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Performance of capillary gel electrophoretic analysis of oligonucleotides coupled on-line with electrospray mass spectrometry

Alexander von Brocke*, Thomas Freudemann, Ernst Bayer[1]

Research Center for Nucleic Acid and Peptide Chemistry, Institute of Organic Chemistry, University of Tübingen, Auf der Morgenstelle 18, 72076 Tübingen, Germany

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Abstract

Synthetic oligonucleotides (ODNs) are routinely analyzed using capillary gel electrophoresis (CGE) for size-sieving based separations as well as electrospray mass spectrometry (ESI-MS) for identification. On-line coupling of these methods is therefore desired in order to combine the analytical capabilities provided by both methods. Performance of on-line CGE–ESI-MS systems is influenced by various parameters, and choice of optimal conditions is crucial for successful coupling experiments. In this study, we explore characteristics of the on-line coupled CGE–ESI-MS system for ODN analysis. Effects of CGE buffer concentration, capillary length, separation and orifice voltage on CGE separation and MS detection of a phosphodiester ODN mixture were examined. Attention was paid to the influence of the interface, such as geometry of capillary alignment, sheath liquid flow-rate and sheath liquid composition on performance of the system.

Keywords: Background electrolyte composition; Sheath liquid effects; Oligonucleotides; Phosphodiesters

1. Introduction

With its high resolving power, capillary gel electrophoresis (CGE) has developed into a widely employed tool for analysis of synthetic oligonucleotides (ODNs) [1–6]. CGE needs only small sample amounts, is easily automated and thus routinely used for determination of the purity of synthesized oligonucleotides. Characterization of pharmacokinetic and metabolic properties are of particular interest in the growing field of antisense ODNs [7,8].

Since ODNs longer than 10–12 nucleotide units possess very similar charge-to-size ratios and thus electrophoretic mobilities, separation by capillary zone electrophoresis is not possible [8]. Oligonucleotide mixtures are separated in polymer-filled capillaries due to a size-sieving mechanism [5,6,9]. Polyacrylamide-filled capillaries were employed for ODN separations [2], but showed several disadvantages: the preparation of the capillaries is delicate and their lifetime limited. To overcome these problems, replenishable polymer solutions have been applied for ODN separations [3–5,10,11].

CGE is usually carried out using UV, diode array,

*Corresponding author. Fax: +49-7071-295-034.
E-mail address: alexander.von.brocke@uni-tuebingen.de (A. von Brocke).
[1]Author deceased.

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or laser-induced fluorescence detectors. Unfortunately, no information regarding the identity of the examined ODNs is given by these detectors.

Molecular mass determination of ODNs is often accomplished using electrospray mass spectrometry (ESI-MS) [12]. ODNs form a distribution of different charge states upon electrospray ionization which can be deconvoluted to yield an accurate molecular mass [13]. Characterization of single ODN probes is easily achieved, but becomes a difficult task when mixtures of oligonucleotides, creating complex signal patterns upon ESI-MS detection, are examined. Thus, on-line coupling of an efficient separation method like CGE with the identification provided by ESI-MS yields a powerful tool for analysis of synthetic ODNs [14].

Despite its potential applications, only few examples for coupling separations in polymer-filled capillaries with on-line ESI-MS detection [18] have been published yet [14–17]. A reason might be the difficulties accompanying this on-line coupling approach: non-volatile buffer salts required for well-resolved CGE separations are not compatible with ESI-MS, giving lower detection sensitivity in the negative ion mode. Furthermore, no electroosmotic flow exists in gel-filled and wall-coated capillaries. Thus, eluting analyte molecules must be delivered to the mass spectrometer exclusively by the interface system.

In this paper we explore the effect of CGE and ESI-MS operating parameters, such as CGE buffer concentration, capillary length and separation voltage as well as the influence of the orifice voltage on separation and MS detection of a phosphodiester ODN mixture. The influence of sheath liquid–flow interface parameters, such as capillary position, flow-rate and composition on performance of the system was investigated in order to further improve CGE–ESI-MS coupling for analysis of oligonucleotides.

2. Experimental

2.1. Chemicals

Chemicals for solid-phase synthesis of ODNs were purchased from Proligo Biochemie (Hamburg, Germany). Fused-silica capillaries were obtained from Ziemer Chromatographie (Mannheim, Germany). Chemicals used for CGE–ESI-MS coupling were purchased from Fluka (Buchs, Switzerland) and Merck (Darmstadt, Germany) in the highest grade available. Purified water was provided by a Millipore Milli-Q185 Plus water-purification system (Eschborn, Germany).

2.2. Oligonucleotides

For all measurements the same oligonucleotide mixture of 5mer (3′-TTT TT-5′), 10mer (3′-CCC CCC CCC C-5′), 15mer (3′-AAG AAC AGA CGA GAA-5′) and 20mer (3′-CCT CCT CTC TCG CTC CCT CT-5′) were used. Oligodeoxyribonucleotides were prepared on an ABI 394 DNA/RNA synthesizer (Applied Biosystems, Perkin-Elmer, Foster City, CA, USA) utilizing standard phosphoramidite chemistry on controlled-pore glass. Purification was carried out by reversed-phase HPLC using a solvent delivery system S 1000, a low-pressure gradient mixer S 8110 (Sykam, Gilching, Germany), and a UV–Vis spectrometer UVIS 205 (Linear, Reno, NV, USA). Analytical and preparative columns (250×4.6 mm and 250×25 mm) were packed with Nucleosil C_{18}, 5 μm (Grom, Herrenberg, Germany).

2.3. Capillary gel electrophoresis

Fused-silica capillaries (100 μm I.D.×164 μm O.D.) were permanently coated with poly(vinyl alcohol) (PVA) according to the method described by Schomburg et al. [19]. Separations were performed using a capillary length of 50 cm. Replaceable gel was prepared by dissolution of poly(ethylene glycol) 35000 (PEG) 20% (w/v), 20 m*M* bis(2-hydroxyethyl)aminotris(hydroxymethyl)methane (BisTris), 20 m*M* boric acid and 20% (v/v) acetonitrile (ACN) in high-purity water followed by overnight shaking; the buffer used in the injection block buffer reservoir consisted of 20 m*M* BisTris–borate. Exceptions to these settings are stated in the Results and discussion.

2.4. Capillary gel electrophoresis–electrospray mass spectrometry

PVA-coated capillaries were connected to the ESI

source steel needle using a coaxial sheath liquid interface. The central CGE separation capillary of 164 μm O.D. was surrounded first by a 10-cm stainless steel needle (Hamilton, Darmstadt, Germany), 210 μm I.D.×410 μm O.D., supplying the sheath flow solution, and a second steel tube delivering the nebulizer gas. The sheath flow solution was introduced into the interface by a Model 22 syringe pump (Haward Apparatus, South Natick, MA, USA). The capillary tip was allowed to stick out of the needle for ~1 mm. A Kapillar-Elektrophorese System 100 (Grom) was used as power supply. Fig. 1 shows a schematic drawing of the set-up.

Gel was replaced manually by application of pressure to a gel-filled gas-tight syringe connected to the injection block before each run. For sample injection, the capillary and the electrode were moved to an external 2-μl steel sample vial, and oligonucleotides were injected electrophoretically by application of −10 kV for 30 s. Inlet electrode and capillary end were moved back to the inlet block after injection. The CE block was rinsed with 0.5 ml of running buffer before starting separations. A constant voltage of −25 kV was applied during electrophoretic runs (denoted as ''applied voltage'' in the Results and discussion), while the ESI needle voltage of the capillary outlet was kept at −4.2 kV, resulting in an overall voltage of −20.8 kV (denoted as ''effective voltage''). Sheath liquid was allowed to flow during injection and separation at a rate of 15 μl/min. No attempt was made to cool the capillary during electrophoretic runs. Other experimental parameters are mentioned in the Results and discussion section.

2.5. Electrospray mass spectrometry

An API III TAGA 6000E triple-quadrupole mass spectrometer (Sciex, Perkin-Elmer, Toronto, Canada) with an electrospray ion source and an m/z range of 2400 was used for mass spectrometric measurements.

Calibration was carried out with CsI solution. The negative ion detection mode was employed, with a needle voltage of −4.2 kV. An orifice voltage of −80 V was applied for all runs, except for the experiments in Section 3.3.1. The ionisation needle–orifice distance was kept at maximum; nebulizer gas flow, curtain gas flow and curtain gas temperature were kept at standard parameters. Mass spectral acquisition was performed using dwell times of 1.5 ms per step of 0.5 u, scanning a molecular mass range of 1500. For data acquisition, data processing and the control of the mass spectrometer, a MacIntosh IIx was employed using MacSpec 3.3 software.

3. Results and discussion

Our CGE on-line coupling set-up can be divided

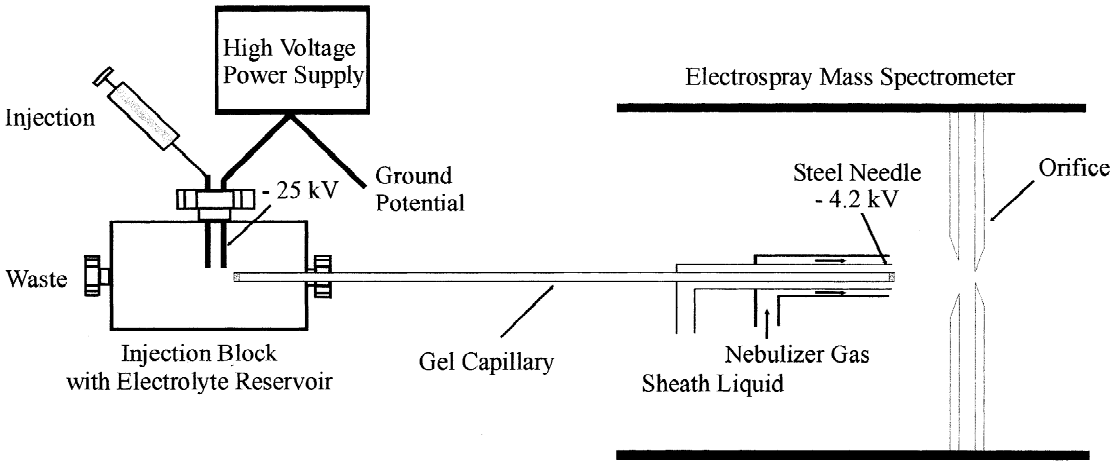

Fig. 1. Scheme of the on-line CGE–ESI-MS coupling set-up.

into three apparative parts: capillary gel electrophoretical separation system, sheath-flow interface and ESI-MS detector. For reasons of clarity, varied working parameters in this study are ordered following this scheme. Of course, working conditions of all parts must harmonize for successful performance of the set-up. While variation of special parameters only affect performance of its corresponding part (e.g. separation voltage influences only CGE separation), other changes may alter performance of the entire set-up. This is especially true for settings of the sheath-flow interface.

3.1. Variation of CGE parameters

3.1.1. Capillary length

Two contrary factors generally influence separations in capillaries of different lengths: separation efficiency is improved using longer capillaries due to prolonged separating interactions. In contrast, peak broadening is caused by diffusion, especially as temperature increases owing to Joule heating in the case of non-cooled capillaries as employed in these experiments. However, large biomolecules like ODNs have small diffusion coefficients and should show only minor peak broadening effects.

The utilized capillary was successively cut at the injection end, not altering the ESI-MS interface geometry for the test series. All measurements were performed at −25 kV applied voltage in order to obtain fast separations (see Section 3.1.2), thus effective electrical field strength increased with shortened capillary length.

Since no ODN signals are observed in the total ion current (TIC) using our initial working conditions, reconstructed ion electropherograms (RIEs) of ODN mass signals were used for graphical clarification. Because maximal signal intensity is automatically set as 100% relative intensity, all ODN peaks seem to possess equally intense peak maxima. Absolute intensities of ODN peaks may differ from these diagrams, but performance of the CGE–ESI-MS runs can be overviewed qualitatively. Extracted mass traces of the 5mer, 10mer, 15mer and 20mer of the same coupling runs are overlaid in order to allow the best overview. Fig. 2 shows overlaid RIEs extracted from CGE–ESI-MS runs using different capillary lengths in comparison.

Separation efficiency as well as analysis time decreased with capillary length shortening. In case of the 30 cm capillary, run time can be lowered to 15 min, but ODN species are not well separated. Using 60 cm, the run time is about 1 h, and efficient separation is achieved. A compromise between well-resolved separation and reasonable run time could be found using 40–50 cm capillaries.

3.1.2. Separation voltage

Using a 50-cm-long capillary, applied voltages between −15 and −27.5 kV were used. Since ESI needle voltage of the capillary outlet was kept constant at −4.2 kV, effective voltages were −10.8 to −23.3 kV. In Fig. 3, the effect of electrical field on separation of the ODN mixture is illustrated for −15, −22.5 and −27.5 kV applied voltage. Migration times of the 20mer varied from 33.6 min using −27.5 kV to more than 80 min in the case of −15 kV applied voltage. Unfortunately, the 20mer peak could be detected only partially in case of −15 kV applied voltage, since 80 min was our maximal possible analysis time. Since the effect of varied voltage on separation efficiency was only minor in these experiments, the highest possible voltage should be applied in order to obtain short analysis times. Applied voltages of more than −25 kV raised problems by flashovers of the high voltage and had to be avoided. Therefore, −25 kV applied voltage performed best.

3.1.3. Background electrolyte

Performance of size-sieving-based separations depend primarily on the sieving media (type of polymer and its concentration), but the type of background electrolyte is another crucial factor. For CGE of ODNs using UV detection, nonvolatile borate-based buffers are usually preferred. However, these buffers are not very compatible with ESI-MS detection, for which volatile buffers are used. Coupling experiments using triethylammoniumacetate (TEAA) as a volatile buffer showed intense but very broad ODN peaks, and separation of ODNs of similar size was not possible. Since BisTris–borate buffers perform well for CGE separations of ODNs, this buffer composition was optimized for on-line ESI-MS detection. For this reason, concentration of the BisTris–borate buffer was varied from 5 m*M* to 200

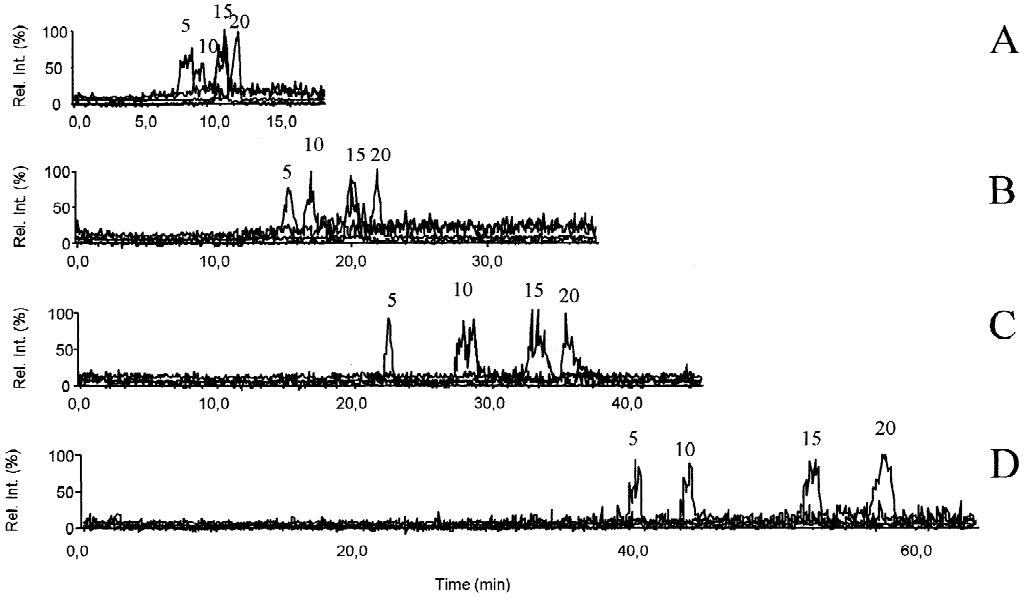

Fig. 2. Effect of capillary length on CGE–ESI-MS runs, reconstructed ion electropherogram (RIE) extracted from runs using a successively shortened capillary with (A) 30 cm, (B) 40 cm, (C) 50 cm and (D) 60 cm length for separation of the 5mer, 10mer (twofold negatively charged species), 15mer and 20mer (threefold negatively charged species) ODN mixture are overlaid. Experimental conditions: gel composition 20% (w/v) PEG 35000, 20% (v/v) acetonitrile and 20 m*M* BisTris–borate; CE inlet buffer 20 m*M* BisTris–borate; sheath flow composition of 50% acetonitrile and 50% 20 m*M* triethylammoniumacetate (pH 7.3); sheath flow of 15 μl/min. Effective CE voltage of −20.8 kV was applied and MS detection in the negative mode with an ionization voltage of −4.2 kV.

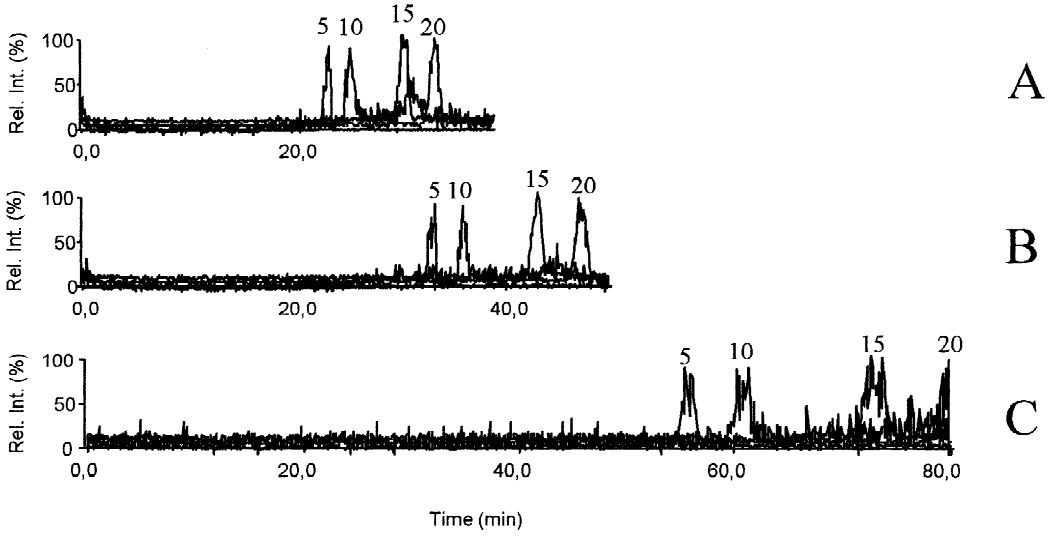

Fig. 3. Effect of separation voltage on CGE–ESI-MS runs, overlaid RIEs extracted from runs using (A) 27.5 kV, (B) 22.5 kV and 15 kV applied voltage (effective voltage is reduced by −4.2 kV ESI needle voltage to (A) 23.3 kV, (B) 18.3 kV and (C) 10.8 kV) are shown. Other experimental conditions as in Fig. 2.

m*M*. Usually, the same background electrolyte is used in the separating gel and the outer buffer reservoirs. Because BisTris–borate is not compatible with ESI-MS detection, it could not be used as sheath liquid buffer (see Section 3.2.1.3). Separations obtained for buffer concentrations of 10, 20, 40 and 100 m*M* are shown in Fig. 4. No successful size-based separations were obtained using 10 m*M* buffer or less. The 5mer was eluting even later than the comigrating 10mer, 15mer and 20mer. Migration order was according to molecular size using 20 m*M* buffer, although the 5mer and the 10mer almost comigrated. Using 40 m*M* and higher buffer concentrations, the 5mer and 10mer were well separated. Although the extracted ion spectra show separated and well-shaped ODN signals in case of a 100 m*M* buffer, it is difficult to assign ODN peaks in extracted single scans due to the high noise level (Fig. 5). Using 200 m*M* buffer, no successful CGE–ESI-MS runs were possible.

3.2. Interface parameters

3.2.1. Sheath liquid

Electroosmotic flow (EOF) is suppressed in PVA-coated and gel-filled capillaries, and transport of analytes is driven by electromigration only. Sheath liquid acts as a counterion buffer reservoir for electrophoresis. Furthermore, it must efficiently remove eluting ODN from the capillary tip and form a stable spray. Electrospray detection of ODNs performs best when employing sheath liquids with organic content and low salt concentration. Volatile buffers are preferred. Thus, sheath liquid composition differs from optimal outlet buffer as employed for CGE without ESI-MS detection. Optimization of

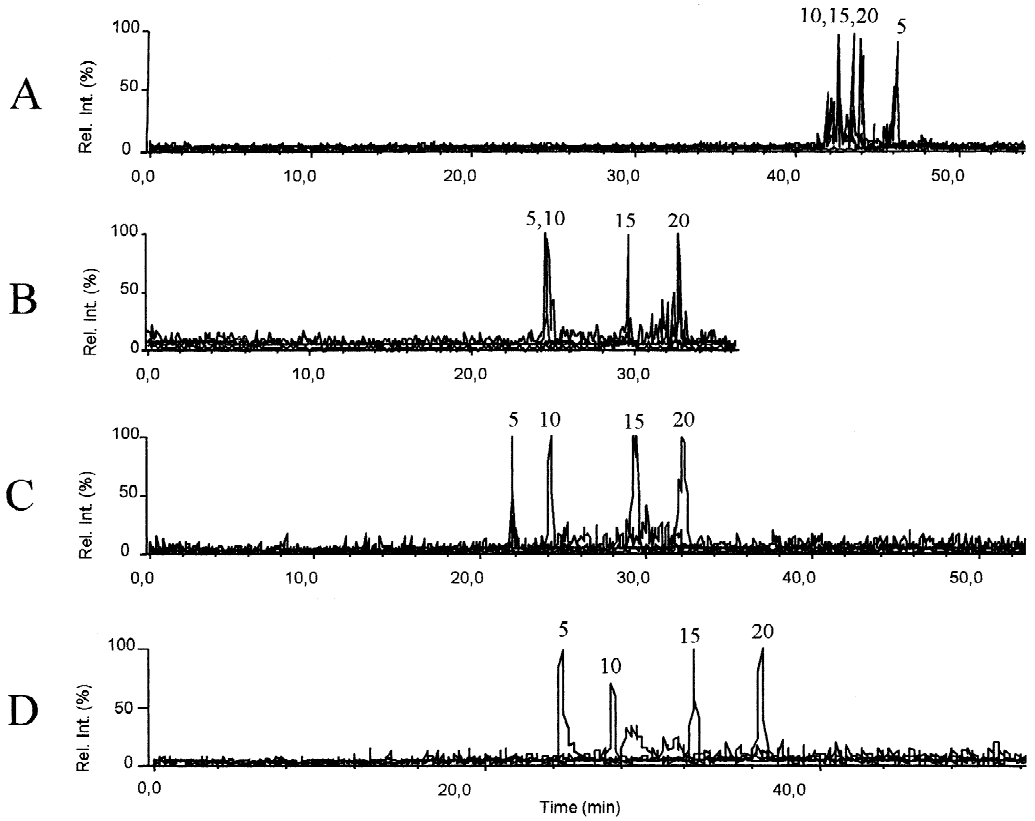

Fig. 4. Effect of background electrolyte on CGE–ESI-MS runs (RIEs), data from runs using (A) 10 m*M*, (B) 20 m*M*, (C) 40 m*M* and (D) 100 m*M* BisTris–borate buffer is depicted. Other experimental conditions as in Fig. 2.

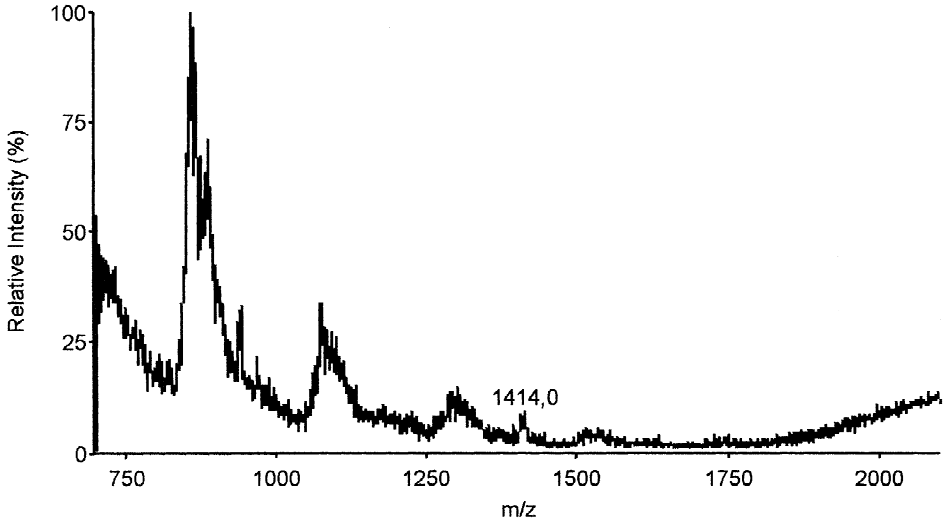

Fig. 5. Spectrum extracted from the coupling run using 100 m*M* BisTris–borate as background electrolyte for separation (Fig. 4D); extensive noise and clustering disable ODN analysis.

sheath liquid parameters is crucial for successful on-line coupling of capillary separation methods with ESI-MS.

3.2.1.1. Sheath liquid flow-rate

The optimal sheath liquid flow-rate should provide a stable spray for ESI-MS detection, but should not cause negative effects like peak broadening or sample dilution. RIEs of CGE–ESI-MS runs using sheath liquid (ACN–20 m*M* TEAA; 1:1, v/v) flow-rates of 3, 6, 9, 12 and 15 μl/min are depicted in Fig. 6.

A flow-rate of 3 μl/min was not sufficient to create a stable spray, and no ODN signals could be detected. Using 6 μl/min, a steady spray was obtained, and weak ODN peaks were detected. At a flow of 9 μl/min, stronger ODN signals were observed. Raising the flow-rate successively to 12 μl/min and 15 μl/min further improved detectability. No peak broadening or sample dilution effects were observed using still higher flow-rates up to 21 μl/min (data not shown). Accelerated deterioration of the MS vacuum due to the increased quantity of solvent penetrating the mass spectrometer occurred at high flow-rates, and only a few runs could be performed until recycling of the MS vacuum system was necessary. Thus, a flow-rate of 15 μl/min performed optimally.

3.2.1.2. Acetonitrile content

Organic sheath flow modifiers like acetonitrile are used to improve the electrospray ionization process and thus detectability of ODNs [20]. Acetonitrile content of the sheath liquid was raised from 0% to 90%, as depicted in Fig. 7. Using 0% ACN, barely any signals of the exemplary 20mer signals are visible. Detectability of the ODNs improved considerably raising the ACN-content to 30% and 50%, and unequivocal assignment of two charge species was possible. In case of 90% ACN, three charge species of the 20mer could be clearly assigned. Due to the increased MS detectability, ODN peaks in the extracted RIEs appeared wider, and thus separation efficiency seemed to be lowered (data not shown). Additionally, migration times were elongated raising the ACN content, in case of the 20mer from 32.2 min (0%) to 38.0 min (90%). Rapid soaking of the CGE capillaries outer polyimide hull was observed using high ACN contents, leading to obstruction of the interface channel delivering sheath liquid. Capillaries had to be replaced in such cases. Using a medium ACN content of 50%, soaking of the

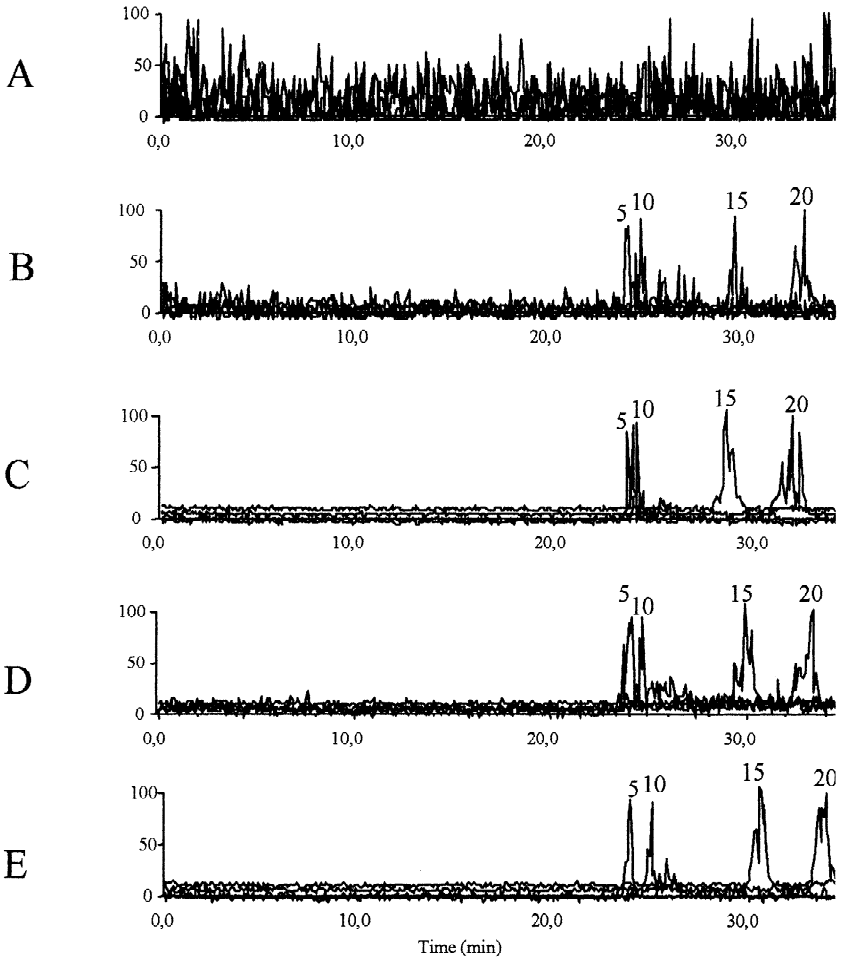

Fig. 6. Effect of sheath liquid flow-rate on CGE–ESI-MS runs (RIEs); measurements using (A) 3 μl/min, (B) 6 μl/min, (C) 9 μl/min, (D) 12 μl/min and (E) 15 μl/min sheath liquid (ACN–water (1:1, v/v), 10 m*M* TEAA) flow are depicted. Other experimental conditions as in Fig. 2.

polyimide hull was slow enough to allow measurements for a working day.

3.2.1.3. TEAA buffer content

TEAA buffer was added to the sheath liquid solution in order to provide a CGE buffer outlet. Triethylamine is frequently added to enhance ESI-MS detectability of ODNs measuring off-line. On the other hand, charge competition between TEAA buffer ions and ODNs may result in signal intensity loss.

Using buffer contents of 0 and 1 m*M* TEAA in the sheath liquid, only weak ODN signals could be detected. Raising the TEAA molarity to 2.5 m*M* improved detectability, and due to the low ion current noise, ODN signals were already visible in the TIC, see Fig. 8. Upon further addition of TEAA buffer to 5–20 m*M*, ODN signals diminished in the TIC, but were still well visible examining RIEs or single scans, respectively. In case of 40 m*M* TEAA buffer, no ODN signals could be detected any more.

3.2.2. Capillary alignment

The effect of the separation capillary position in the interface with respect to the surrounding steel needle was examined. RIEs obtained from these

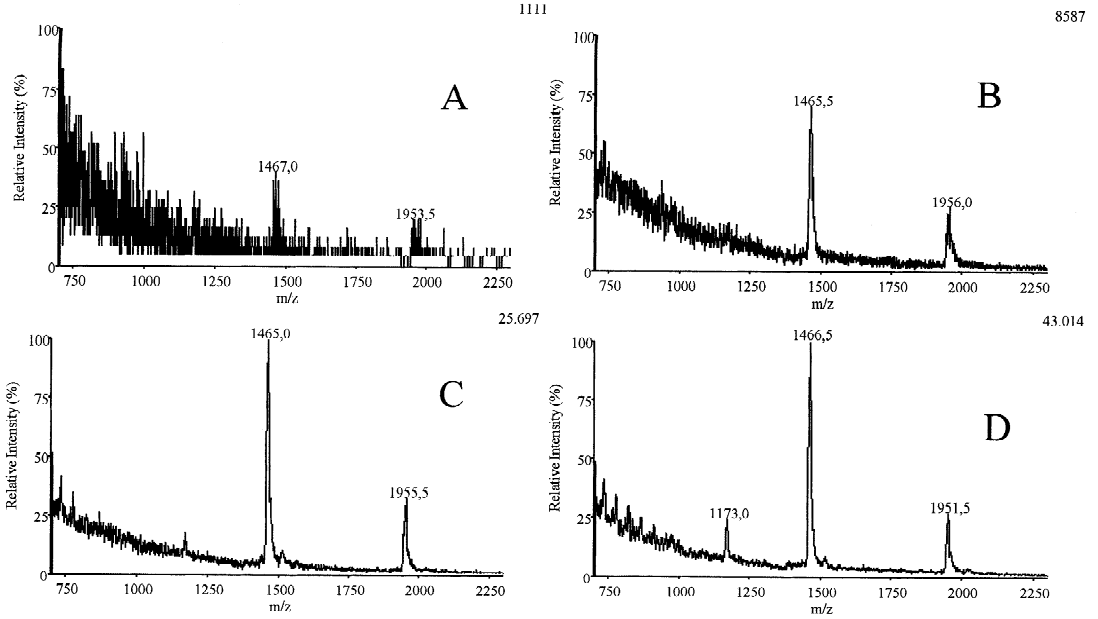

Fig. 7. Effect of sheath liquid composition (ACN content) on MS detection of CGE–ESI-MS on-line coupling runs; single scans of the 20mer ODN extracted from runs using (A) 0%, (B) 30%, (C) 50% and (D) 90% ACN mixed with water (TEAA concentration kept at 10 m*M*) buffer are shown.

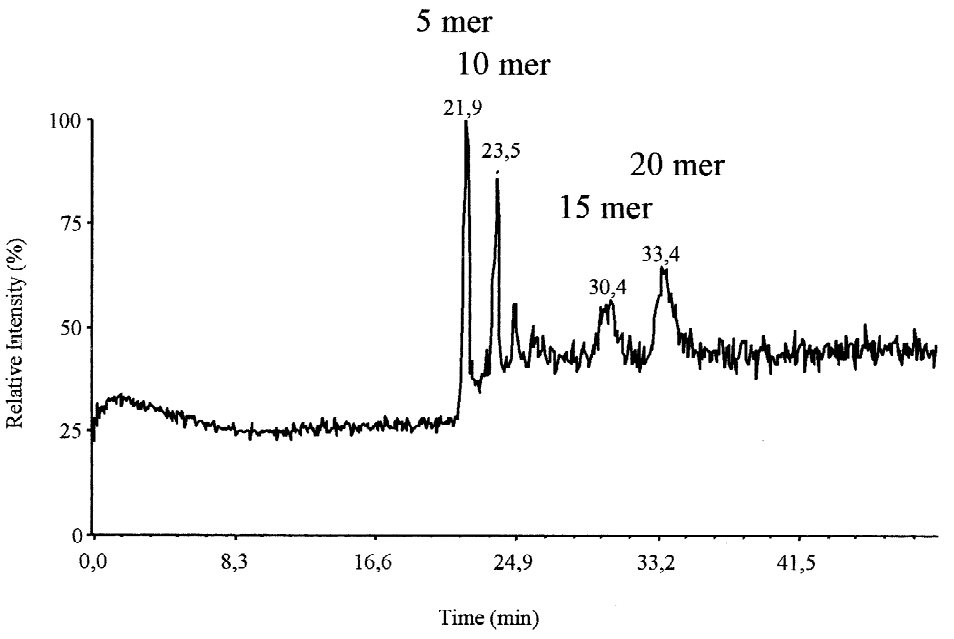

Fig. 8. Total ion current (TIC) of a CGE–ESI-MS run using 2.5 m*M* TEAA in an ACN–water (1:1; v/v) sheath liquid. Other experimental conditions as in Fig. 2.

different alignments are shown in Fig. 9. If the capillary was sticking about 2 mm out of the steel needle, no stable spray was obtained and no ODN signals could be detected. Using shorter distances of capillary and needle tips between about 1 mm outstanding or no distance between capillary and needle tip, coupling runs performed well. Peak broadening of eluting ODNs was observed if the capillary was sticking in the needle for about 1 mm. This result underlines the importance to consider carefully geometrical parameters of CE–ESI-MS coupling interfaces.

3.3. ESI-MS parameters

3.3.1. Orifice voltage

Voltage applied to the MS orifice controls which ions are allowed to penetrate the MS vacuum chamber. Using high orifice voltages, only few ions manage to enter the detection chamber, and only low ion currents are observed. In contrast, low orifice voltages do not efficiently reject entering molecules, and background noise is raised.

Analyzing ODNs, typically orifice voltages between −80 V and −120 V are utilized. In Fig. 10, single scans of the 20mer extracted from CGE–ESI-MS runs using orifice voltages of −60, −80, −100, −120 and −150 V are compared. Numbers of total ion counts decrease with increasing orifice voltage, mostly because of background noise effects. For best MS detection, intense analyte mass signals compared to the noise level are required. Optimal performance is found in case of −100 V orifice voltage.

Note that intensities of different ODN charge states are dependent on the applied orifice voltage. The fourfold negatively charged signal of the 20mer

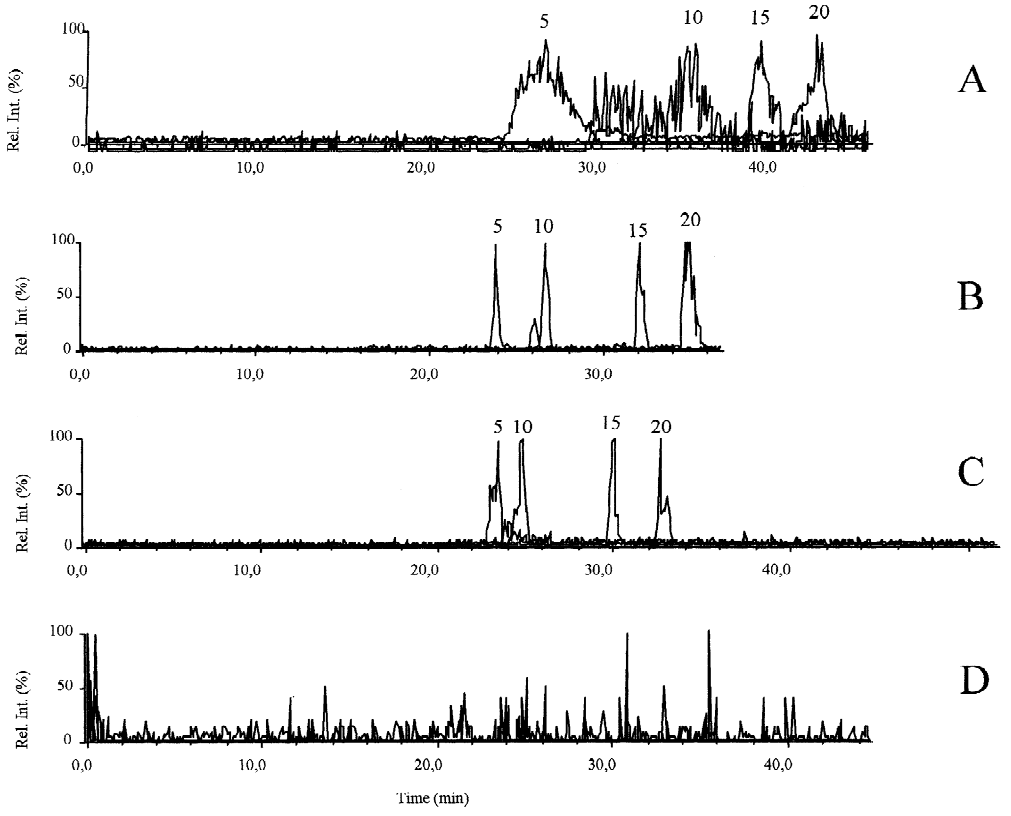

Fig. 9. Effect of capillary position in the sheath flow interface on on-line coupling performance; overlaid RIEs of measurements with the capillary position being (A) sticking ~1 mm in the interface, (B) no distance between the capillary and needle tip, (C) sticking ~1 mm out of the steel needle and (D) sticking ~2 mm out of the steel needle. Other experimental conditions as in Fig. 2.

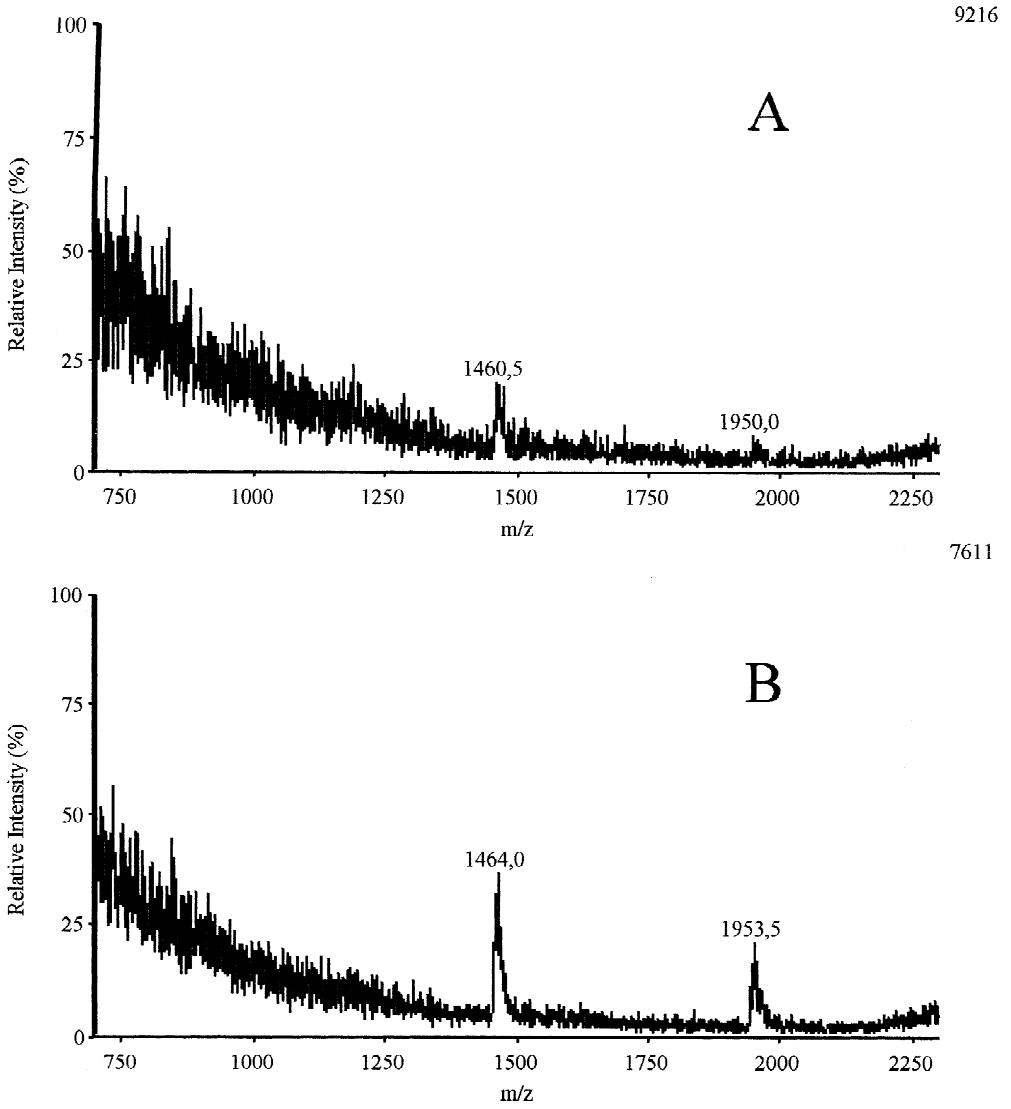

Fig. 10. Effect of orifice voltage on CGE–ESI-MS detection of the 20mer ODN; extracted single scans using (A) −60 V, (B) −80 V, (C) −100 V, (D) −120 V and (E) −150 V are shown.

is more intense than the corresponding threefold charged signal using lower orifice voltages, while the signal ratio is inverted using −150 V.

3.3.2. Limit of detection

Detectability of ODNs using ESI-MS in the negative ion detection mode is dependent upon various factors. Firstly, signal intensities vary with the chemical structures of the examined ODNs, e.g. with length, base composition, and chemical modification of the ODNs. Detection properties are limited by the utilized MS apparatus, and ESI-MS parameters that are hardly quantifiable put their impact on detectability, e.g. needle–orifice distance and its alignment.

Using our standard CGE–ESI-MS parameters in the full-scan negative ion detection mode, the limit of detection was reached at an ODN sample con-

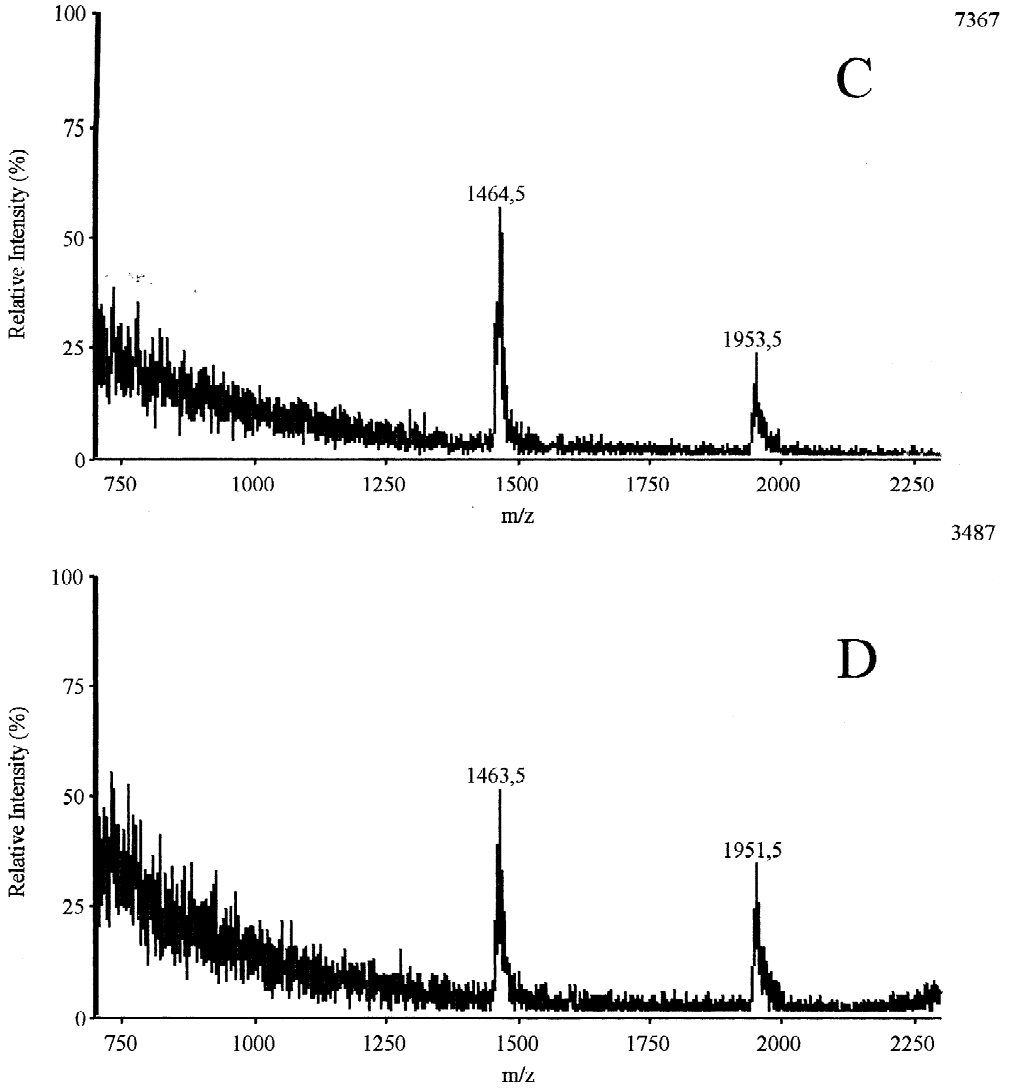

Fig. 10. (*continued*)

centration of 100 n*M*. The ODN solution was injected using a 2-μl vial, calculating for a total amount of 2 pmol ODN in the sample. The signal-to-noise ratio (S/N) for the measurements to determine the detection limit was for all experiments not below 3.

Detecting in the single ion monitoring (SIM) mode, MS sensitivity is enhanced. ODN concentrations as low as 10 n*M*, corresponding to 200 fmol ODN, could be detected.

4. Conclusions

The study described in this publication explores variations of CGE–ESI-MS parameters and consequences for performance of the on-line coupling system for ODN separation and detection. The results can be exploited in order to find optimal conditions for CGE–ESI-MS analysis of ODNs. Choice of optimized interface parameters like sheath liquid flow-rate and composition as well as geometry

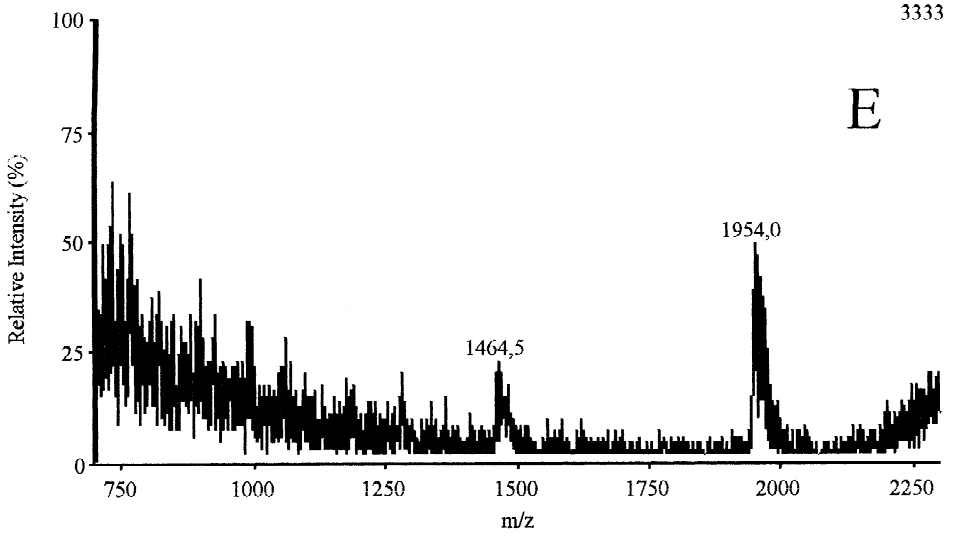

Fig. 10. (*continued*)

of the separation capillary alignment in the interface are crucial for successful coupling experiments.

Since we concentrated in our study on analysis of phosphodiester ODNs in the range from 5 to 20 bases in length, hyphenation of this technique for analysis of chemically modified ODNs as well as for longer DNA fragments represents the next challenge for on-line coupling of size-separating CGE with ESI-MS characterization.

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