# Fast analysis of oligonucleotides by capillary liquid chromatography-nano-electrospray mass spectrometry combined with column switching.

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## 1. Introduction

Polymerase chain reaction (PCR) technology is widely used in DNA sequencing and DNA analysis. In that respect, the availability of PCR primers and synthetic oligonucleotides is very important. These oligonucleotides have to be very pure and a defect in length or sequence is not tolerated. The only reliable way to operate is to test each oligonucleotide after synthesis. The quality control method used for this purpose must be rapid, low cost and reliable, and use minimal labor (1, 2). Mass spectrometry provides a basis for detecting both length and sequence variations of oligonucleotides, based on a difference in mass. Electrospray ionization mass spectrometry (ESI-MS) has become one of the most important mass spectrometric techniques for the analysis of oligonucleotides. ESI with high resolution MS analysis, such as ESI-Q-TOF MS analysis, allows differentiation of single base substitutions, resulting in a mass change between 9 and 40 Da (A-T and G-C switch respectively) (3). However, the major difficulties using ESI arise from the adduction of non-volatile cations such as sodium or potassium to the polynucleotide backbone resulting in mass spectra of poor quality. Most of the sample pretreatment procedures used for the removal of these metal ions are off-line approaches and time consuming, thus difficult to reconcile with the presented analytical demand. The essence of such procedures involves the replacement of sodium ions with ammonium to reduce sodium adduct formation. These ammonium ions are less tightly bound to the oligonucleotide when ionized. Consequently, a capillary liquid chromatography (LC)-nano-ESI-MS method combined with column switching for the on-line removal of salt ions for the analysis of oligonucleotides was developed.

## 2. Experimental

Oligonucleotide: 5'-CCCTGGGCTCTGTAAAGAATAGTG-3' Applied Biosystems, Foster City, CA, USA) (MW = 7392.8, concentration = 25 pmol/µl). Before analysis, the samples were diluted 1 to 1000.

LC conditions:

- Column: microguard column: C18 Pepmap®, 300 µm i.d., 5 µm particle (LC Packings, The Netherlands)
- Gradient: In the injection phase, the column, which is used for compound trapping and separation, was loaded using 0.4 M 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP; Sigma, St. Louis, MO, USA) in water (adjusted to pH 7.0 with triethylamine (TEA), including 10 mM ammonium acetate) at a flow rate of 12 µl/min. After the switch, the separation was started using 0.4 M HFIP in 50/50 methanol/water (adjusted to pH 7.0 with TEA) at a flow rate of 0.8 µl/min.
- Autosampler: Famos (LC Packings, The Netherlands)
- Column switching: Switchos (LC Packings, The Netherlands)
- Pump: Ultimate (LC Packings, The Netherlands)

MS conditions:

- Mass Spectrometer: Q-TOF hybrid mass spectrometer (Micromass, Manchester, UK)
- Ion Source: nano-electrospray source (Z-spray®) in negative ion mode
- Capillary voltage: 2200 V
- Cone voltage: 50 V

## 3. Results and discussion

Anion-exchange HPLC and ion-pair reversed-phase HPLC are the most popular modes for the separation of oligonucleotides. Unfortunately, the use of anion-exchange chromatography is incompatible with ESI due to the presence of nonvolatile inorganic salts. Ion pair chromatographic systems that employ volatile eluent components can be coupled to ESI-MS. Therefore, a LC mobile phase containing 1,1,1,3,3,3-HFIP adjusted to pH 7.0 with TEA, which results in efficient LC separation and high-sensitivity ESI with a minimum of cation adduction, was used (4, 5).

In a first set of experiments, the samples were diluted in HPLC water and the column was first loaded using 0.4 M HFIP in water (pH 7.0) during 4 minutes. In this way, the oligonucleotide was captured at the top of the column and impurities, including salt ions, were removed. After the switch, the micro-trapping column was eluted with 0.4 M HFIP in 50/50 methanol/water (pH 7.0) into the mass spectrometer, with concurrent rudimentary separation of the oligonucleotide. In this system, the retention time of the oligonucleotide was around 8.5 minutes. The resulting mass spectrum contained an envelope of peaks, which corresponded to the multiple charge states. Sodium and potassium adducts were, however, markedly present (Figure 1). The deconvoluted spectrum (zero charge state) showed the molecular mass 7393 of the oligonucleotide and also a lot of salt adducts in even greater intensity compared to the mass 7393 peak (Figure 2). These bound cations clearly reduce the sensitivity for the analyte. Reduction of the amount of adducts is critical to obtain high quality spectra.

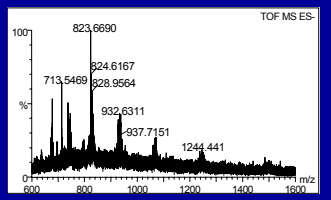


Figure 1. Spectrum of the oligonucleotide sample (with salt ions).

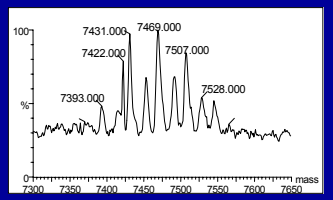


Figure 2. Deconvulted spectrum of the oligonucleotide sample.

The strategy used for reducing the amount of sodium adducts involved the replacement of sodium ions by ammonium ions, which are less tightly bound to the oligonucleotide when ionized. Therefore, the samples were diluted in ammonium acetate (pH 7.0) instead of pure HPLC water. Partial displacement of sodium adducts was observed, resulting in improved mass spectra. An end concentration of 0.3 M ammonium acetate gave the best results. Nevertheless, the potassium adducts remained visible, and an obvious impediment to sound analytical results.

Subsequent additional make up with 10 mM ammonium acetate pH 7.0 of the solvent used for loading the column, before the column switching, provided some reduction of the potassium ions. If, however, in addition the loading time was optimized from 4 to 8 minutes, a marked reduction of the potassium adducts was observed due to profoundly enhanced potassium to ammonium counterion exchange (Figure 3 and 4).

As such, a suitable procedure was established allowing fast and efficient testing of oligonucleotides.

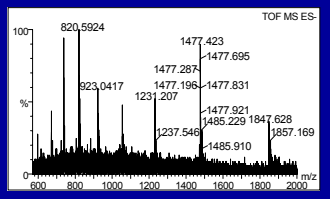


Figure 3: Spectrum of the oligonucleotide sample (no salt ions).

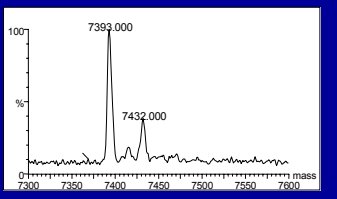


Figure 4: Deconvulted spectrum of the oligonucleotide sample.

## 4. Conclusion

It is concluded that the capillary-LC-nano-ESI-MS method with column switching can remove salt ions, deleterious for mass spectrometric oligonucleotide length and sequence analysis. The procedure, which combines trapping and separation in a single step, is rapid and fully automated, so it is ideally suited for the quality control of oligonucleotides.

## 5. Acknowledgements

We wish to thank Ing. S. Vande Casteele for all her help with the analysis of the samples. This work was supported by grant GOA99-120501-99 (Eigen OnderZoeksFonds).

## 6. References

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