the desalting process are given in Figure 3. Using the less acidic proton source **2e**, ACN, ethyl acetate and 1,4-dioxiane were also found effective. Selected spectra are given in supporting information. Besides these three solvents, several other solvents were also screened, but found not suitable for the purpose for reasons indicated in Table 1.

We next screened several different proton sources (**2a**-**g**) for the desalting method. To be a suitable proton source, the compound should meet the following criteria: (1) Suitable acidity so that it can protonate the phosphate groups of ON in an organic solvent but does not damage the ON. (2) Suitable solubility in organic solvents so that it can protonate the phosphate groups of ON and bring the ON into solution. (3) Volatile under vacuum so that if excess is used, it can be removed and thus does not affect the MALDI process. This criterion is not a requirement because

the use of excess proton source is optional. (4) Readily available and inexpensive. The proton sources **2a**-**g** meet all the requirements except that **2b** and **2g** are not easy to evaporate under vacuum due to the high boiling points of their conjugate base (190 ºC and 261 ºC, respectively). Besides **2a** and **2e** described above, **2b** and **2f** were tested using ACN as the solvent, and **2c**-**d** and **2f**-**g** were tested using ethyl acetate as the solvent. Salted ODN **4a** was used as the sample. In all the cases, sweet points giving high quality spectra with little or no salt adduct peaks could be found without much trouble. Selected spectra with acceptable quality are given in the supporting information. Among the proton sources tested, their pKa values in ACN ranged widely from 7.74 of **2c** to 24.31 of **2g**,[15] but they seemed not affect the MALDI MS results very much. One possible explanation could be that in ACN, some of the phosphate groups in the ON might not have been protonated, and instead they exist in the form of **1j** with the cation of **2** as the counter cation. After the organic solvent was evaporated and the ON was dissolved in water for sample preparation, either the same cation of **2** or more likely a different ammonium cation from the matrix mixture is associated with the phosphate group. Once in the gas phase during MALDI, the strong tendency toward neutralization via proton transfer converts form **1j** to **1e**.[17]

To test the dissolve-spin method for desalting ORN for MALDI MS, a 30-mer ORN (**4b**, 5'-rCrUrCrGr^{pseudo}UrArGrArCrUrArUrArArCrUrUrArArUrCrUrCrArCrArUrArGrC-3') was synthesized on an automated synthesizer using standard phosphoramidite chemistry. The standard dissolve-spin desalting and MALDI MS procedure described for ODN was used. Ethyl acetate was used as the organic solvent, and **1a** was used as the proton source. The ORN was salted similarly for salting ODN **4a**. The MALDI MS results were similar as ODN analysis, and sweet spots giving molecular peaks of **4b** devoid of any salt adducts were easy to find. A selected spectrum is shown in Figure 3. A full spectrum is given in the supporting information.

Besides the conditions used in the standard desalting procedure described above, we also tested several other conditions. In the absence of intentionally added proton sources (**2**), using ACN as the organic solvent, under otherwise identical sample preparation and MALDI MS conditions described earlier, ODN was detectable as well possibly due to the residue ammonium salts from ODN ammonium hydroxide deprotection or ammonium salts from the buffers used for RP-HPLC purification. For this reason, it is possible for the dissolve-spin desalting method to function without intentionally added proton source. However, in our hands, this simpler procedure gave less consistent results. We also tested if TFA and HCl could be used as the proton source. Using dilute TFA (0.012 M) as the proton source and ACN as the organic solvent, sweet spots giving ODN peaks could be found. However, trifluoroacetate salts such as sodium and potassium trifluoroacetate have significant solubility in organic solvents. Therefore, TFA is not an option for the desalting method. Hydrogen chloride would be an excellent proton source because it is volatile and its sodium and potassium salts are insoluble in organic solvents. However, we found that significant DNA damage occurred even solutions of HCl in ACN as dilute as 0.020 M was used.

Although we hypothesized that the phosphate groups of ONs in the organic layer exist in the neutral forms such as **1e**, they could also exist in ionic forms with their counter cations being the relatively hydrophobic **2a**-**g**. With some phosphate groups exist in these ionic forms and some exist in the neutral forms, the ONs might have sufficient solubility in the organic solvents.

Predicting which of the two types of forms predominates or estimating their relative quantities is challenging. However, we observed that the more hydrophobic cations **2f-g** did not give more intense molecular peaks than the cases involving the less hydrophobic cations such as **2a** and **2e** under similar MALDI MS conditions. This provides evidence of neutral nucleotides in ON. In addition, the fact that TFA could bring the ONs into the organic phase as discussed above also supports the hypothesis of neutral nucleotides in ON. With these analyses, it is reasonable to believe that at least some of the nucleotides in ONs in the organic phase may indeed exist in the neutral forms such as **1e**.

Several features of the new method for ON desalting are notable. First, the method does not require any special materials such as carbon-18 silica, ion exchange resin or magnetic beads.[3, 7-9] All the needed materials such as organic solvents and proton sources are readily available and inexpensive. Second, the method is well suited for desalting samples that contain a large amount of inorganic salts such as those from ion-exchange HPLC. This is a significant advantage over known methods such as reversed phase filtration, which has limits on the amount of salts that can be removed. Third, the method is well suited for desalting ON samples that require high throughput analysis. It is predicted that using pipettes with multiple channels, desalting multiple samples simultaneously using the method is achievable. This feature is important in areas such as DNA and RNA sequencing, and SNP analysis using MALDI MS.[18-22] Fourth, the method could be readily adopted to desalt samples that contain organic materials including organic buffers because most organic impurities are soluble in *n*-butanol and could be simply removed by precipitating ON from slightly basic aqueous solution with *n*-butanol. The precipitated ON can then be desalted using the new dissolve-spin method.

Conclusion

In summary, a new method for desalting ON for MALDI MS analysis has been developed. By mixing an ON with a nitrogen based proton source in the presence of an organic solvent followed by centrifugation, the ON can be separated from inorganic salts with the ON in the supernatant and inorganic salts in the precipitate. The method is simple, does not require expensive materials, can tolerate large amount of salts, and is expected to be suitable for high throughput desalting and MALDI MS analysis. The method has been demonstrated for desalting both DNA and RNA. High quality MALDI MS spectra could be reliably obtained even for intentionally salted samples.

Experimental

Preparation solutions of ON and proton sources: ODN **4a** (5'-TCATTGCTGCTTAGACC GCT-3') and ORN **4b** (5'-rCrUrCrGr^{pseudo}UrArGrArCrUrArUrArArCrUrUrArArUrCrUrCrArCr ArUrArGrC-3') were synthesized on a MerMade 6 synthesizer using standard phosphoramidite chemistry and purified using trityl-on reversed phase HPLC as described elsewhere.[23] For the preparation of the solution of **4a**, to ODN (1.0 μg, 0.1650 nmol) in a 1.5 mL centrifuge tube was added water (2 μL), and NaCl solution (20.81 mM, 8 μL, 0.1664 μmol). This gives the salted solution of **4a** with a concentration of 16.50 μM ODN and 16.64 mM NaCl. The solution of ORN **4b** (10.58 μM ORN and 16.64 mM NaCl) was prepared in the same manner. The proton sources

2a-**g** were prepared by mixing their conjugate base with HCl in 1,4-dioxane. The purified salts were dissolved in an organic solvent to give the solutions of proton source (12.11 mM).

Desalting ON and MALDI MS analysis: ODN **4a** (1 μg, 0.1650 μmol ODN, more or less can be used; in the cases of salted sample, 16.50 μM ODN, 16.64 mM NaCl, 10 μL, 1 μg, 0.1650 nmol ODN, 9.644 μg, 0.1664 μmol NaCl, more or less NaCl does not affect MS analysis results) in a 1.5 mL centrifuge tube was dried under vacuum. To the tube was added the solution of pyridinium chloride (**2a**) in dry EtOAc (12.11 mM, 20 μL, 0.2423 μmol). The tube was capped tightly and the cap was secured by wrapping with parafilm and a clamp. The materials were mixed by vortex for 1 min. The liquid was brought down by a brief spin, and mixing was carried out further by sonicating for 2.5 min at room temperature. After standing for 30 min, the tube was centrifuged at 14.5 rpm for 5 min. The supernatant was carefully transferred to another 1.5 mL centrifuge tube without agitating the precipitate, and evaporated to dryness in a centrifugal evaporator. Deionized water (2 μL) was added, and the tube was vortexed and spun briefly giving the desalted ODN solution. On a piece of parafilm, the desalted ODN solution (0.5 μL) and a matrix solution (0.5 μL), which was saturated 3-hydroxypicolinic acid (3-HPA) in the solvent mixture containing equal volumes of ACN and 0.1% aqueous TFA with 10 mg/mL diammonium hydrogen citrate, were mixed. The combined solution was loaded onto a stainless steel MALDI substrate, and allowed to air dry and crystallize. MALDI MS was then carried out on a Bruker MALDI-TOF Microflex LRF mass spectrometer using negative mode, 0.139 laser bean attenuation, 30 Hz laser repetition rate, 100 shots for each spectrum, 100 ns delay time, and 19 kV and 15.55 kV ion source voltages. For the case of salted ODN **4a**, the precipitate was also dried after the organic supernatant was removed, loaded on the stainless steel substrate and analyzed with MALDI MS under the same conditions as the supernatant. For the cases of using organic solvents other than EtOAc (ACN and 1,4-dioxane) and different proton sources (**2a**-**g**), as well as for the analysis of ORN **4b** (10.58 μM ORN, 16.64 mM NaCl, 10 μL, 0.1058 nmol, 1 μg ORN, more or less can be used; 0.1664 μmol, 9.664 μg NaCl,, more or less does not affect MS analysis results; EtOAc was used as the organic solvents, and **2a** was used as the proton source), the same sample preparation, sample loading and MS conditions were used. For the two alternative loading methods, one involved loading the matrix solution (same composition as described above, 0.5 μL) onto the substrate to form crystals first followed by loading the ON solution in water (0.5 μL) over the crystal. The other involved loading the organic supernatant of ON (20 μL) from the desalting procedure over the matrix crystal on the substrate slowly with the caution of retaining the ON on the crystal. MALDI MS conditions were the same as described above.

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