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Sheath liquid effects in capillary high-performance liquid chromatography–electrospray mass spectrometry of oligonucleotides

Christian G. Huber*, Alexander Krajete

Institute of Analytical Chemistry and Radiochemistry, Leopold-Franzens-University, Innrain 52a, A-6020 Innsbruck, Austria

Abstract

Fused-silica capillary columns of 200 μm inner diameter were packed with micropellicular, octadecylated, 2.3 μm poly(styrene–divinylbenzene) particles and applied to the separation of oligonucleotides by ion-pair reversed-phase high-performance liquid chromatography. Oligonucleotides were eluted at 50°C with gradients of 3–13% acetonitrile in 50 m*M* triethylammonium bicarbonate. Addition of sheath liquid to the column effluent allowed the detection of oligonucleotides by electrospray ionization mass spectrometry using full-scan data acquisition with a detectability comparable to that obtained with UV detection. The signal-to-noise ratios with different sheath liquids increased in the order isopropanol< methanol<acetonitrile. The incorporation of volatile acids or bases such as triethylamine or hexafluoroisopropanol into the sheath liquid was found to influence the charge state distribution of oligonucleotides longer than 20 nucleotide units whereas no significant effect was observed with shorter oligonucleotides. Organic acids and bases in the sheath liquid generally deteriorated the signal-to-noise ratios in the chromatograms and mass spectra mainly because of increased background noise. Only a few charge states were observed in the mass spectra of oligonucleotides because of charge state reduction due to the presence of carbonic acid in the eluent. With triethylammonium hydrogencarbonate as chromatographic eluent and acetonitrile as sheath liquid, very few cation adducts of oligonucleotides were observed in the mass spectra. However, the presence of small amounts of monopotassium adducts enabled the calculation of the charge state of multiply charged ions. With acetonitrile as sheath liquid, 710 amol of a 16-mer oligonucleotide were detected using selected ion monitoring data acquisition with a signal-to-noise ratio of 3:1. Finally, capillary ion-pair reversed-phase high-performance liquid chromatography–electrospray ionization mass spectrometry was applied to the separation and characterization of heterooligonucleotide primers. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Sheath liquid effects; Oligonucleotides

1. Introduction

Synthetic oligodeoxyribonucleotides are extensively applied in biotechnology and biochemistry as hybridization probes, primers for DNA amplification by polymerase chain reaction, adaptors for cloning, and templates for the construction of deletions, insertions and site-specific mutations [1]. Since the introduction of phosphoramidite chemistry, rapid solid-phase synthesis of oligomers of 100 or more nucleotide units is feasible. Nonetheless, the crude products that come from solid-phase synthesis usually are contaminated with variable amounts of truncated and partially protected sequences, and hence, purification and quality control are essential [2]. One of the more popular separation techniques for oligonucleotides is ion-pair reversed-phase high-performance liquid chromatography (IP-RP-HPLC) using

*Corresponding author. Tel.: +43-512-507-5176; fax: +43-512-507-2767.

E-mail address: christian.huber@uibk.ac.at (C.G. Huber)

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columns packed with a reversed-phase stationary phase, triethylammonium acetate (TEAA) as ion-pair reagent, and acetonitrile gradients to elute the oligonucleotides from the column [3–5]. Mass spectrometry (MS) became another important tool for the analysis of high-molecular-mass biopolymers, such as oligo- and polynucleotides [6–10] through the introduction of electrospray ionization (ESI) by Whitehouse et al. as a soft ionization technique [11]. Moreover, ionization of analytes from a liquid phase makes ESI ideally suited for direct interfacing of HPLC with MS [12]. HPLC using the ion-pair reversed-phase mode for separation, on the other hand, can be conveniently coupled to ESI-MS because the mobile phase comprises exclusively volatile components.

However, for optimum performance of HPLC–ESI-MS, the flow of sample solution introduced into the ion source of the mass spectrometer should be in the microliter or nanoliter per minute range [13]. This requires either the post-column splitting of the column effluent [14] or miniaturization of the chromatographic separation system by using columns in the capillary format [15]. Another important issue in the conjugation of HPLC and ESI-MS is the proper choice of a suitable chromatographic phase system which allows the efficient mass spectrometric detection of the separated analytes. Generally, the choice of mobile phase compositions compatible with ESI-MS detection is restricted to components of high volatility, low surface tension and low conductivity. The effects of the most important solution parameters such as concentration of organic solvent, type and concentration of ion-pair reagent, and solution pH on the performance of IP-RP-HPLC and negative-ion ESI-MS have been shown to be usually contrary, i.e., conditions ideal for one method are non-ideal for the other [16]. Bleicher and Bayer as well as Apffel et al. found that triethylammonium acetate, which is the optimal mobile phase additive for IP-RP-HPLC of nucleic acids, resulted in drastic reduction of ion-formation during ESI [14,17]. However, utilization of 2.2 m*M* triethylamine–400 m*M* 1,1,1,3,3,3-hexafluoro-2-propanol as an ion-pair reagent enabled efficient HPLC separation and detection of oligonucleotides in the low picomol range [18,19]. Nevertheless, compared to triethylammonium acetate, separation efficiencies for larger oligomers were impaired with eluents containing triethylammonium hexafluoroisopropanolate.

Through the addition of a coaxial flow of sheath liquid to the column effluent solvent conditions otherwise inappropriate for ESI-MS can be used for the separation and detection of the analytes. The idea of using coaxial flow was originally developed for the post-column derivatization of analytes [20,21]. Later, addition of a sheath liquid was proposed for the coupling of capillary electrophoresis (CE) and ESI-MS [22], where it assists in formation of the electrospray itself and completes the electrical circuit. Moreover, sheath liquids that contain volatile acids or bases offer the possibility of manipulating the charge state distribution of multiply charged ions [23]. Witters et al. utilized the concept of adding a sheath liquid to enhance the detectabilities of cyclic nucleotides after separation by IP-RP-HPLC [24]. They observed an approximately 10-fold increase in the ESI-MS signal of cyclic cytidine monophosphate upon addition of an isopropanol sheath to the column effluent comprising 50 μ*M* tetrabutylammonium bromide, 15% methanol and 0.25% acetic acid.

Recently, we demonstrated that capillary IP-RP-HPLC employing a micropellicular, octadecylated poly(styrene–divinylbenzene) stationary phase (PS–DVB–C_{18}) and triethylammonium hydrogencarbonate as ion-pair reagent can be efficiently coupled on-line to ESI-MS for the separation and detection of nucleic acids, if acetonitrile is added as a sheath liquid to the column effluent, resulting in a more than seven-fold increase in signal intensity compared to detection without sheath liquid [16]. Since in that study only acetonitrile at a fixed flow-rate was applied as a sheath liquid, we now investigated in detail the influence of different sheath liquid compositions, acids or bases as additives, and sheath liquid flow-rate on the detectability, charge state distribution, and cation adduct formation in ESI-MS detection of oligonucleotides that have been separated by IP-RP-HPLC.

2. Experimental

2.1. Chemicals and oligonucleotide samples

Acetonitrile (HPLC gradient-grade), methanol

(HPLC gradient-grade), and isopropanol (analytical reagent grade) were obtained from Merck (Darmstadt, Germany). Triethylamine (analytical reagent grade) and 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, analytical reagent grade) were purchased from Fluka (Buchs, Switzerland). A 1.0 *M* stock solution of triethylammonium hydrogencarbonate (TEAB), pH 8.4–8.8, was prepared by passing carbon dioxide gas (AGA, Vienna, Austria) into a 1.0 *M* aqueous solution of triethylamine at 5°C until the desired pH was reached. 400 m*M* triethylammonium hexafluoroisopropanolate, pH 6.90, was prepared by titration of 400 m*M* hexafluoroisopropanol with a 5.0% solution of triethylamine in water. For preparation of all aqueous solutions, high-purity water (Epure, Barnstead, Newton, MA, USA) was used. The pH of the eluents was adjusted with an accuracy of ± 0.05 units and the given pH values always correspond to the neat aqueous solutions before addition of organic solvent. The standards of phosphorylated and non-phosphorylated oligonucleotides [$p(dT)_{12-18}$, $p(dT)_{19-24}$, $p(dT)_{25-30}$, $(dT)_{16}$] were purchased as sodium salts from Pharmacia (Uppsala, Sweden) or Sigma–Aldrich (St. Louis, MO, USA). The synthetic oligonucleotides $(dT)_8$ (M_r 2371.59), $(dT)_{16}$ (M_r 4805.15), $(dT)_{24}$ (M_r 7238.71) were ordered from Microsynth (Balgach, Switzerland) and used without further purification. Oligonucleotides of mixed sequence (19-mer, 20-mer and 21-mer) were synthesized on a DNA synthesizer (Model 381-A, Applied Biosystems, San Jose, CA, USA). Subsequently, they were purified by means of oligonucleotide purification cartridges (Applied Biosystems).

2.2. High-performance liquid chromatography

The HPLC system consisted of a low-pressure gradient micro pump (Model Rheos 2000, Flux Instruments, Karlskoga, Sweden) controlled by a personal computer, a vacuum degasser (Knauer, Berlin, Germany), a column thermostat made from 3.3 mm O.D. copper tubing which was heated by means of a circulating water bath (Model K 20 KP, Lauda, Lauda-Königshofen, Germany), a microinjector (Model C4-1004-.02, Valco Instruments, Houston, TX, USA) with a 20-nl internal sample loop, a variable-wavelength detector (Model Linear UV–VIS 200, Linear Instruments, Fremont, CA, USA) with a capillary detector cell (Grom, Herrenberg, Germany), and a personal computer-based data system (GynkoSoft, Version 5.22, Gynkotek, Germering, Germany). Octadecylated PS–DVB–C_{18} particles (2.3 μm) were prepared according to the previously published protocol [5] and packed into polyimide coated fused-silica capillary tubing of 350 μm O.D.×200 μm I.D. (Polymicro Technologies, Phoenix, AZ, USA) as described in Ref. [16].

2.3. Electrospray ionization mass spectrometry and coupling with capillary liquid chromatography

ESI-MS was performed on a Finnigan MAT LCQ quadrupole ion trap mass spectrometer (Finnigan MAT, San Jose, CA, USA) equipped with the electrospray ion source. The capillary column was directly connected to the spray capillary (fused-silica, 150 μm O.D.×50 μm I.D.) by means of a small piece of PTFE tubing. A syringe pump equipped with a 500-, 250-, or 100-μl glass syringe (Unimetrics, Shorewood, IL, USA) was used for continuous infusion experiments and for pumping sheath liquid. For analysis with pneumatically-assisted ESI, an electrospray voltage of 3.7–4.0 kV and a nitrogen sheath gas flow of 60–80 arbitrary units were employed. The temperature of the heated capillary was set to 200°C. Total ion chromatograms and mass spectra were recorded on a personal computer with the LCQ Navigator software version 1.2 (Finnigan). Mass calibration and coarse tuning was performed in the positive ion mode by direct infusion of a solution of caffeine (Sigma, St. Louis, MO, USA), methionyl–arginyl–phenylalanyl–alanine (Finnigan), and Ultramark 1621 (Finnigan). Fine tuning for ESI-MS of oligonucleotides in the negative ion mode was performed with a 0.36 mg/ml solution of $(dT)_{24}$ (Microsynth) in 10 m*M* triethylamine in water–acetonitrile (50:50, v/v). For all direct infusion experiments, cations present in the oligonucleotide samples were removed by on-line cation-exchange using a 20×0.50 mm I.D. cation-exchange microcolumn packed with 38–75 μm Dowex 50W-X8 particles (Serva, Heidelberg, Germany) [25]. Usually, 20 scans were acquired and averaged to determine the signal intensities. For determination of the total ion current (TIC)-to-noise ratios, the background noise in the mass spectrum of

$(dT)_{24}$ was measured between m/z 2344 and 2353. For IP-HPLC–ESI-MS analysis, oligonucleotides were injected without prior cation removal.

3. Results and discussion

3.1. Investigation of sheath liquid effects by direct infusion experiments

It has been shown that the solvent composition controls the chromatographic and mass spectrometric performances in IP-RP-HPLC–ESI-MS in opposite ways: high acetonitrile concentration is optimal for ESI-MS but not practicable for IP-RP-HPLC, high concentration of ion-pair reagent improves chromatographic resolution but suppresses the signal in ESI-MS, and oligonucleotides are better ionized, but not retained by the stationary phase at high pH [16]. The post-column addition of a sheath liquid to the column effluent through the triaxial electrospray probe was supposed to improve the mass spectrometric performance basically by three different effects: (1) the surface tension is reduced through addition of an organic solvent which enables the formation of smaller droplets during electrospray resulting in faster evaporation of the solvent and increased ion yield. (2) The conductivity of the electrosprayed solution is lowered because of dilution of the eluate leaving the column by the sheath liquid. (3) The pH of the electrosprayed solution can be manipulated through addition of volatile acids or bases to the sheath liquid.

Direct infusion experiments were performed to investigate the effect of different sheath liquids and sheath liquid flow-rate on mass spectrometric detectability without the complexity of liquid chromatographic separation. A solution of 0.36 mg/ml $(dT)_{24}$ in 50 m*M* TEAB–10% acetonitrile, a typical eluent for IP-RP-HPLC, was infused at a flow-rate of 3.0 μl/min. Upon addition of organic sheath liquids the total ion current (TIC, calculated as the sum of the signal intensities of all individual charge states) increased in the order: methanol<isopropanol< hexafluoroisopropanol<50 m*M* triethylamine in acetonitrile<acetonitrile. The total ion current changed from 112 795 counts without sheath liquid by a factor of 9.5 to 1 068 640 counts with a coaxial flow of 1.2 μl/min acetonitrile. However, depending on the applied solvent, the addition of sheath liquid also entailed an increase in the background noise level by a factor of 2–5, so that the total ion current-to-noise ratio is the proper parameter to describe the gain in detectability with different sheath liquids.

Table 1 shows that the total ion current-to-noise ratios increased in the order: no sheath liquid< hexafluoroisopropanol<isopropanol<50 m*M* triethylamine in acetonitrile<methanol<acetonitrile. Acetonitrile and methanol enhanced the total ion current-to-noise ratios of $(dT)_{24}$ by a factor of 2.4 and 2.2, respectively, compared to sheathless operation and were both equivalent with respect to their capability of improving the detectability of oligonucleotides in ESI-MS. The addition of an organic base (50 m*M* triethylamine) to acetonitrile as the sheath liquid considerably decreased the total ion current-to-noise ratio, mainly due to increased background noise, although it has been shown that an improvement in signal intensity could be achieved

Table 1
Influence of different sheath liquids on total ion current-to-noise ratio in direct infusion ESI-MS of oligonucleotides

Sheath liquid	Total ion current-to-noise ratio[a]
No sheath	320
Hexafluoroisopropanol	404
Isopropanol	594
50 m*M* triethylamine in acetonitrile	597
Methanol	717
Acetonitrile	756

[a] Conditions: cation-exchange microcolumn, Dowex 50W-X8, 20×0.50 mm I.D.; scan, 400–4000 u; electrospray voltage, 4.0–4.5 kV; sheath gas, 60–70 units; direct infusion of 0.36 mg/ml $(dT)_{24}$ in 50 m*M* TEAB, pH 8.80, 10% acetonitrile; flow-rate of sample solution, 3.0 μl/min; flow-rate of sheath liquid, 1.2 μl/min.

with sample solutions adjusted to pH values higher than 9.00 (compare with Fig. 5 in Ref. [16]). The influence of acidic conditions on signal intensity was evaluated by using hexafluoroisopropanol as sheath liquid. Whereas the background noise level with hexafluoroisopropanol was comparable to that of acetonitrile, the total ion current-to-noise ratio was significantly lower.

The charge state distribution of $(dT)_{24}$ with different sheath liquids can be deduced from Fig. 1 where the fractions of the signal intensities of all observed charge states normalized to the total ion current (=100%) are plotted. In all experiments, the 3− and 4− charge states were the most abundant and represented 96.5–97.5% of the total ion current. The distribution of the m/z signals of oligonucleotides among only few multiply charged ions is the result of efficient charge state reduction in the presence of acids such as carbonic acid [26,27]. Without addition of sheath liquid, charge states from 3− to 8− were observed (Fig. 1a). Addition of methanol or acetonitrile as sheath liquid did not significantly affect the charge state distribution (Fig. 1b and c), whereas isopropanol slightly shifted the distribution towards

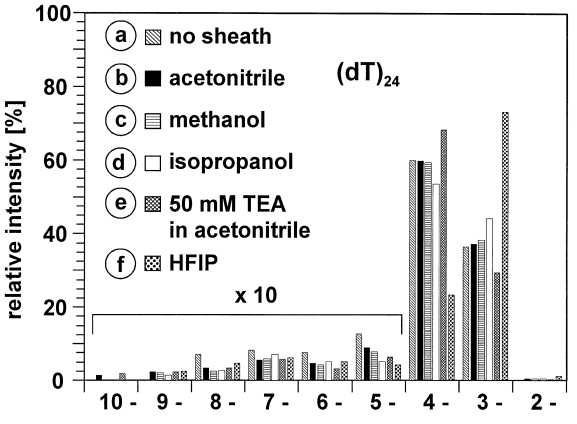

Fig. 1. Influence of different sheath liquids on charge state distribution in direct infusion ESI-MS of oligonucleotides. All plotted signal intensities represent the average of 20 individual measurements with a relative standard deviation of 2.2–5.6%. Cation-exchange microcolumn, Dowex 50W-X8, 20×0.50 mm I.D.; scan, 400–4000 u; electrospray voltage, 4.0–4.5 kV; sheath gas, 60–70 units; direct infusion of 0.36 mg/ml $(dT)_{24}$ in 50 m*M* TEAB, pH 8.80, 10% acetonitrile; flow-rate 3.0 μl/min; no sheath liquid in (a); sheath liquid, (b) acetonitrile, (c) methanol, (d) isopropanol, (e) 50 m*M* triethylamine in acetonitrile, and (f) hexafluoisopropanol; flow-rate, 1.2 μl/min.

lower charge states (Fig. 1d). Addition of 50 m*M* triethylamine in acetonitrile caused a significant shift to higher charge states, as shown in Fig. 1e by the decrease in the intensity of the 3− and the increase in the intensity of the 4− charge state. Hexafluoroisopropanol shifted the distribution to lower charge states, so that the 3− charge state is the most abundant in the spectrum of $(dT)_{24}$ (Fig. 1f). The total number of detectable charge states was highest with hexafluoroisopropanol and acetonitrile where charge states from 2− to 10− were observed. From these results it is concluded that the charge state distribution with neutral, acidic and basis sheath liquids is shifted in the expected direction but the variation in the most abundant charge states is only moderate.

The coaxial flow of sheath liquid is expected to dilute the analyte as it mixes with the column effluent at the tip of the electrospray probe. A linear decrease in signal intensity as a function of sheath liquid flow-rate was observed by Banks in CE–ESI-MS of peptides [28]. The effect of sheath liquid flow-rate on oligonucleotide signals was evaluated by varying the sheath flow-rate from 0–6 μl/min and measuring the total ion current generated by a flow of 3.0 μl/min of $(dT)_{24}$ dissolved in two solvents suitable for IP-HPLC–ESI-MS, namely 50 m*M* triethylammonium hydrogencarbonate–10% acetonitrile [16] and 2.2 m*M* triethylamine–400 m*M* hexafluoroisopropanol–20% methanol [18]. Because hexafluoroisopropanol is immiscible with acetonitrile, isopropanol was used as sheath liquid in combination with the latter solvent. For the same reason methanol was used as organic modifier for IP-RP-HPLC and its concentration had to be increased to 20% in order to enable gradient elution of oligonucleotides. Table 2 illustrates that with 50 m*M* triethylammonium hydrogencarbonate–10% acetonitrile as solvent and a sheath liquid flow of 0–6 μl/min acetonitrile the total ion current initially increased by a factor of 9.5 and then remained almost constant at flow-rates higher than 1.2 μl/min (Table 2, second column). Because the optimal sheath gas flow-rate and electrospray voltage vary with the flow of liquid introduced into in the electrospray ion source, both parameters were tuned for maximum signal intensity at all investigated sheath flow-rates. The influence of sheath liquid on

Table 2
Influence of sheath liquid flow-rate on total ion current and total ion current-to-noise ratio in direct infusion ESI-MS of oligonucleotides

Sheath liquid flow-rate (μl/min)	Sample solvent			
	50 m*M* TEAB–10% acetonitrile, pH 8.80	2.2 m*M* Triethylamine–400 m*M* HFIP, pH 6.90	50 m*M* TEAB–10% acetonitrile, pH 8.80	2.2 m*M* Triethylamine–400 m*M* HFIP, pH 6.90
	Sheath liquid			
	Acetonitrile TIC[a] (counts)	Isopropanol TIC[a] (counts)	Acetonitrile TIC-to-noise ratio[a]	Isopropanol TIC-to-noise ratio[a]
0	112 975	4 685 250	320.5	855
1.2	1 068 640	4 236 300	945.7	1077.9
3	1 060 000	–	702	–
6	1 191 000	4 816 750	555	1199.7

[a] Conditions as in Table 1.

signal intensity was notably different when using 2.2 m*M* triethylamine–400 m*M* hexafluoroisopropanol–20% methanol as solvent for the oligonucleotide sample. Compared to 50 m*M* triethylammonium hydrogencarbonate–10% acetonitrile, the total ion current was 41 times higher without addition of sheath liquid and did not change significantly upon the addition of isopropanol as sheath liquid (Table 2, third column). The lower conductivity (2.2 m*M* versus 50 m*M* triethylamine, the degree of dissociation of hexafluoroisopropanol is very low) and a higher portion of organic solvent (20% acetonitrile plus 400 m*M* corresponding to 4.2% hexafluoroisopropanol) are responsible for the increase in signal intensity compared to 50 m*M* triethyammonium hydrogencarbonate–10% acetonitrile.

The difference between the performance of both solvent systems narrows when the total ion current-to-noise ratios are compared (Table 2, fourth and fifth column). Without sheath liquid, the total ion current-to-noise ratios using 50 m*M* triethylammonium hydrogencarbonate–10% acetonitrile and 2.2 m*M* triethylamine–400 m*M* hexafluoroisopropanol–20% methanol differed by a factor of 2.7. At a sheath liquid flow of 1.2 μl/min the factor was reduced to 1.1, suggesting that under these conditions both eluent systems allow the detection of oligonucleotides with equivalent detectability. With 50 m*M* triethylammonium hydrogencarbonate–10% acetonitrile as solvent and acetonitrile as sheath liquid, the total ion current-to-noise ratio gradually decreased at higher flow-rates (Table 2, fourth column), an effect that is not related to the dilution of the sample but to the increase in background noise produced by the sheath liquid. The use of higher quality acetonitrile might alleviate this problem.

3.2. Comparison of UV and ESI-MS detection for the separation of oligonucleotides

A high-resolution liquid chromatographic separation technique such as IP-RP-HPLC yields very narrow eluting peaks with peak widths at half height of less than 5 s. Interfacing this separation technique to mass spectrometry has an influence on the separation efficiency which is an important issue to consider when coupling IP-RP-HPLC to ESI-MS. Fig. 2 compares the chromatographic signals obtained from 0.57–1.33 ng injections each of $p(dT)_{12}$ to $p(dT)_{30}$ with UV and ESI-MS detection. A gradient of 5.0–7.0% acetonitrile in 2.0 min, followed by 7.0–10% in 5.0 min in 50 m*M* aqueous triethylammonium hydrogencarbonate was used to separate the oligonucleotides. The flow-rates of the chromatographic eluent and the acetonitrile sheath liquid were 2.9 and 3.0 μl/min, respectively. The extra column band broadening through volumes in the connecting capillaries was kept to a minimum by using 25 μm and 50 μm I.D. fused-silica capillaries, respectively, to connect the column to the capillary detector cell or to conduct the column effluent into the electrospray ion source. Both detection methods recorded the eluting 19 oligonucleotide peaks with similar signal-to-noise ratios. With ESI-MS detection, the identity of the separated oligonucleotides was readily established on the basis of their molecu-

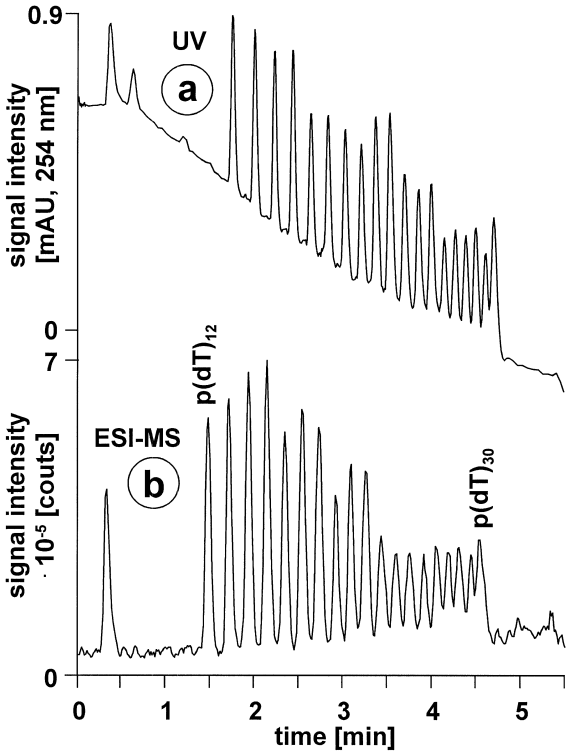

Fig. 2. Comparison of UV and ESI-MS detection in the capillary IP-RP-HPLC separation of phosphorylated oligonucleotide ladders. Column, PS–DVB–C_{18}, 2.1 μm, 60×0.20 mm I.D.; mobile phase, (A) 50 m*M* TEAB, pH 8.40, (B) 50 m*M* TEAB, pH 8.40, 20% acetonitrile; linear gradient, 25–35% B in 2.0 min, 35–50% B in 5.0 min; flow-rate, 2.9 μl/min; temperature, 50°C; detection, (a) UV, 254 nm, (b) ESI-MS, 1000–3100 u, electrospray voltage, 3.7 kV; sheath gas, 80 units; sample, $p(dT)_{12-18}$, (a) 0.57 ng, (b) 0.94 ng each, $p(dT)_{19-24}$, (a) 1.33 ng, (b) 1.1 ng each, and $p(dT)_{25-30}$, (a) 1.33 ng, (b) 1.1 ng each.

lar masses, which could be calculated from the extracted mass spectra with an average mass accuracy of 0.011%. Whereas a strong baseline drift is observed during gradient elution with UV detection (Fig. 2a) the baseline in ESI-MS is not affected by the acetonitrile gradient (Fig. 2b). The peak widths at half height with UV detection ranged from 2.9 s for $p(dT)_{12}$ to 3.8 s for $p(dT)_{21}$, those with ESI-MS detection from 4.2 s to 4.8 s for the same components. This increase in peak widths of 26–45% is primarily caused by the low data acquisition rate of 1.3 data points per second during ESI-MS detection, which becomes significant especially with the narrow peaks obtained on micropellicular PS–DVB–C_{18} as stationary phase [29].

One of the major problems associated with ESI-MS of nucleic acids is the tendency of these polyanions to form quite stable adducts with cations, such as sodium, potassium, magnesium and iron ions, resulting in complication of mass spectra, low signal-to-noise ratios, and poor mass accuracy [25,30,31]. Therefore, the utility of IP-RP-HPLC as separation technique prior to mass analysis by ESI-MS largely depends on its ability to remove cations from oligonucleotide samples. Fig. 3 depicts the separation of three oligodeoxythymidylic acids of different chain lengths as well as the mass spectra extracted from the reconstructed ion chromatogram. Because of charge state reduction one charge state dominates the mass spectra of short oligonucleotides, such as $(dT)_8$ (Fig. 3b), which precludes the calculation of the molecular mass from a series of multiply charged ions. However, it can be seen in the mass spectra shown in Fig. 3b–d that a low relative abundance of potassium adducts is detected by ESI-MS, even after IP-RP-HPLC separation. The incomplete removal of adducts during IP-RP-HPLC is highly advantageous because the charge state of an m/z signal can be easily determined from the difference in m/z between different adduct peaks, such as the signals of $(M-2H)^{2-}$ and $(M-3H+K)^{2-}$ observed in Fig. 3b. The measured difference of 18.7 u between the two signals readily led to the conclusion that the charge state of this signal is $2-$. With longer oligonucleotides, a higher number of charge states can be observed (Fig. 3c and d), enabling the calculation of the charge state either from series of multiply charged ions or from cation adduct peaks.

3.3. Influence of sheath liquid on IP-HPLC–ESI-MS of oligonucleotides

The mixture of three oligodeoxythymidylic acids was also used to characterize the influence of sheath liquids under chromatographic conditions. The major differences between direct infusion ESI-MS and IP-HPLC–ESI-MS lie in the different amounts of sample that are consumed during a single analysis (typically a few hundred nanograms for direct infusion ESI-MS and a few nanograms for IP-RP-HPLC–ESI-MS), the concentration of the analyte in

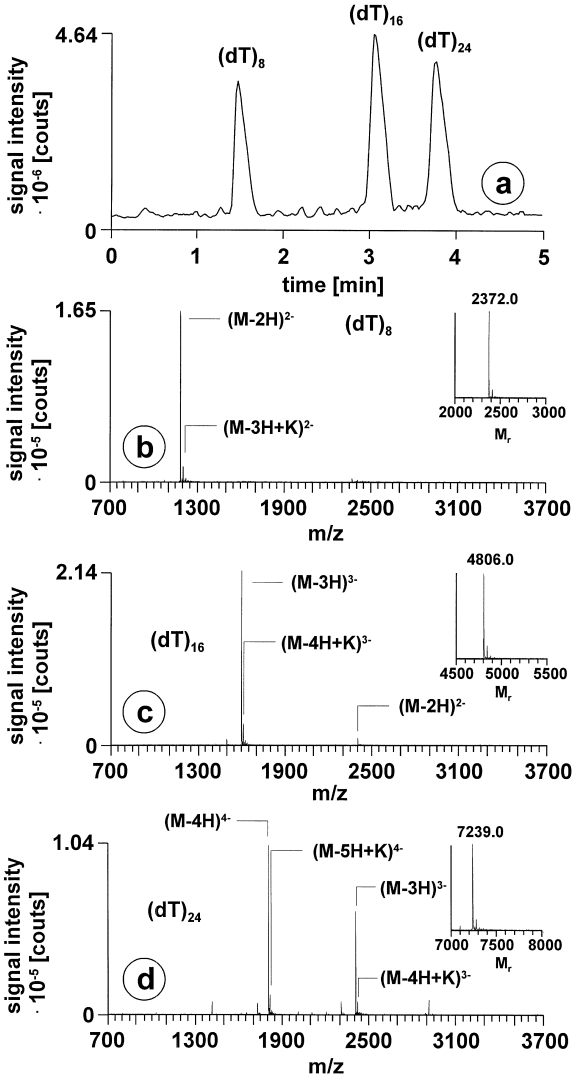

Fig. 3. IP-RP-HPLC–ESI-MS analysis of three oligodeoxythymidylic acids, (a) reconstructed ion chromatogram, (b–d) extracted and deconvoluted mass spectra. Column, PS–DVB–C_{18}, 2.1 μm, 60×0.20 mm I.D.; mobile phase, (A) 50 m*M* TEAB, pH 8.40, (B) 50 m*M* TEAB, pH 8.40, 20% acetonitrile; linear gradient, 20–65% B in 5.0 min; flow-rate, 2.9 μl/min; temperature, 50°C; detection, ESI-MS, 700–3700 u, electrospray voltage, 3.7 kV; sheath gas, 68 units; sheath liquid, acetonitrile; flow-rate, 6 μl/min; sample, 5.22 ng $(dT)_8$, 10.6 ng $(dT)_{16}$, 15.9 ng $(dT)_{24}$.

the electrosprayed solution, and the number of available spectra that can be averaged to improve the signal-to-noise ratio (typically 60–90 scans for direct infusion ESI-MS and 5–15 scans for IP-RP-HPLC–ESI-MS). The influence of neutral, basic and acidic sheath liquids on the performance of IP-RP-HPLC–ESI-MS is summarized in Fig. 4. With acetonitrile as sheath liquid, 10.6 ng $(dT)_{16}$ were detected in the reconstructed ion chromatogram with a signal-to-noise ratio of 13.5:1 (Fig. 4a). Addition of triethylamine as base or hexafluoroisopropanol as acid to the sheath liquids resulted in deterioration of the signal-to-noise ratios, as reflected in the chromatograms depicted in Fig. 4b and c with ratios of 11.3:1 and 8.9:1, respectively. The deterioration of oligonucleotide signals was even more profound when pure hexafluoroisopropanol or triethylamine were applied as sheath liquids. With hexafluoroisopropanol, the chemical background became sufficiently high that no oligonucleotide peaks could be detected in the chromatogram (not shown). This was most probably due to the immiscibility of hexafluoroisopropanol with eluents containing acetonitrile. Triethylamine increased the chemical background to a level of $1.6 \cdot 10^6$ counts (Fig. 4d). Moreover, the baseline was very noisy leading to the detection of 10.6 ng $(dT)_{16}$ with a signal-to-noise ratio of only 4.6:1. As expected, the chromatographic peak widths were independent of the different sheath liquids that were added post-column.

Fig. 5 shows the influence of four different sheath liquids, namely acetonitrile, 100 m*M* triethylamine in acetonitrile, triethylamine, and 400 m*M* hexafluoroisopropanol in methanol, on charge state distribution in the mass spectra extracted from the reconstructed ion chromatograms of Fig. 4. The general trend that longer oligonucleotides preferred higher charge states as well as a higher number of charge states can be readily seen. The spectrum of $(dT)_8$ was characterized by a predominant 2− charge state contributing to more than 96% of the total ion current with all sheath liquids except triethylamine, where the 2− charge state contributed to 88% of the total ion current. Among the four tested sheath liquids, only pure triethylamine was able to shift the distribution slightly towards the 3− charge state (Fig. 5a). The picture is very similar with $(dT)_{16}$, where a total of five charge states was observed, the 3− charge state being the most abundant (Fig. 5b). Again, only pure triethylamine shifted the distribution slightly towards higher charge states. The spectrum of $(dT)_{24}$ has two dominant charge states, 3− and 4−. A total of seven charge states from 3− to 9− were detected with acetonitrile as sheath liquid. Triethylamine significantly shifted the distribution to

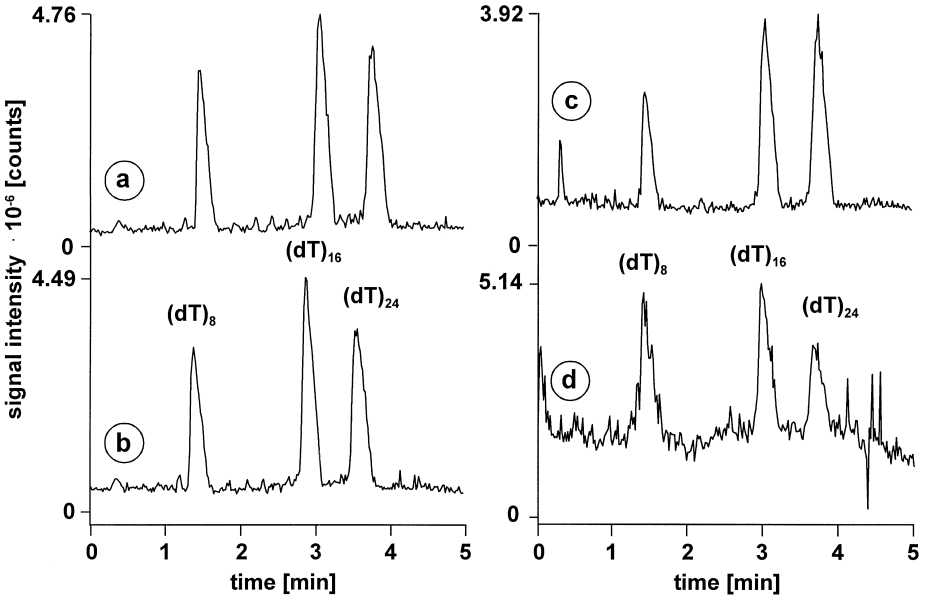

Fig. 4. Influence of sheath liquid composition on chromatographic detectability of oligonucleotides. Column, PS–DVB–C_{18}, 2.1 μm, 60×0.20 mm I.D.; mobile phase, (A) 50 m*M* TEAB, pH 8.40, (B) 50 m*M* TEAB, pH 8.40, 20% acetonitrile; linear gradient, 20–65% B in 5.0 min; flow-rate, 2.9 μl/min; temperature, 50°C; detection, ESI-MS, 700–3700 u, electrospray voltage, 3.7 kV; sheath gas, 68 units; sheath liquid, (a) acetonitrile, (b) 100 m*M* triethylamine in acetonitrile, (c) 400 m*M* hexafluoroisopropanol in methanol, (d) triethylamine; flow-rate, 6.0 μl/min; sample, 5.22 ng $(dT)_8$, 10.6 ng $(dT)_{16}$, 15.9 ng $(dT)_{24}$.

higher charge states, whereas 400 m*M* hexafluoroisopropanol increased the intensity of lower charge states (Fig. 5c). These findings suggest, that the charge state distribution is mainly determined by the composition of the column effluent that dissolves the oligonucleotide. Moreover, the sheath liquid as well as acids or bases added to the sheath liquid have only a minor influence on the charge state distribution of small oligonucleotides. However, with longer oligonucleotides such as $(dT)_{24}$, acids or bases induce significant shifts in the charge distribution and are therefore a proper means to manipulate the charge state distribution of large nucleic acids.

3.4. Adduct formation with different sheath liquids

As already discussed in Section 3.2, suppression of cation adduct formation is a critical issue in ESI-MS of oligonucleotides. In a recent report we demonstrated, that the eluent used for IP-RP-HPLC separation has a substantial impact on cation adduction: whereas only monosodium and monopotassium adducts were found with an eluent comprising 50 m*M* triethylammonium hydrogencarbonate, extensive cation adducts up to the tetrasodium–monopotassium adduct were observed with 2.2 m*M* triethylamine–400 m*M* hexafluoroisopropanol [16]. In the course of this study, we discovered a surprising effect of sheath liquid on cation adduct formation in IP-RP-HPLC–ESI-MS of oligonucleotides. With 50 m*M* triethylammonium hydrogencarbonate as the eluent, adduct formation was strongly influenced by the type of sheath liquid used (Fig. 6). Very little adduction was present in the signal for the 4− charge state of $(dT)_{24}$ with acetonitrile as sheath liquid (Fig. 6a). However, when 400 m*M* hexafluoroisopropanol was used as sheath liquid, various adducts were detected in the mass spectrum even after chromatographic separation (Fig. 6b). The introduction of cations through the sheath liquid could be excluded because no adducts were detected in the direct infusion experiments with methanol or hexafluoroisopropanol as sheath liquid. One explanation compatible with this observation is that the adducts endure the chromatographic separation process and are dissociated subsequently during the electrospray process. Obviously, hexafluoroisopropanol inhibits the exchange of metal cations with

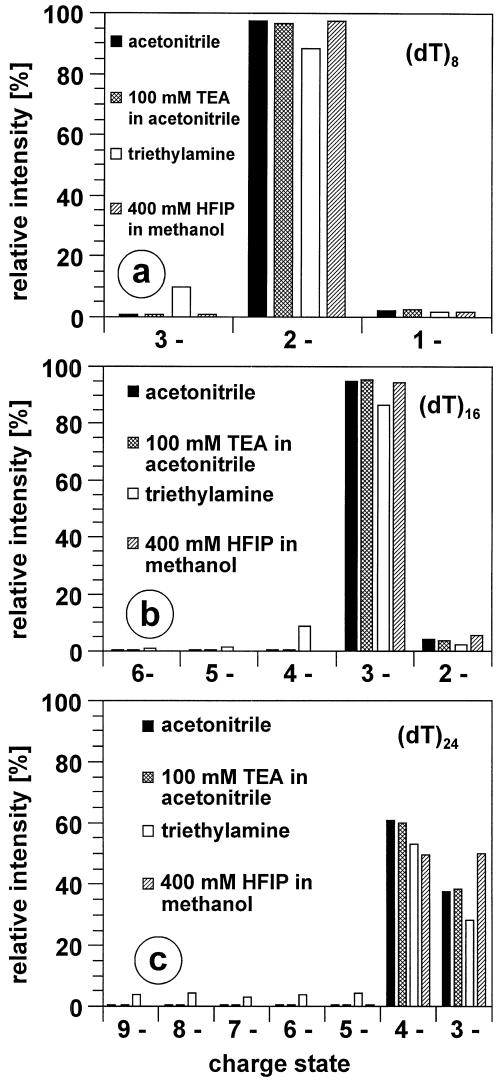

Fig. 5. Influence of sheath liquid composition on charge state distribution and signal intensity in the mass spectra of (a) $(dT)_8$, (b) $(dT)_{16}$, and (c) $(dT)_{24}$ extracted from the chromatograms in Fig. 4.

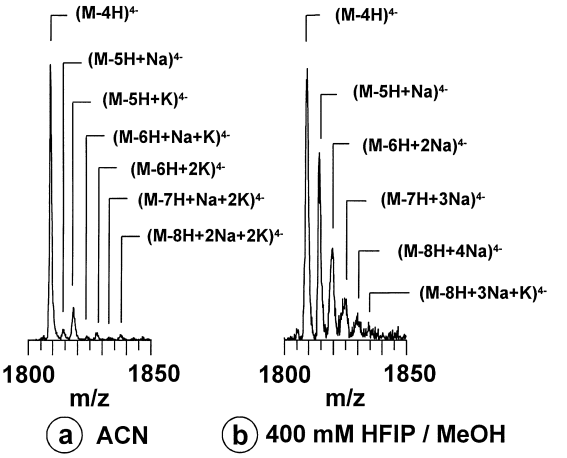

Fig. 6. Influence of sheath liquid composition on cation adduction in the 4− charge state of $(dT)_{24}$. Sheath liquid, (a) acetonitrile, (b) 400 m*M* hexafluoroisopropanol in methanol; flow-rate, 6.0 μl/min; other conditions as in Fig. 4.

protons in the gas phase during the electrospray process.

3.5. Detection limits with full-scan and selected ion monitoring data acquisition

The detection limits in full scan and selected ion monitoring mode were determined by chromatographic analysis of a $(dT)_{16}$ standard with ESI-MS detection. Fig. 7a illustrates the reconstructed ion chromatogram of 104 fmol (25 pg/nl, 20 nl injected) $(dT)_{16}$ at the limit of detection (signal-to-noise ratio of 3:1). The mass spectrum shown in Fig. 7b was extracted from the chromatogram in Fig. 7a. A charge state of 3− was calculated for the signal at m/z 1600.8 from the potassium adduct peak. The calculated molecular mass of 4805.4 correlates well with the theoretical molecular mass of 4805.15. Monitoring the chromatographic separation by selected ion monitoring of the 3− charge state at m/z 1600.72 allowed the detection of 870 amol (208 fg/nl, 20 nl injected) with an average signal-to-noise ratio of 3.7:1 (average of five injections) which corresponds to a lower limit of detection of 710 amol at a signal-to-noise ratio of 3:1. Compared to full scan data acquisition, this represents an improvement in detectability by a factor of 146 in selected ion monitoring mode.

3.6. Application of IP-HPLC–ESI-MS to the separation of heterooligonucleotides

The ability of IP-RP-HPLC–ESI-MS to separate and characterize heterooligonucleotides of molecular biological relevance was evaluated by analysis of a mixture of three oligonucleotide primers differing in length by one nucleotide. The 20-mer is a possible failure sequence of the 21-mer with one guanosine

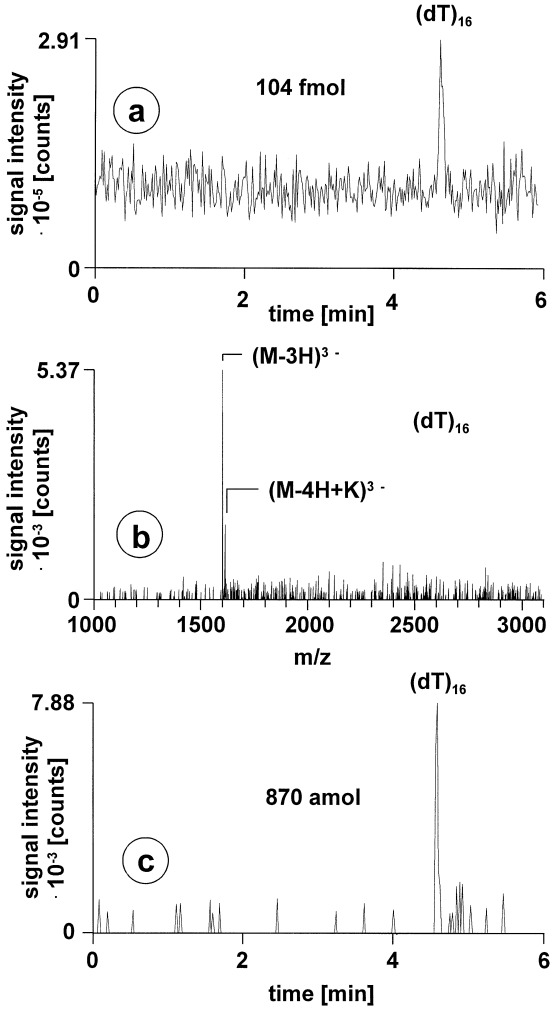

Fig. 7. Lower limits of detection for a 16-mer oligonucleotide in (a and b) full scan and (c) selected ion monitoring mode of data acquisition. Column, PS–DVB–C_{18}, 2.1 μm, 60×0.20 mm I.D.; mobile phase, (A) 50 m*M* TEAB, pH 8.40, (B) 50 m*M* TEAB, pH 8.40, 20% acetonitrile; linear gradient, 25–60% B in 10 min; flow-rate, 2.9 μl/min; temperature, 50°C; detection, ESI-MS, (a) 1000–3100 u, (b) SIM at *m*/*z* 1599–1603, electrospray voltage, 3.7 kV; sheath gas, 84 units; sheath liquid, (a) acetonitrile; flow-rate, 3.0 μl/min; sample, (a and b) 104 fmol and (c) 870 amol $(dT)_{16}$.

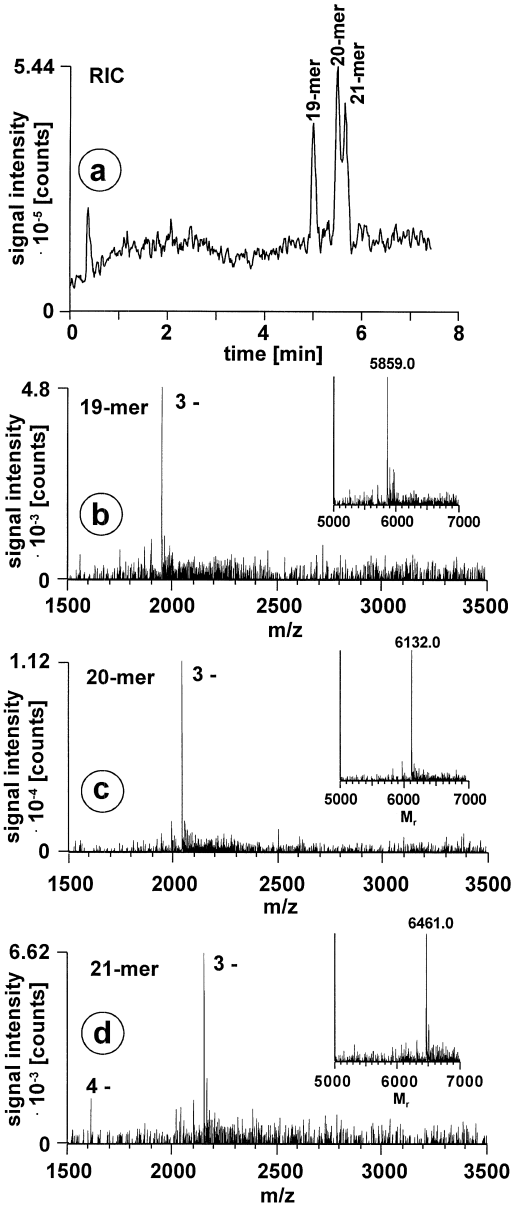

Fig. 8. Characterization of synthetic heterooligonucleotides. Column: PS–DVB–C_{18}, 2.1 μm, 60×0.20 mm I.D.; mobile phase, (A) 50 m*M* TEAB, pH 8.40, (B) 50 m*M* TEAB, pH 8.40, 20% acetonitrile; linear gradient, 15–40% B in 10 min; flow-rate, 2.9 μl/min; temperature, 50°C; scan, 1500–3500 u; electrospray voltage, 3.7 kV; sheath gas, 80 units; sheath liquid, acetonitrile, 6.0 μl/min; sample, 19-mer, 20-mer, 21-mer.

missing at the 5′-end, the 19-mer (sequence, GTG CTC AGT GTA GGA TGC C, theoretical mass, 5 859.90) differs from the 20-mer (sequence, TGC TCA GTG TAG CCC AGG AT, theoretical mass, 6 133.08) and 21-mer (sequence, GTG CTC AGT GTA GCC CAG GAT, theoretical mass, 6462.30) both in length and sequence. Fig. 8a shows the reconstructed ion chromatogram that was obtained by applying a gradient of 3.0–8.0% acetonitrile in 50 m*M* triethylammonium hydrogencarbonate at a flow-

rate of 2.9 μl/min in 10 min. A flow of 6.0 μl/min acetonitrile was added as sheath liquid to improve the detection sensitivity. The unequal retention differences between the 19- and 20-mer and the 20- and 21-mer reflect the influence of base sequence in oligonucleotides of mixed sequence on retention in IP-RP-HPLC. The mass spectra of the three oligonucleotides are depicted in Fig. 8b–d. For the 19- and 20-mer, only the 3− charge state could be detected whereas the 21-mer showed signals for the 3− and 4− charge state. The molecular masses of the oligonucleotides could be calculated from the spectra with high accuracy, yielding masses of 5859.0, 6132.0 and 6461.0 with relative mass errors of 0.015, 0.018 and 0.020%, respectively.

4. Conclusions

It is concluded that the addition of organic sheath liquids to column effluents from capillary IP-RP-HPLC allows the detection and characterization of oligonucleotides by ESI-MS with enhanced detectability. Volatile acids or bases can be added to the sheath liquids to manipulate the charge state distribution of oligonucleotides longer than approximately 20 nucleotide units whereas the distribution for shorter oligonucleotides remains essentially unaffected. By virtue of its high resolving power and detection limits in the lower femtomol range in full scan mode and in the upper attomole range in the selected ion monitoring mode IP-RP-HPLC–ESI-MS proves to be very useful for the separation, identification and characterization of oligonucleotides to be used in molecular biological experiments.

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