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Rapid characterization of oligonucleotides by capillary liquid chromatography–nano electrospray quadrupole time-of-flight mass spectrometry

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Abstract

A fast quality control method is developed allowing the desalting and characterization of oligonucleotides by capillary liquid chromatography and on-line nano-electrospray ionization quadrupole time-of-flight mass spectrometry using column switching. The influence of addition of ammonium acetate, *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid, formic acid or acetic acid to the sample, addition of ammonium acetate to the trapping solvent and variation of the trapping time on the further reduction of cation adduction was studied. Final conditions were the addition of 0.1 M ammonium acetate to the sample, the use of a trapping solvent consisting of 0.4 M aqueous 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) adjusted to pH 7.0 with triethylamine plus 10 mM ammonium acetate during 8 min and the elution of the oligonucleotides with 0.4 M HFIP in 50% methanol. The potential of the optimized procedure is demonstrated for different synthetic oligonucleotides.

Keywords: Oligonucleotides; Quality control; Capillary ion-pair reversed-phase chromatography; Nano-electrospray ionization mass spectrometry; Quadrupole time-of-flight mass spectrometer

1. Introduction

Nowadays, synthetic oligonucleotides have become indispensable tools in modern molecular biology. They are extensively used as primers for DNA amplification by the polymerase chain reaction (PCR), as probes for in situ hybridization and as antisense oligonucleotide therapeutic agents for the treatment of several viral infections and cancer [1–5]. These oligonucleotides have to be of high purity and a defect in length or sequence is not tolerated. Therefore, the quality control is a fundamental step in their synthesis and is usually accomplished by high-performance liquid chromatography (HPLC), gel electrophoresis or capillary gel electrophoresis. These methods are based on the separation of the oligonucleotides according to their length, regardless of their base sequence or composition and are thus not sufficient for absolute identification. Furthermore, gel-electrophoretic-based methods are laborious, time-consuming, and require the use of fluorescent or radioisotopic labels or staining [1–4,6,7].

During the past decade, mass spectrometry (MS) has become another important tool in the analysis of oligonucleotides, mainly as a consequence of the development of electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) as very soft ionization techniques for large biomolecules [8,9]. This way, MS provides a basis for detecting both length and sequence variations of oligonucleotides, based on a difference in mass. Under ESI

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conditions, multiply charged ions are produced, resulting in a mass spectrum containing an envelope of peaks which correspond to ions with various charge states, having a relatively low mass-to-charge ratio ($m/z < 2500$). Computer algorithms can transform this spectrum to a zero charge spectrum [6,10].

However, oligonucleotide mass measurements are very much complicated by the affinity of the polyanionic backbone for ubiquitous cations such as sodium and potassium. These cations lower the sensitivity for the analyte by dispersing the ion abundance among multiple adducted ions. In the case of ESI-MS, these multiple cation adduct ions result in highly complex spectra, which decreases the ability to characterize mixtures of oligonucleotides. Moreover, accurate mass measurements are hampered. Therefore, effective removal of these cations is required to obtain better interpretable mass spectra, high mass accuracy, and satisfactory sensitivity [11,12].

Several off- and on-line strategies were developed for the reduction of cation adduction. Removal of these metal ions can be accomplished by the multiple ammonium acetate precipitation method [2,12,13], on-line microdialysis [11], on-line cation exchange [12] or the use of molecular weight cut-off desalting columns [14]. A lot of these procedures involve the replacement of sodium ions with ammonium ions, which are less tightly bound to the oligonucleotide when ionized. Unfortunately, these methods are rather time-consuming and require relatively large amounts of sample.

Greig and Griffey [15] reported that the 1:1 co-addition of 2.5–25 mM imidazole and a strong base such as piperidine or triethylamine (TEA) to the spray solution yielded significant suppression of adduct formation and an enhancement of signal abundance for ESI of smaller oligonucleotides. Several other reports also have suggested the use of TEA to moderate sodium and potassium adduction [16,17]. Limbach et al. [18] concluded that the addition of *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid (CDTA) and TEA to solutions of precipitated tRNAs and 5S RNA reduced magnesium and sodium adduct ions to levels which permit accurate mass determination.

Cheng et al. [19] and Muddiman et al. [20] investigated the effects of the addition of acids to the analyte solution on cation adduct formation using ESI. Direct addition of acids to the oligonucleotide solution shifted the center of the charge state distribution to higher m/z (called charge state reduction) without appreciably affecting the sensitivity. Acetic and formic acids were observed to be better reagents than inorganic acids. The main argument for reducing the charge states of oligonucleotide ions in ESI-MS is to increase the signal-to-noise ratio and to decrease the spectral complexity in order to enable or to simplify mixture analysis.

Also high-performance liquid chromatographic methods can be used for the purification of oligonucleotides. It has the added advantage of separation of mixtures. Anion-exchange HPLC and reversed-phase HPLC, which can be done with or without an ion-pairing reagent, are the most popular chromatographic modes. Because ESI forms ions directly from a liquid solution, it is ideally suited for the direct interfacing to HPLC. An advantage of the coupling of HPLC to ESI-MS is that it combines desalting, separation and characterization of the oligonucleotide and possible oligonucleotide impurities (such as incomplete or adventitious synthesis products) [21]. This combination requires the choice of volatile mobile phase additives, which allows the efficient mass spectrometric detection of the analytes.

Unfortunately, anion-exchange HPLC applies gradients of increasing salt concentrations to elute the oligonucleotides from the columns, which makes this mode incompatible with ESI-MS. Bleicher and Bayer [22] and Apffel et al. [4,23] reported that the coupling of the conventional reversed-phase separation method for oligonucleotides, based on the use of 100 mM triethylammonium acetate (TEAA) buffer solution as mobile phase, with ESI-MS results in a drastic reduction of electrospray signal abundance. Reducing the concentration of TEAA improved the signal abundance in ESI-MS, but significantly deteriorated the chromatographic separation performance.

Therefore, several research groups looked for MS-friendly mobile phases, which result in efficient HPLC separation and high ESI-MS sensitivity. Several reviews on the analysis of nucleic acids by on-line liquid chromatography (LC)–MS have recently been published [10,24,25]. Solvent systems consisting of ammonium acetate [22], diisopropylammonium acetate [26], triethylammonium bicarbonate [8,27] and butyldimethylammonium bicarbonate [28] with an acetonitrile gradient for the HPLC–ESI-MS analysis of oligonucleotides were reported. In 1997, Apffel et al. [4,23] recommended the use of 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) adjusted to pH 7.0 with TEA as ion-pairing reagent and methanol as organic modifier, which resulted in efficient HPLC separation of oligonucleotides up to 75 nucleotides and high sensitivity ESI-MS with a minimum of adduct formation. This eluent combination is nowadays often used, although with varying success with respect to adduct formation [8,21].

Methods utilized for the quality control of oligonucleotides after synthesis should be rapid and easy to automate as well as sensitive and informative. Therefore, the desalting step should be fast, not labor-intensive and easy to automate. In this paper, we describe an optimized, fast capillary liquid chromatographic method coupled to nano-ESI-quadrupole time-of-flight (Q-TOF) for the single-step desalting and separation as well as characterization of oligonucleotides using column switching. HFIP adjusted to pH 7.0 with TEA was used as mobile phase additive with methanol as organic modifier. The influence of the addition of ammonium acetate, CDTA, formic acid or acetic acid to the sample, the addition of ammonium acetate to the trapping solvent and the variation of the trapping time on maximizing reduction of cation adduction was studied. The optimized ion-pair LC–ESI-MS method was finally used to analyse different synthetic oligonucleotides.

Table 1
Oligonucleotides used in this study

No.	Base composition	Base length	Theoretical mass	Measured mass (S.D.) (n = 3)	Relative error (ppm)
1	5′-CCA CCA TGC CAC CTC CT-3′	17	5011.3092	5011.50 (0.05)	39
2	5′-GGT GCT CCA GGT GCC CAT-3′	18	5491.6027	5491.98 (0.03)	69
3	5′-CCC TGG GCT CTG TAA AGA ATA GTG-3′	24	7392.8587	7393.00 (0.11)	20
4	5′-ATC AGA GCT TAA ACT GGG AAG CTG-3′	24	7425.8973	7426.06 (0.05)	20
5	5′-AAT AAG CTT CCA CCA TGC CAC CTC CT-3′	26	7795.1307	7795.44 (0.15)	39
6	5′-ATT GTC GAC GGT GCT CCA GGT GCC CA-3′	26	7963.2024	7963.47 (0.09)	34
7	5′-ATT GTC GAC GCT CTT CAT CCT CGT TCT CA-3′	29	8745.7164	8745.94 (0.12)	25
8	5′-ATT GTC GAC CAC AGC TGA GAC CTT CCA GCC-3′	30	9111.9673	9112.35 (0.05)	42

2. Experimental

2.1. Chemicals and samples

Ammonium acetate (98%) and acetic acid (99.8%) were obtained from Aldrich (St. Louis, MO, USA). Formic acid (analytical reagent grade) was purchased from Fluka (Buchs, Switserland). Methanol (HPLC gradient grade) was from Merck (Darmstadt, Germany). 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP, analytical reagent grade), triethylamine (TEA, 99%) and *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid (CDTA, >99%) were from Sigma (St. Louis, MO, USA). For preparation of all aqueous solutions, high-purity water, provided from a Synergy 185 system (Millipore, Bedford, MA, USA), was used.

The oligonucleotides were synthesized by Applied Biosystems (Warrington, Cheshire, UK) and were used without further purification (Table 1). The concentration of the oligonucleotides was between 125 and 180 pmol/μL.

2.2. Liquid chromatography

The capillary liquid chromatography system consisted of a low-pressure gradient micropump (Ultimate, LC Packings, Amsterdam, The Netherlands), a Famos autosampler with a 10 μL sample loop (LC Packings) and a Switchos column switching system (LC Packings). Compound trapping and separation were performed using one single microguard column packed with C_{18} Pepmap (1 mm × 300 μm i.d., 5 μm particle, LC Packings).

The HFIP mobile phase was prepared as an aqueous stock solution of 0.8 M, adjusted to pH 7.0 with TEA. This stock solution was diluted to 0.4 M with water (when used as loading solvent) or methanol (separation solvent). All solvents were degassed using helium gas.

In a first set of experiments, the oligonucleotide sample 3 was diluted 1 to 500 with 0.4 M HFIP in water (adjusted to pH 7.0 with TEA). After the injection of 10 μL of the sample, the column was loaded/washed/desalted using 0.4 M HFIP in water (adjusted to pH 7.0 with TEA) at a flow rate of 12 μL/min. After a preset loading time, a valve switch was initiated and the elution/separation was started with 0.4 M HFIP in methanol–water (50:50) (adjusted to pH 7.0 with TEA) at a flow rate of 0.8 μL/min. Unfortunately, a lot of cation adducts were visible using these conditions. Therefore, in a second set of experiments, the samples were diluted with 0.4 M HFIP in water supplemented with varying concentrations of ammonium acetate, CDTA, or formic or acetic acid. In order to further improve the quality of the spectra, the loading solvent was supplemented with 10 mM ammonium acetate. If, in addition, the loading time was optimized, a further reduction of the cation adducts was accomplished. This optimization procedure resulted in a final experimental protocol in which the oligonucleotide sample was diluted in 0.4 M aqueous HFIP + 0.1 M ammonium acetate (pH 7.0). The loading solvent used was 0.4 M aqueous HFIP + 10 mM ammonium acetate (pH 7.0) with a loading cycle time of 8 min.

2.3. Mass spectrometry

ESI-MS experiments were performed on a quadrupole time-of-flight (Q-TOF) mass spectrometer (Waters, Manchester, UK) equipped with a nano-electrospray source (Waters, Manchester, UK). The capillary HPLC system was connected to the spray capillary (on-line PicoTip emitter, coated SilicaTip, 360 μm o.d., 20 μm i.d., 10 μm i.d. at the tip; New Objective, Woburn, MA, USA) by means of fused silica (280 μm o.d. × 20 μm i.d.).

Data were collected and analyzed using the MassLynx software (Waters, Manchester, UK). The expected average molecular masses of all oligonucleotides were calculated using Biolynx software, which is part of the MassLynx software package. Using the MaxEnt algorithm (Waters, Manchester, UK), deconvoluted spectra were calculated, displaying the observed masses of the uncharged molecules (Table 1).

Negative ESI was performed using an ionization voltage of −2.5 to −2.8 kV. The sample cone voltage was set at 35−45 V and the source temperature was controlled at 80 °C. The N_2 desolvation gas was maintained at 120 °C at a flow rate of 50 L/h and the N_2 nebuliser gas pressure was optimized at 0.3 bar. Full scan spectra were acquired over the m/z 510–2500 range at a scan accumulation rate of 2.5 s/scan and an interscan delay of 0.1 s. All spectra were collected in continuum mode.

3. Results and discussion

After synthesis, all oligonucleotides should be tested for a defect in length or sequence. MS provides a basis for detecting both length and sequence variations of oligonucleotides, based on a difference in mass. ESI-MS analysis of oligonucleotides with high resolution instruments, such as the Q-TOF or the Fourier transform ion cyclotron resonance (FT-ICR) mass analyzers, allows differentiation of single base substitutions, resulting in a mass change between 9 and 40 Da (A–T and G–C switch, respectively) [10,29–31].

However, the detection and characterization of oligonucleotides by ESI-MS is associated with difficulties arising from the adduction of non-volatile cations, such as sodium or potassium, to the polynucleotide backbone resulting in highly complex mass spectra, decreased sensitivity and poor mass accuracy. Therefore, efficient desalting of oligonucleotides is imperative [12].

Ion-pair reversed-phase HPLC (RP-HPIPC), which is one of the most popular modes for the desalting and separation of oligonucleotides, can easily be coupled to ESI-MS, if volatile eluent components are employed. As an alternative to TEAA, which is the optimal mobile phase additive for IP-RP-HPLC of oligonucleotides but results in drastic reduction of ion formation during ESI, a mobile phase containing 1,1,1,3,3,3-HFIP was introduced by Apffel et al. [4].

Using HFIP at the 0.4 M level when the solution was adjusted to pH 7.0 with TEA, separations comparable to those obtained with TEAA as mobile phase could be obtained. Nevertheless, compared to TEAA, separation efficiency for long-chain oligonucleotides was impaired with eluents containing triethylammonium hexafluoroisopropanolate, mainly because the concentration of TEA is very low in the TEA/HFIP eluents [8]. The separation of the oligonucleotides is based on a gradient using methanol as an organic modifier.

According to Apffel et al. [4], the mechanism proposed for the behavior of this system is based on the dynamic adjustment of the pH in the electrospray droplet as a function of the preferential removal of anionic counterion from the droplet by evaporation. Comparing the two solvent systems aqueous triethylamine/acetic acid and aqueous triethylamine/hexafluoro-2-propanol, the key physicochemical parameters involved are the relative volatilities of the species and the relative dissociation constants. HFIP (b.p. = 57 °C) is more volatile than TEA (b.p. = 89 °C), while acetic acid is much less volatile (b.p. = 118 °C). As a buffer system for HPLC, the weak acid/base system of HFIP/TEA maintains a stable pH at around 7.0. During the separation, the TEA ions ion-pair with the negatively charged phosphate groups of the oligonucleotide backbone. However, as the column effluent is electrosprayed and desolvated, the volatile HFIP is depleted at the droplet surface (if not the bulk) and the pH at the surface rises toward 10. At the higher pH, the oligonucleotide–TEA ion pairs dissociate, and the oligonucleotides can be desorbed into the gas phase. The other important parameter involved is the dissociation constants in the two buffer systems. The pK_a values of acetic acid, hexafluoro-2-propanol and triethylamine are 4.8, 9.3 and 11.0, respectively. Thus, at pH 7.0, acetic acid is virtually completely dissociated and consequently cannot be efficiently removed by evaporation. The HFIP is not charged at pH 7.0 and can be evaporated freely [4]. However, later literature has clearly shown that TEA/HFIP eluents facilitate a very efficient detection by ESI-MS, mainly because of the low conductivity of this solvent system [8].

For optimum compatibility of HPLC with ESI-MS and low detection limits, the flow of the sample solution introduced into the ion source of the mass spectrometer should be in the microliter or nanoliter per minute range. Therefore, miniaturized chromatographic separation systems applying capillary columns of 10–500 μm inner diameter and low flow-rate electrospray sources are the method of choice for the seperation and characterization of oligonucleotides [24,25,27,32].

The aim of this research is to develop an on-line capillary liquid chromatographic ESI-MS method which combines the efficient desalting and characterization of oligonucleotides and is less complicated and faster than the existing HPLC–ESI-MS methods. In that respect, a capillary microguard column was selected for the desalting and the separation of the oligonucleotides and coupled to the nano-electrospray source of the Q-TOF mass spectrometer. The HFIP/TEA eluents were selected as mobile phase.

In a first set of experiments, an oligonucleotide (sample 3, Table 1) was diluted with 0.4 M HFIP in water (pH 7.0) to a concentration of 0.36 pmol/μL. After injection of 10 μL of this sample (3.6 pmol on column), the column was loaded using 0.4 M HFIP in water (pH 7.0) during 4 min. This way, the oligonucleotide was captured at the top of the column and impurities, including salt ions, were removed. After the valve switch, the micro-trapping column was eluted with 0.4 M HFIP in methanol–water (50:50) (pH 7.0) into the mass spectrometer. As such, the micro-trapping column is not only eluted but a concurrent rudimentary separation is obtained. Removal of cations during this chromatographic performance takes place in the mobile phase through competition of an excess of triethylammonium ions with metal ions for the negative charges at the sugar–phosphate backbone. These triethylammonium ions dissociate and evaporate during the ESI process, leaving the oligonucleotide molecule in the hydrogen form [8,12].

Fig. 1A shows the total ion chromatogram, Fig. 1B shows the raw electrospray negative ion spectrum for oligonucleotide 3 and Fig. 1D shows its deconvoluted spectrum. As can be seen, the retention time of the oligonucleotide was around 13–14 min. The resulting mass spectrum contained an envelope of peaks, which corresponded to the multiply charge states. However, also sodium and especially potassium adducts were markedly present, with adducts ranging from the monosodium to the monosodium-tetrapotassium adduct (see Fig. 1C, enlargement of charge state 7−). Such an

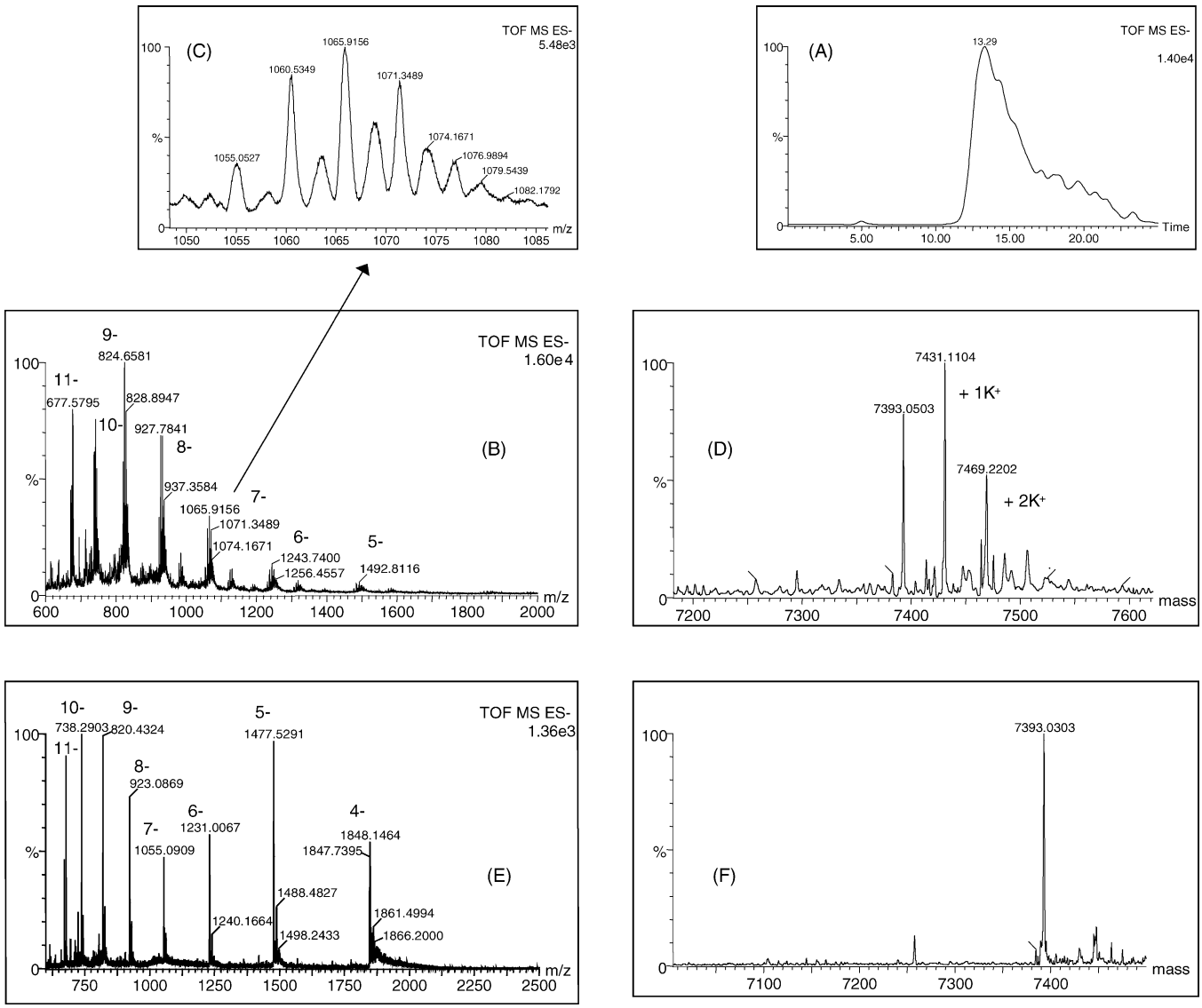

Fig. 1. RP-HPICP-ESI-Q-TOF analysis of oligonucleotide 3 diluted with 0.4 M HFIP in water (pH 7.0): (A) the total ion chromatogram; (B) the raw electrospray negative ion spectrum; (C) enlargement of charge state 7− and (D) the deconvoluted spectrum (7393.0503: oligonucleotide 3 (zero charge state), 7431.1104: K adduct, 7469.2202: 2K adduct); loading/washing solvent, 0.4 M HFIP in water (pH 7.0); loading time, 4 min; elution solvent, 0.4 M HFIP in methanol–water (50:50) (pH 7.0). RP-HPIPC–ESI-Q-TOF analysis of oligonucleotide 3 diluted with 0.4 M HFIP in water (pH 7.0) + 0.1 M ammonium acetate (pH 7.0): (E) the raw electrospray negative ion spectrum and (F) the deconvoluted spectrum (7393.0303: oligonucleotide 3, zero charge state); loading/washing solvent, 0.4 M HFIP in water (pH 7.0) + 10 mM ammonium acetate (pH 7.0); loading time, 8 min; elution solvent, 0.4 M HFIP in methanol–water (50:50) (pH 7.0).

extensive cation adduction, unlike the initial claims made with an HFIP/TEA eluent mixture, was also observed by Huber and Krajete [8], using the same mobile phase. The reason for the low efficiency in reducing cation ion adduction is probably the relatively low concentration of triethylammonium ions present in the mobile phase. The deconvoluted spectrum (zero charge state) showed the molecular mass 7393 of the oligonucleotide and also a lot of salt adducts in even greater abundance compared to the mass 7393 peak. The monopotassium adduct was the most abundant species in the deconvoluted mass spectrum (ratio of 1:0.8 for the intensities of the 7431:7393 signals). The higher abundance of the potassium adducts indicates a higher affinity of potassium for the phosphodiester groups of the oligonucleotides [8]. These bound cations clearly reduce the sensitivity for the analyte by dispersing the signal from any charge state across an envelope of cation-containing species. Reduction of the amount of adducts is critical to obtain high quality spectra and adequate sensitivity.

In an attempt to improve the quality of the spectra, the oligonucleotide sample was diluted with 0.4 M HFIP in water supplemented with varying concentrations of ammonium acetate (0.1, 0.3, 0.5 or 1 M). The strategy used for reducing the amount of adducts involves the competition of an excess

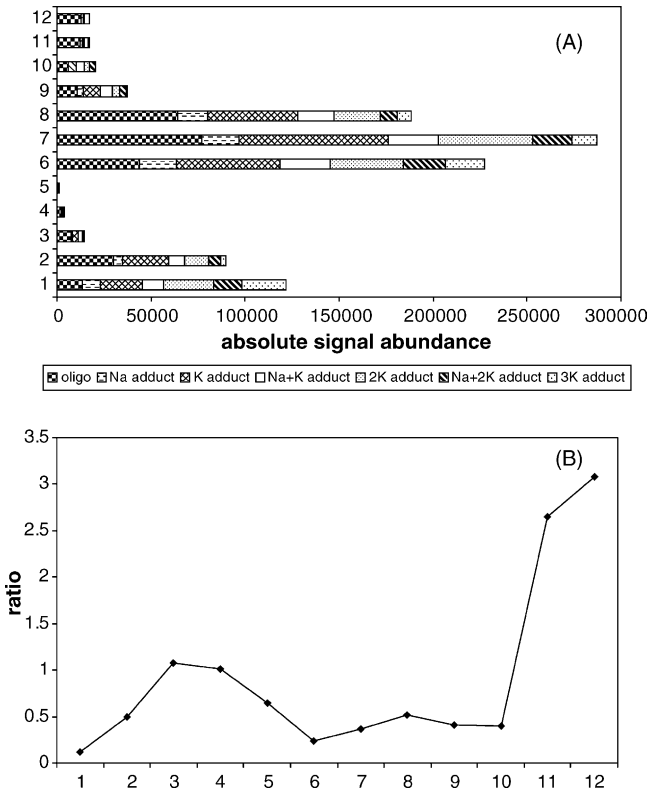

Fig. 2. (A) Comparison of the sum of the signal abundances (peak heights) of the different multiply charged ions of oligonucleotide 3 and of all the observed adducts, extracted from the full scan spectra of the sample diluted with 0.4 M HFIP in water (pH 7.0) (1), 0.4 M HFIP in water (pH 7.0) + 0.1 M (2), 0.3 M (3), 0.5 M (4) or 1 M ammonium acetate (pH 7.0) (5), 0.4 M HFIP in water (pH 7.0) + 1 nmol CDTA/100 pmol oligonucleotide (6), 3 nmol CDTA/100 pmol oligonucleotide (7) or 5 nmol CDTA/100 pmol oligonucleotide (8), 0.4 M HFIP in water (pH 7.0) + 0.5% acetic (9) or formic acid (10), and all loaded/washed with 0.4 M HFIP in water (pH 7.0) during 4 min, or diluted with 0.4 M HFIP in water (pH 7.0) + 0.1 M ammonium acetate (pH 7.0) and loaded/washed with 0.4 M HFIP in water (pH 7.0) + 10 mM ammonium acetate (pH 7.0) during 4 min (11) or 8 min (12). (B) Effect of the mode of dilution, the composition of the solvent for loading/washing and the loading cycle time on the signal abundance of oligonucleotide 3 and its adducts, expressed as the ratio of the sum of the peak heights of the different multiply charged ions of the oligonucleotide and the sum of the peak heights of all the observed adducts extracted from the full scan spectrum of the sample.

of ammonium ions with metal ions for the negative charges at the sugar–phosphate backbone. Ammonium ions appear to be less tightly bound to the phosphodiester groups than sodium or potassium ions and they can dissociate during the electrospray process, leaving one proton with the oligonucleotide [33].

Fig. 2A shows a comparison of the sum of the signal abundances (peak heights) of the different multiply charged ions of the oligonucleotide and of all the observed adducts, extracted from the full scan spectra of the sample diluted with 0.4 M HFIP in water supplemented with, respectively, 0.0, 0.1, 0.3, 0.5 and 1 M ammonium acetate. Fig. 2B represents the ratio of the sum of the peak heights of the different multiply charged ions of the oligonucleotide and the sum of the peak heights of all the observed adducts extracted from the full scan spectrum of the sample. The higher this ratio, the better the oligonucleotide sample was desalted. Partial displacement of sodium and potassium adducts was observed (Fig. 2A), resulting in improved mass spectra. Nevertheless, the potassium adducts remained visible, obviously impeding sound analytical results. According to Fig. 2B, the optimum results were accomplished with the supplementation of 0.3 M ammonium acetate for the dilution of the sample. However, using an ammonium acetate concentration of 0.3 M or more for the dilution of the sample, resulted also in a substantially reduction of the analyte signal (Fig. 2A).

According to Limbach et al. [18], the addition of chelating agents and TEA to the spray solution of ammonium precipitated tRNAs and 5S RNA was extremely effective at reducing mono- and bivalent cation adduction. These chelating agents abstract especially bivalent metal cations, such as magnesium, from the analyte. The best results were accomplished when CDTA was used. In that respect, the sample was, in a next experiment, diluted with the 0.4 M aqueous HFIP solution with varying concentrations of CDTA (1, 3 or 5 nmol/100 pmol oligonucleotide) added. In Fig. 2A, a comparison can be made between the effect of the addition of CDTA to the dilution solvent and simple dilution with 0.4 M HFIP in water expressed as the sum of the absolute abundance of all the charge states of the oligonucleotide sample and its adduct ions. If the sample was diluted with a CDTA containing solvent, the sensitivity of the analyte signal was

about four times higher. But also adduct ions remained visible, especially the potassium adducts (Fig. 2).

If alternatively, the sample was diluted with 0.4 M HFIP in water supplemented either with 0.5% acetic or formic acid, only a reduction of the signal abundance, but no reduction of the amount of adduct ions was seen (Fig. 2). The suppression of the oligonucleotide signal abundance upon addition of an acid is most likely due to the competition of anions for ionization [8].

It can be concluded from these experiments that the best results for cation reduction were obtained if the sample was diluted with 0.4 M HFIP in water supplemented with 0.1 M ammonium acetate.

In a next step, the actual loading process was addressed. An extension of the loading time from 4 min to 8 or 15 min did not yield better results. Ammonium acetate (10 mM, pH 7.0) was added to the loading solvent and evaluated on its possibility to reduce the amount of sodium and especially potassium adducts. The oligonucleotide sample was diluted with 0.4 M HFIP in water containing 0.1 M ammonium acetate. During the loading step, the cations complexed to the oligonucleotide were replaced by ammonium ions by washing with the ammonium acetate solution used in the loading/washing solvent. These ammonium ions, which appear to be less tightly bound to the phosphodiester groups, can dissociate during the electrospray process, leaving one proton with the oligonucleotide [33].

The results are shown in Fig. 2. A comparison can be made between the effect on the absolute abundance of the sum of the charge states of the oligonucleotide and its adducts, if the sample was loaded/washed with the ammonium acetate containing loading solvent and diluted with 0.4 M HFIP in water supplemented with 0.1 M ammonium acetate, and the absolute abundance of the sum of the charge states of the oligonucleotide and its adducts, if the sample was diluted with 0.4 M HFIP in water + 0.1 M ammonium acetate and was loaded/washed with 0.4 M HFIP in water. A reduction of the sodium adducts and an impressive reduction of the potassium adducts was observed. A significant increase of the ratio oligonucleotide/adducts can be observed in Fig. 2B.

If, in addition, the loading time was optimized from 4 to 8 min, a further reduction of the potassium adducts was observed due to profoundly enhanced potassium to ammonium counterion exchange (Fig. 2). Fig. 1E shows the raw electrospray negative ion spectrum for oligonucleotide 3 and Fig. 1F shows its deconvoluted spectrum, which was obtained after the analysis of the sample diluted with 0.4 M HFIP + 0.1 M ammonium acetate and loaded/washed with 0.4 M HFIP + 10 mM ammonium acetate during 8 min. As can be seen, applying this on-line capillary RP-HPIPC–nano-ESI-MS system, the cations were efficiently removed leaving only a very small amount of monopotassium adducts. The molecular mass 7393 was the most abundant species in the deconvoluted mass spectrum (ratio of 1:15 for the intensities of the 7431:7393 signals). The almost complete elimination of adduction allowed a precise molecular mass determination with a measured mass of 7393.03, which compares well to the theoretical mass of 7392.8587.

Several other oligonucleotide preparations ranging in size from 17 to 30 nucleotides were analyzed the same way, to evaluate the ability of the on-line desalting procedure. All the samples were diluted to a concentration between 0.25 and 0.36 pmol/μL. With a 10 μL injection, the amount of oligonucleotide directed into the electrospray source ranged from 2.5 to 3.6 pmol. The theoretical and the mean molecular masses of three measurements with the standard deviations (S.D.s) are summarized in Table 1. It can be seen that all masses were obtained with a mass measurement accuracy between 15 and 70 ppm. For seven oligonucleotides, the maximum error obtained on the mass determination was less than 45 ppm. In the molecular mass range of these oligonucleotides (5000–9000 Da), this corresponds to an error of maximum 0.45 Da.

These values are better than those reported in literature for the mass determination of oligonucleotides by ESI-MS with a Q-TOF mass spectrometer. Deforce et al. [3] reported that the maximum errors obtained on the mass determination of oligonucleotides (5000–9000 Da) using capillary zone electrophoresis–ESI-Q-TOF-MS are about 100 ppm, which corresponds in this molecular mass range to an error of 0.8 Da. Other reports using ESI-Q-TOF-MS analysis of oligonucleotides mention mass errors of less than 0.2 Da (2000–5000 Da) [29,34] and less than 4 Da (about 17,000 Da) [30]. According to Flora et al. [35], the best results for mass accuracy with a Q-TOF instrument can be achieved using internal calibration (dual-micro-ESI-source) with an internal standard of similar size and charge state to that of the analyte. However, in spite of this improved mass accuracy, it is impractical to use an internal calibrant with a molecular mass analogous to the large PCR amplicons. They also pointed out that internally calibrating the multiply charged ions of the oligonucleotide using singly-charged synthetic polymer peaks resulted in a systematic error attributed to the kinetic energy difference induced by the significant difference of size and charge between the calibrant and the analyte compounds. Thus, this may explain some of the poorer mass measurement accuracies, i.e. between 1 and 3 Da for large nucleic acids (14,000–35,000 Da). This would also result if the instrument was externally calibrated with a low-molecular-mass-species.

Previously reported mass accuracies for oligonucleotides and DNA fragments range between 0.1 and 6 Da for small (3000–12,000 Da) and between 0.3 and 45 Da for large nucleic acids (17,000–38,000 Da) by ESI-quadrupole-MS [4,12,18,30,36], between 0.1 and 0.9 Da for small (2000–5000 Da) and between 0.1 and 5 Da for large nucleic acids (19,000–30,000 Da) by ESI-ion trap-MS [27,28,37], between 0.1 and 3.6 Da (8000–34,000 Da) by ESI-orthogonal-TOF-MS [21], and between 0.2 and 3 Da (25,000–70,000 Da) and between 0.04 and 1 Da (4500–50,000 Da) by ESI-FT-ICR-MS with external [2,38] and internal calibration, respectively [39–41].

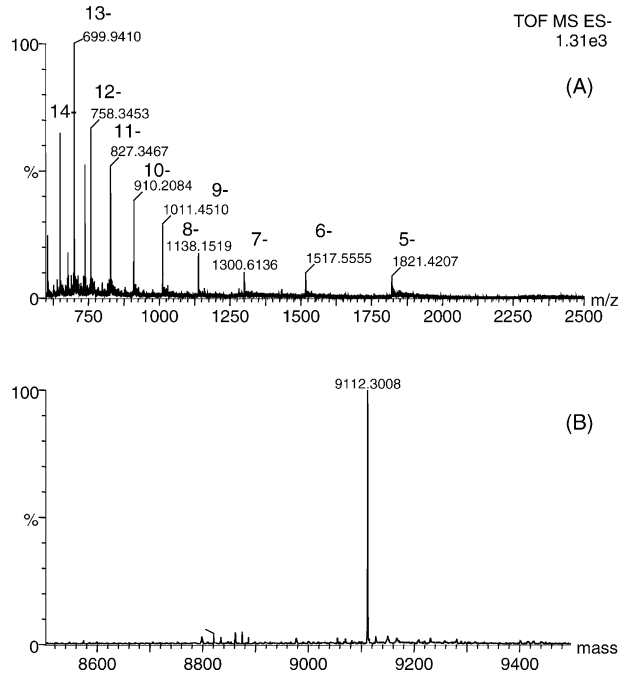

Fig. 3. RP-HPIPC–ESI-Q-TOF analysis of oligonucleotide 8 diluted with 0.4 M HFIP in water (pH 7.0) + 0.1 M ammonium acetate (pH 7.0): (A) the raw electrospray negative ion spectrum; (B) the deconvoluted spectrum (9112.3008: oligonucleotide 8, zero charge state); loading/washing solvent, 0.4 M HFIP in water (pH 7.0) + 10 mM ammonium acetate (pH 7.0); loading time, 8 min; elution solvent, 0.4 M HFIP in methanol–water (50:50) (pH 7.0).

It is obvious from these data that even the smallest difference (A–T switch differing 9 Da in mass) can be detected without any problem. Fig. 3 shows the raw electrospray negative ion spectra and its deconvoluted spectrum for oligonucleotide 8. Note that in this sample there are no adducts observed. This example demonstrates that by using this online capillary RP-HPIPC–ESI-MS method, the unequivocal identification of low picomole amounts of oligonucleotides is feasible on the basis of their molecular masses without interference of cation adducts. As such, a suitable procedure was established allowing fast and efficient testing of oligonucleotides.

4. Conclusions

It is concluded that the capillary LC–nano-ESI-MS method with column switching provides effective suppression of cation adducts deleterious for mass spectrometric oligonucleotide length and sequence analysis, thus eliminating the need for a sample preparation step. The procedure which combines trapping and separation in a single step is rapid, easy and fully automated so it is ideally suited for the quality control of oligonucleotides. The combination of capillary RP-HPIPC and nano-ESI-Q-TOF-MS allows the identification of oligonucleotides differing in length by one nucleotide, so a misincorporation of the smallest mass difference (A–T switch differing 9 Da in mass) can be detected without any problem. The method is useful for oligonucleotide amounts below the 5 pmol level.

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