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Composition dependent separation of oligonucleotides by capillary electrophoresis in acidic buffers with application to the quality control of synthetic oligonucleotides

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Abstract

Oligonucleotides have become a widely used tool in molecular biology and molecular diagnostics. Their parallel synthesis in large numbers has raised the request for fast and informative analytical tools for their quality control. Here we propose an alternative to current analytical methodologies based on capillary electrophoresis at very low pH in free solution. In these non-classical analytical conditions oligonucleotides can be discriminated for their base composition, thus adding a further dimension to the classical electrophoretic sizing performed in sieving media at moderately basic pH. We have tested several separating conditions at various pH values on a very large set of samples (about 200 synthetic oligonucleotides) ranging from 10 to 50 residues and with different terminal modification including amine, phosphate, biotin, fluorescein and other fluorescent dyes. We have been able to characterize the quality of these synthetic products and to detect base redundancies (i.e. the copresence of different bases in a single position of the oligonucleotide chain) in oligonucleotides up to 50 bases long, thus posing the basis for the application of this method to the emerging field of detection mutation in complex genomes analysis.

Keywords: Oligonucleotides

1. Introduction

High-performance capillary electrophoresis (HPCE) is currently being developed as an important separation tool in analytical chemistry. One of the most common applications of this technique is the analysis of nucleic acids either for DNA sequencing [1], DNA sizing [2] or oligonucleotide analysis [3]. Oligonucleotides have became an indispensable tool in modern molecular biology. The fall of their cost has widened the number of potential users and applications. Polymerase chain reaction (PCR), DNA sequencing and all the related techniques rely on the availability of standard or modified oligonucleotides. The use of oligonucleotides is further expanding after the introduction of the DNA chip technology [4–6] based on a microarray of oligonucleotides chemically bound to a suitable flat surface. This

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technology requires the availability of huge numbers of oligonucleotides in very tiny amounts. Parallel synthesizers [7] have been presented to meet the challenge posed by these needs but there is a parallel demand for automated, fast and informative analytical tools for the quality control of the synthetic products.

Different techniques have been employed in the analysis of oligonucleotides including high-performance liquid chromatography (HPLC) [8] and polyacrylamide gel electrophoresis (PAGE), which is regarded as the 'gold standard'. These techniques have the potential for preparative analysis but result expensive or scarcely amenable to automation. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) and electrospray ionization (ESI) mass spectrometry have raised wide interest being very informative analytical tools for macromolecules, although their application to DNA is lagging well behind the analysis of polypeptides due to the highly charged nature of the phosphate backbone in oligonucleotides which poses limitations to their use [9].

The electrophoretic separation of oligonucleotides is traditionally carried out in slightly basic buffers where the mass to charge ratio of these molecules is nearly constant over a wide mass range. Therefore a sieving media is mandatory to achieve a size-dependent separation and thus CE analysis conditions employing crosslinked gel or replenishable liquid high viscosity polymers have been proposed [10,11] for this purpose. Here we present a composition dependent separation of oligonucleotides by capillary electrophoresis in free acidic solution. As we reported previously [12], it is possible to discriminate oligonucleotides at low pH by using CE even without any sieving media. At low pH (<5) in fact the four nucleobases (adenine, cytosine, guanine and thymine) acquire a variable fraction of positive charge due to their different pK_a values [13]. This implies that the mass-to-charge ratio of a nucleic acid (single stranded) depends on its composition in terms of monomeric units. In these conditions the electrophoretic separation of a mixture of nucleic acids is feasible without the need of any sieving media, being based solely on the differential overall charge carried by each oligonucleotide of different composition. This concept, derived from the chemical properties of oligonucleotides, have been exploited in the early days of analytical molecular biology on very short stretches of polyribonucleotides in bidimensional gel electrophoresis in an attempt to determine their sequence [14,15].

After the publication of our previous report another group presented data and theoretical considerations regarding the feasibility of oligonucleotides separation by capillary electrophoresis in acidic buffers [16], coming to the conclusion that a pH window between 3 and 4 would be optimal for sequence discrimination. We now report here the results gained expanding our observations on a very large set of oligonucleotides (about 200 oligonucleotides) of variable length and composition (ranging from 10 to 50 mers), with a wide range of modifications including amine, phosphate, biotin and several fluorescent moieties exploring acidic buffers with and without denaturing agents. Our findings demonstrate that HPCE can be used as an automated analytical tool for the quality control of synthetic oligonucleotides due to its flexibility and robustness. In addition we also envision its applicability in the expanding field of mutation detection and single nucleotide polymorphism analysis thanks to the very promising discriminative properties in terms of separation of oligonucleotides of equal sequence differing by a single base.

2. Materials and methods

Instrumentation. All electrophoretic separations were performed on a BioFocus 3000 system (Bio–Rad Labs., Hercules, CA, USA) working in reversed polarity. Separations were monitored on-column at 260 nm. The liquid cooling system provided with the instrument controlled the temperature of the capillary column. CE analysis was performed in coated fused-silica capillary tubing (J&W, DB-Wax, I.D. 50 μm) with a column length of 50 cm.

2.1. Reagents

The oligonucleotides were purchased from Genset (France), Genosys (USA), MWG (Germany) and Pharmacia Biotech (Sweden). Most of these were simply desalted by the manufacturer. Several modi-

Table 1
Composition of 12 oligonucleotides analysed in CE at low pH

No.	Sequence	Length	R=(A+C)/(G+T)
1	GCTCCAAATGTCTTTGTAAGG	21	0.75
2	CCCTGCCACTCTCATCAATC	20	2.33
3	GAATTGTGTTTAGGTGCTGTG	21	0.24
4	TTGTGTCACGGTTCTGGTGG	20	0.25
5	CAGAGTTCTACAGAGAAACAG	21	1.63
6	CCTCAGTGTGAGATACCAAG	20	1.22
7	ACGGACTTGAGGGTCAACAG	20	1.00
8	TAAACAGTAGCTGCTGTTCC	20	1.00
9	GAGGCACAGTGAAATGAGAG	20	1.00
10	CCATCAATACTCCATGAGCG	20	1.86
11	CTCATGGAGTATTGATGGGC	20	0.54
12	CCATCTCACAAATAGACCTTG	21	2.00

fied oligonucleotides were purified by RP-HPLC. Iminodiacetic acid was from Aldrich. Other reagents were from Fluka (analytical grade or better).

2.2. Procedures

All the oligonucleotides tested were diluted with

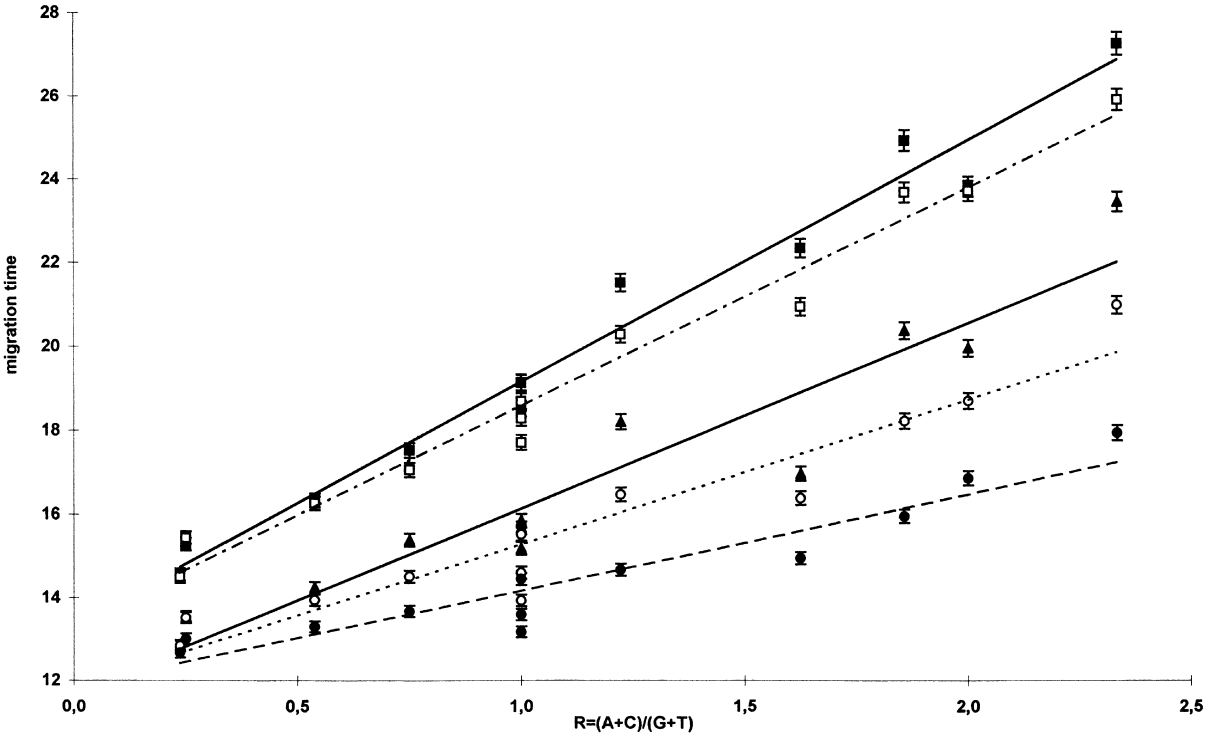

Fig. 1. Relationship between ratio R=(A+C)/(G+T) and migration time of 12 oligonucleotides analyzed by HPCE at different pH in 50 mM phosphoric acid titrated with NaOH. Sample injected by pressure at 20 p.s.i. s and run at 10 kV, and 20°C. ■=pH 2.3; □=pH 2.5; ▲=pH 2.8; ○=pH 3.0; ●=pH 3.3. Error bars (RSD less than 1%) are shown.

water to 4 pmol/μl and stored at +4°C when not in use. They were injected by pressure into the capillary column (20 p.s.i·s=137.8 MPa·s) and electrophoresed at a constant voltage (10 kV) at 20°C unless otherwise stated in figure legends.

Denaturing solutions (formamide, methylformamide, urea) were prepared as concentrated stock solution and stored at 4°C. The buffer solutions were filtered through a 0.45 μm pore size filter and stored at +4°C. Iminodiacetic buffers were prepared fresh every week. In fact we found that the separation performance worsened steadily with older stock solutions.

3. Results and discussion

The present work aimed at demonstrating the robustness and flexibility of a previously proposed procedure [12] for oligonucleotide analysis. This procedure is based on the differential protonation of nucleobases at a given pH. Tabulated [13] pK_a values for protonated nitrogen atoms of nucleobases in nucleotides are 3.8 for adenine; 4.5 for cytosine and 2.4 for guanine. Thymine is not protonated even at very low pH. The phosphate on the backbone has a pK_a=1.0. Following theoretical considerations based on the reminded pK_a values, the best pH

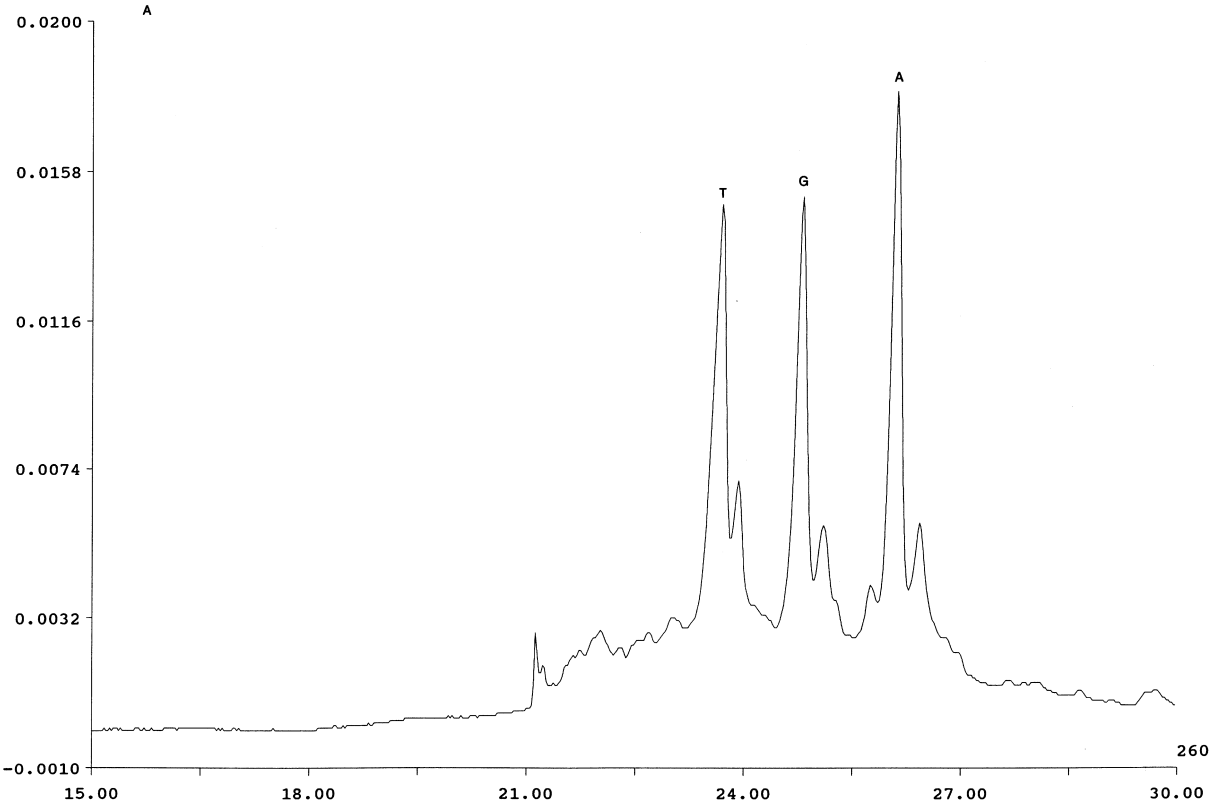

Fig. 2. CE separation in 50 m*M* iminodiacetic acid pH 2.3 of a mixture of three 20mer (A) and two 19mer (B) 5′ aminated oligonucleotides bearing a redundant base in a single position (the last base at 3' end). Sequences as follows: (A) CACCGTCCAGAGGATGTA**T**; CACCGTCCAGAGGATGTA**G**; CACCGTCCAGAGGATGTA**A**. (B) TTCTCACACCGTCCAGA**A**; TTCTCACACCGTCCAGA**C**. Peaks are labeled with the corresponding nucleobases in the redundant position. Note the presence of small peaks as a result of oligo synthesis by products. Sample injected by pressure at 20 p.s.i. s, run at 10 kV and 20°C. RSD less than 1% for all peaks.

region to maximise CE separation based on composition differences seemed the 3–4 window as recently proposed [16]. Despite of this, we have previously reported very good separation even at pH 2.5 [12], with remarkable results in term of parent sequences discrimination (i.e. truncated oligonucleotides sequences coming from synthesis failure). To ascertain the behaviour of oligonucleotides in acidic buffers at very low pH we compared different acids, counterions and denaturants at different pH values (ranging from 2.3 to 3.3) thus exploring potential limits and drawbacks or specificity in these conditions, uncommon in oligonucleotides analysis.

We applied a simplified model already proposed in the analysis of RNA fragments in bidimensional electrophoresis [14] to correlate migration time with composition of each oligo expressed as ratio (R) between the number of A+C and the number of G +T. We chose 12 oligonucleotides (Table 1) with $0.24<R<2.33$ and examined this mixture at different conditions: we varied pH (2.3; 2.5; 2.8; 3.0; 3.3) using two different acids [50 m*M* iminodiacetic acid (IDA) and 50 m*M* phosphoric acid] titrated with different bases (NaOH; NH_4OH; tetrapentylammonium hydroxide). We tested the need for denaturing agents in the acidic buffers by using 8 *M* urea, 30% *N*-methylformamide and 30% formamide.

Fig. 1 shows an example of data gained by using phosphoric acid titrated with NaOH. Relevant differences emerged in the analysis at different pHs: linear regression analysis demonstrate that slope of straight lines that best fit our data increased continuously from pH 3.3 to pH 2.3 and we got a relevant increase in resolution from pH 3.3 to pH 2.3. In particular at pH 2.3 either phosphoric acid and IDA (data not shown) performed very well. Correlation coefficient ranged from 0.93 to 0.99 thus demonstrating a good relationship between migration time and R in all

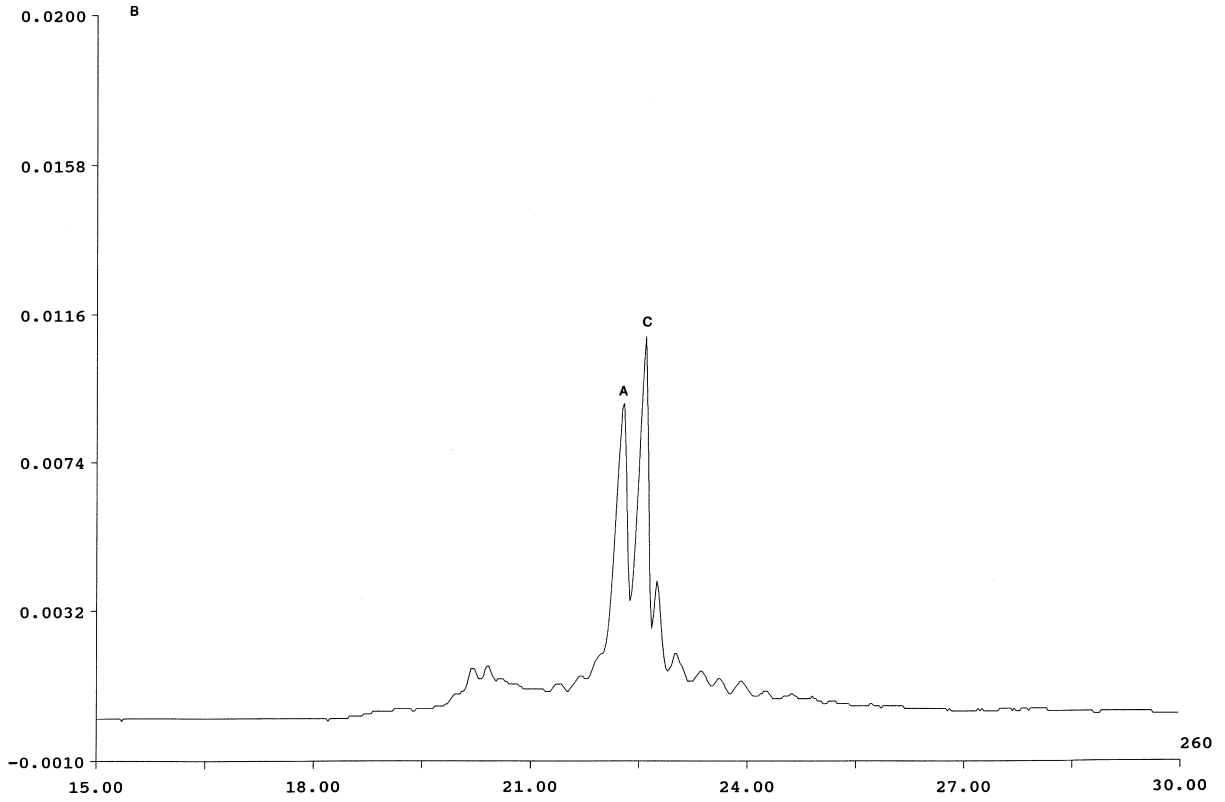

Fig. 2. (*continued*)

tested conditions. We performed the analysis in triplicate with a relative standard deviation (RSD) less than 1%.

Different bases were employed to detect the potential effect of counterions on the separation at low pH. No major differences emerged using either sodium or ammonium hydroxide as titrating bases for IDA and phosporic acid. Even tetrapentylammonium hydroxide, which has been recently reported for dsDNA sizing in gels when using intercalating dyes such as TOTO [17], performed similarly when used to titrate phosphoric acid (data not shown).

These results are somehow in contrast with the prediction of an optimal pH window for the separation at pH 3–4 [16] and suggest a different protonation behaviour of nucleobases included in a oligonucleotide chain respect to free nucleotides. Tabulated pK_a values could not be corrected values in these complex molecular environment. We hypotesize that, due to close proximity of the protonable groups within the polynucleotide chain, pK_a values could be considerably lowered with respect to those tabulated for free nucleobases, thus lowering the optimal pH window for separation.

Regarding the analysis in presence of denaturing agents, either formamide and *N*-methylformamide gave very poor results when added either at IDA or phosphate at pH 2.3 or 2.8. These two molecules may undergo rapid hydrolysis in these very acidic solutions and were subsequently discarded as denaturing media. On the contrary, urea remains stable even after long storage period at these pH values.

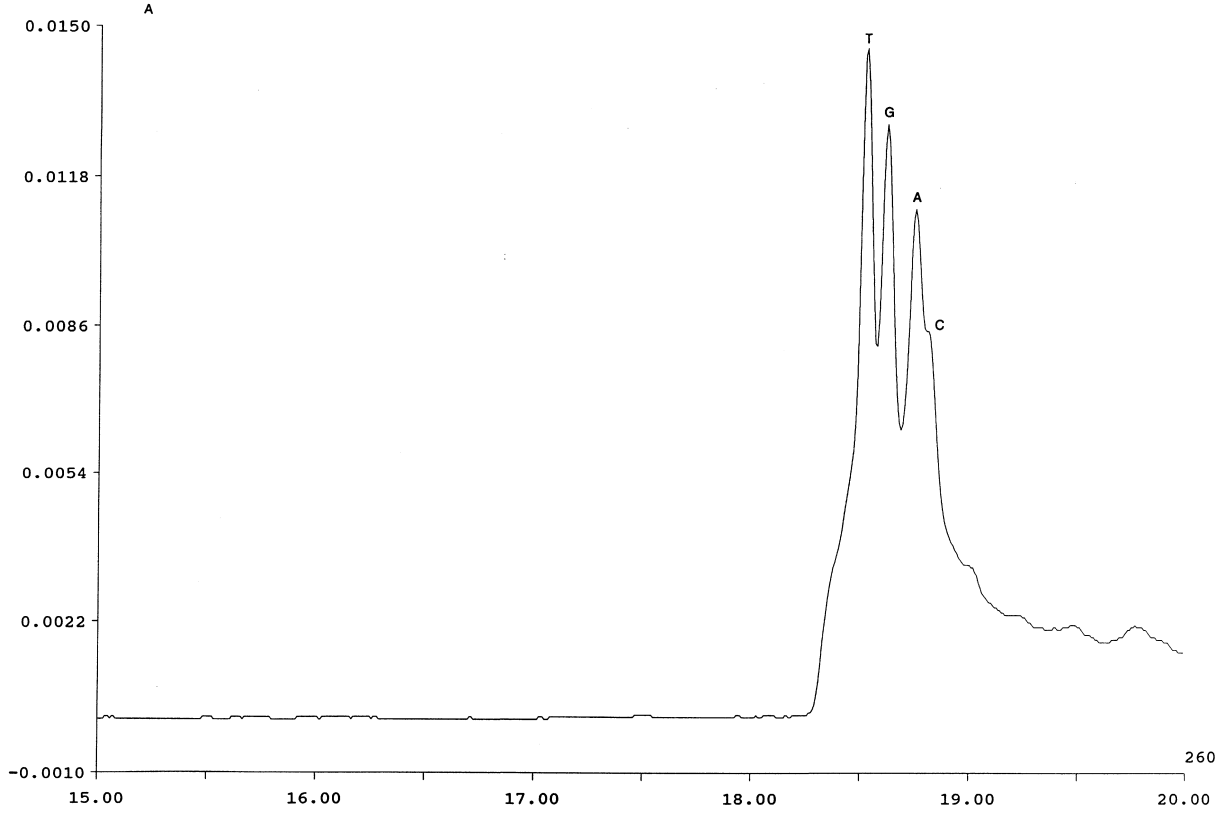

Fig. 3. CE separation in 50 m*M* phosphoric acid (pH=2.8) of a 50mer oligonucleotide bearing all four bases in a single position. (GCACTTGACATTAGATCT(**ACGT**)GTTCAAGTCACGTTACATCCTCTGGACGGTG). Peaks labeled with the corresponding nucleobases in the redundant position. Note the background in the electropherogram due to oligo synthesis by products. The analysis was performed in two different days (A and B) with different buffer preparations (RSD less than 1% for all peaks). Sample injected by pressure (20 p.s.i. s) and run at 10 kV, 20°C.

Urea seems not to alter significantly the separation but retention times increased significantly (data not shown). It should be noted however that, analysing longer oligonucleotides, urea may play a significant role in denaturing intrastrand loops, thus allowing separation only on the basis of composition and not on the conformation. As reported below, we have not noted anything similar even analysing a 50mer.We can suppose that the protonation of titrable groups (namely amine) on the nucleobases disrupt efficiently any possible inter- or intra- strand base pairing at these very low pH. Thus we conclude that denaturing agents are not necessary using the proposed condition for oligonucleotide analysis.

Following these results we started analysing the applicability of this method for routine quality control of standard and modified oligonucleotides. In particular we tested about 200 oligonucleotides purchased from different manufacturers. Among them about 80 standard oligonucleotides, which are the same of those reported in our previous work [12], were analysed again and over 120 samples including modified oligonucleotides carrying biotin, amine (as reported in our paper in press [18]), phosphate and fluorescein moiety or with other fluorescent labels.

Furthermore, to explore the real potential of this innovative technique we tested several oligonucleotides bearing redundant bases in a single position. The proposed technique seemed ideal for the discrimination of the presence of multiple bases in a single position of the oligonucleotide chain. Results in Fig. 2 were gained with oligonucleotides bearing a A/G/T (2A) and a A/C (2B) redundancy respectively. These results are especially remarkable because suggest the feasibility of sequence dependent separation of homologous oligonucleotides differing for a single base change. We have previously reported [12]the separation of a 49mer bearing a C/T substitution. In Fig. 3 we show the separation of a 50mer with all four bases in a single position. We

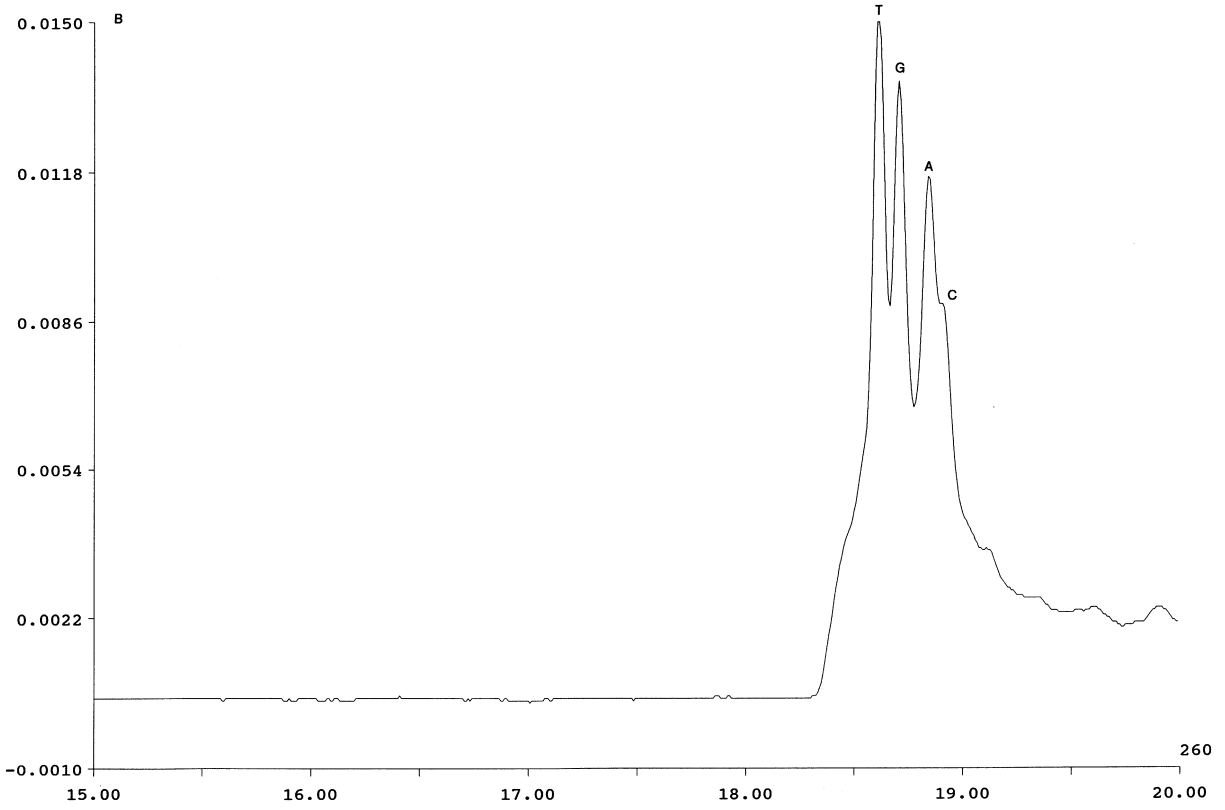

Fig. 3. (*continued*)

tested all buffers previously employed with and without urea. It is noteworthy that only phosphate buffers can discriminate three out of four molecular species. IDA buffers gave only two peaks whatever the pH. Urea is not mandatory in longer oligonucleotides analysis as an intraloop denaturing agent. In fact the only effect obtained by adding this additive was the increase of migration times, without any improvement in resolution (data not shown). Differences emerged when performing the analysis with phosphate at the various pH values. This behaviour would suggest that for demanding separations (i.e. large oligonucleotides) a careful optimization of the separation conditions is mandatory to achieve the expected result. It should be noted that all these analysis were performed in triplicate in different days and changing buffer preparations. In this way we tested the reproducibility of this method (compare Fig. 3A with 3B); we gained a RSD less than 1% for all peaks.

4. Conclusion

We presented a fast, inexpensive and automated method for oligonucleotides quality control by capillary electrophoresis in free solution at low pH. A large trial using about 200 oligonucleotides confirmed the robustness of this procedure either in terms of reproducibility and of discriminative capability of related sequences. We have applied successfully the method for the analysis of modified oligonucleotides, gaining very good results in the range 10–50mers. In particular in 50mer oligonucleotide analysis it is remarkable that migration times are not dependent, on a first approximation, on length. Composition in terms of nucleobases, instead, plays the central role for the separation. Even long oligonucleotides are separated within few min, as their shorter counterparts of similar composition.

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