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Separation of double-stranded DNA in conventional and isoelectric buffers: studies on stability and separation performance

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Abstract

In the capillary electrophoresis of double-stranded DNA in isoelectric buffers, worsening of resolution was observed in electropherograms as a function of time passed from the preparation of the separation solution, which consisted of 0.7% hydroxypropylcellulose, HPC, M_r 10^6, diluted in 150 m*M* histidine buffer. The DNA standards used were: kilobase pair-ladder, Marker V and Marker VI. In order to understand what happens in the histidine–HPC solution with ageing, the absorbance spectrum (200–500 nm), the conductivity and the pH of the solutions as a function of time were monitored. Fresh His gave a distinct peak at 206 nm. For all the solutions a significant diminution in the maximum absorbance value at 206 nm was observed as a function of ageing, with the concomitant appearance of a peak at 278 nm as the solutions became older. Also the conductivity increases dramatically with the ageing of the solutions and seemed to reach a plateau after ca. 40 days. In concomitance with the conductivity increments with time, the pH of the His solution (isoelectric point, p*I*=7.6) grew slowly up to pH 7.9; these combined data indicated that a new species contributing to the conductivity and altering the pH was formed from the His molecule, suggesting that His degraded in time. When the dipeptide His–Gly was used instead, a similar ageing phenomenon was observed, but with much reduced kinetics. Mass spectrometry, coupled to RP-HPLC, detected, in aged His solutions, in addition to intact His, two main degradation products: a 110.1 u species and a 93.2 u compound. The mass of the former coincides with the protonated species derived from the formation of a Schiff base on the α-amino group of His and subsequent decarboxylation without transformation of the final Schiff base into a chetonic group (a histamine-like molecule terminating with an imino, rather than with an amino group).

Keywords: Isoelectric buffers; Buffer composition; DNA

1. Introduction

The buffers used in capillary electrophoresis (CZE) function as an electrolyte solution by providing a conductive medium through which charged analyte molecules can migrate freely in an electric field. More important, however, the buffers also provide selectivity in the electrophoretic process by allowing the manipulation of sample analyte mobility. The quality of the separation, as measured by

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efficiency and resolution, is dependent on the running current and the resulting Joule heating inside the capillary, whereas the reproducibility of a series of analyses is dependent on the buffering strength at a given pH value [1]. In recent times, isoelectric buffers (i.e. amphoteric ions used as sole background electrolytes in CZE, at pH=isoelectric point, p*I*) have gained importance in CZE separations, mostly due to the fact that, by virtue of their very low conductivity, they allow very high voltage gradients, resulting in fast separations and high resolution (for a review, see [2]). For protein and peptide separations, a number of low p*I*, isoelectric buffers, have been described, operating in the pH 2–3 interval [3–6]. For DNA separations, two approaches were described: zone electrophoresis against a stationary pH gradient, created by carrier ampholytes, and CZE in isoelectric His buffer (pH=p*I*=7.6) [7,8]. The latter was found to give remarkable separations of single-stranded (ss) DNA: oligonucleotides, when analysed in a highly concentrated polymer solutions (8% polyacrylamide), could be fully resolved in only 4 min at 800 V/cm.

However, when analysing double-stranded (ds) DNA, a more complex picture emerged: there seemed to be a dual behaviour of DNA fragments, by which resolution is augmented for the smaller sizes while worsening for the larger ones [9]. The cut-off point seemed to be located at around 150 base pairs (bp) (i.e. one persistence length for a dsDNA). Above that, there was a rapid deterioration of resolution in His buffer, but below this critical length there was in fact an improvement of resolution, as additionally proved in the analysis of the poly-T tract allelic variants in intron 8 of the cystic fibrosis gene [10]. In this case, two DNA fragments of 59 and 63 bp, which could not possibly be resolved in standard buffers (e.g., Tris–borate–EDTA, Tris–acetate) could be separated only in 200 m*M* His buffer. On the other hand, above this critical length of 150 bp, resolution rapidly worsened. We assumed that a direct binding of His would occur on the phosphodiester bridges on the outside of the double helix until full saturation (i.e. that His would occupy every single charged oxygen on both strands; this binding was also proven by analysis of purified DNA fragments in free-solution CZE [11]). This could lead to a stiffening of the double helix (in addition to markedly alter the DNA mass and diameter of the double helix). A rigid DNA rod could start reptating much earlier than a standard DNA helix (for which the persistence length is assessed to extend over a stretch of ca. 150 bp). We have in fact already shown that, in CZE in sieving liquid polymers, the transition from the Ogston to the reptation-without-stretching regimes occurs already at 200 bp; reptation with partial stretching starting already at 3000–4000 bp [12]. In addition, the phenomenon of reptation is strongly dependent on the applied field strength. We notice in fact a worsening of resolution at higher voltages and for larger DNA fragments. Thus, our apparent loss of resolution in His buffer might simply be due to an earlier onset of reptation. On the other hand, highly beneficial effects were reported by Viovy's group in the analysis of very large DNAs in presence of the same His buffer [13]. Although they had reported, using pulsed fields, field-induced aggregation of large DNA, when adopting zwitter-ionic buffers (His, 178 m*M*) such aggregation was abolished in the 72 to 166 kilobase pair (kb) range and unique, highly resolved patterns could be elicited in less than 10 min. These authors hypothesised the following mechanism: upon electrophoresis in a standard background electrolyte (composed of free cations and anions) a non-uniform ion distribution forms inside the aggregate; upon electric transport, there is a continuous accumulation of co- and counter-ions at the right side of the aggregate and a depletion on its left side. This non-uniform ion transport leads to a net salt depletion in the region of higher DNA concentration, creating a polarisation and hence a slight violation of the electroneutrality in the proximity of the aggregate. This leads to further DNA condensation to the point of yielding macroscopic aggregates. Conversely, when adopting a zwitter-ionic buffer, due to its almost null mobility in the electric field, its concentration remains highly constant everywhere along the migration path, thus preventing ion depletion and DNA aggregation.

In the present report, we have further investigated these aspects and found additional evidence which suggests that, in addition to the phenomena described above, there is another important one leading to

DNA pattern degradation: the time-dependent decomposition of the His buffer.

2. Experimental

2.1. Reagents

Histidine, histidyl–glycine, *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid (TAPS) and Tris were purchased from Sigma–Aldrich (St. Louis, MO, USA). DNA samples: Marker V [containing the following 21 fragments in bp (only 17 peaks are detected): 8, 11, 18, 21, 51, 57, 64, 80, 89, 104, 123, 124, 184, 192, 213, 234, 267, 434, 458, 504, 540] and Marker VI [containing the following 12 fragments in bp (usually the two last fragments are eluted together): 154, 220, 234, 298, 394, 453, 517, 653, 1033, 1230, 1766, 2176] were from Boehringer Mannheim, Germany. The 1-kb ladder [containing the following 23 fragments: 72, 134, 154, 201, 220, 298, 344, 396, 506, 517, 1018, 1636, 2036, 3054, 4072, 5090, 6108, 7126, 8144, 9162, 10180, 11198, 12216] was purchased from Life Technologies, Glasgow, UK.

2.2. Capillary zone electrophoresis

The separations were performed in a Bio-Rad BioFocus capillary electrophoresis instrument by using a poly(acryloylamino etoxyethanol, AAP) [21] coated capillary, total length 30 cm (25.4 cm to the detector)×100 μm I.D. Non-coated capillaries [in the case of poly(*N*,*N*-dimethylacrylamide, DMA) polymer] were of a total length of 24 cm (19.4 cm to the detector)×100 μm I.D. 0.7% hydroxypropylcellulose (HPC) (M_r 10^6 u), 1.5% long-chain poly(DMA), 2.5% short-chain poly(DMA) (kindly provided by C. Heller) and 6% short-chain polyacrylamide were used as sieving polymers. Separations were performed in 100 m*M* Tris–TAPS (pH 8.3, conductivity 1.7 mS/cm), or in 87 m*M* TBE (Tris–borate, EDTA), pH 8.3, or in 150 m*M* His (pH 7.6, conductivity 138 μS/cm), or in 50 m*M* histidine–glycine (pH 7.3, conductivity 237 μS/cm). Note that 150 m*M* His and 50 m*M* His–Gly have comparable buffering capacity (as calculated with the program of Giaffreda et al. [14]).

2.3. Absorbance and conductivity measurements (ageing studies)

The absorbance was monitored by an ATI Unicam UV–VIS spectrophotometer. A solution containing 150 m*M* His and 0.7% HPC was diluted 1/500 resulting in 0.3 m*M* His and negligible amounts of polymer. This solution was let to age and aliquots were taken from that solution for absorbance monitoring. The same was done for conductivity measurements (CDM 92 conductivity meter), but the original solution was diluted to 10 m*M* histidine.

2.4. Liquid chromatography–mass spectrometry (LC–MS)

These measurements were performed by using a single quadrupole instrument (VG platform from Micromass, UK) coupled to an HP1100 LC binary pump (Hewlett-Packard, Palo Alto, CA, USA). The analysis conditions were: column, C_{18} ODS3 (150× 4.6 mm, Chrompack, Netherlands), flow-rate 1 ml/min, which was split to allow 10 μl/min into the ion source. The eluent consisted of 99.9% water containing 0.1% trifluoroacetic acid TFA (isocratic).

3. Results

3.1. Performance of isoelectric and conventional buffers in different sieving polymers

Fig. 1 shows the separation in fresh 150 m*M* His (pH=p*I*=7.6) of dsDNA fragments, from Marker V (1A), Marker VI (1B) and 1-kb ladder (1C), in 1.5%, long chain, poly(*N*,*N*-dimethyl acrylamide, DMA). These fragments range in size from 8 bp up to 12 216 bp. It is seen that base-line resolution is obtained for most fragments up to ca. 3000 bp; above this size (see Fig. 1C), DNA peaks are still separated, although not to baseline.

Fig. 2 displays the same data, for the same set of DNA fragments in the same poly(DMA) polymer, in presence of another isoelectric background elec-

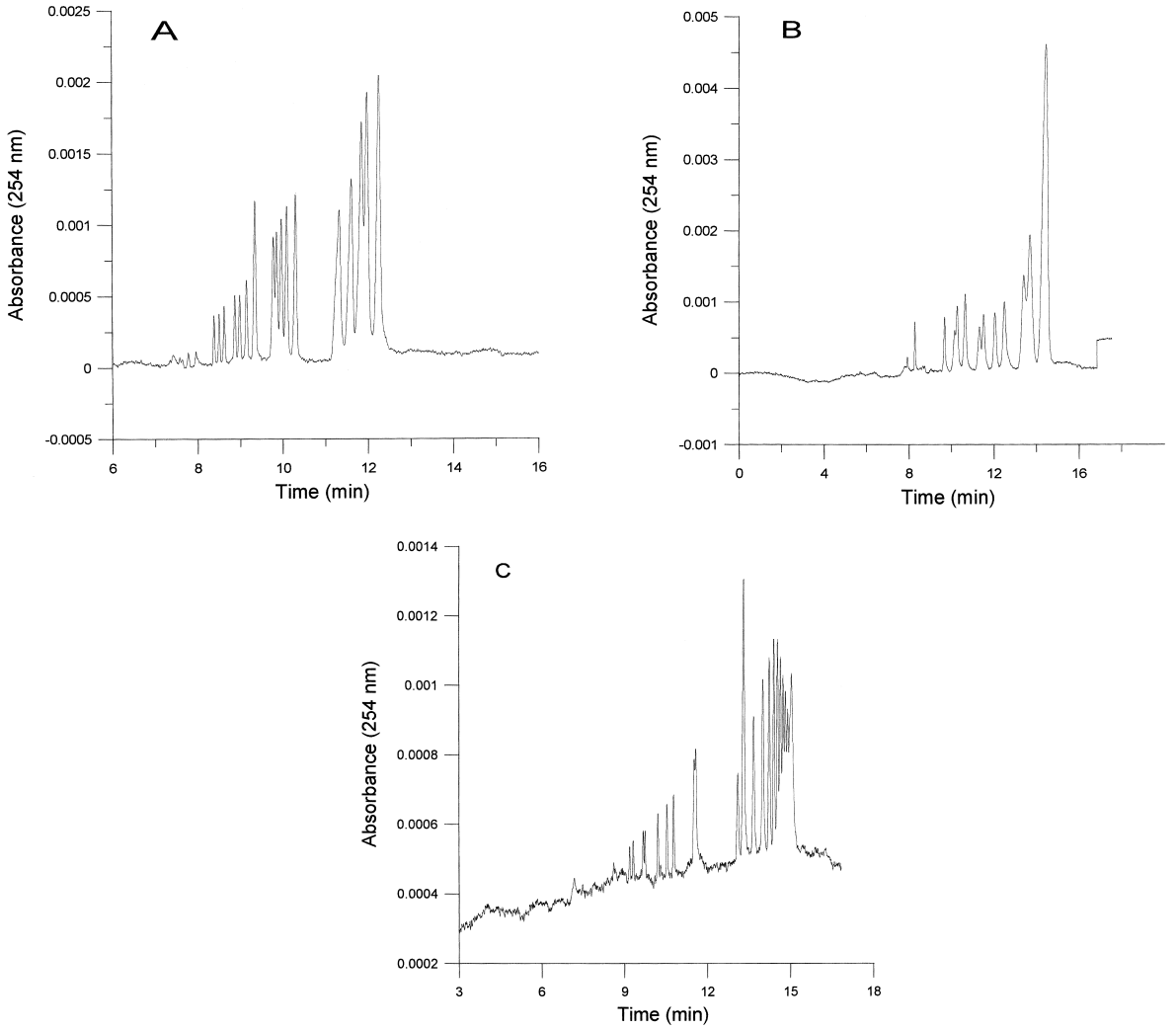

Fig. 1. CZE of different DNA markers in isoelectric buffers. Conditions: 150 m*M* His buffer, pH = p*I* = 7.6 in presence of 1.5% poly(DMA); poly(AAP) coated capillary, total length 30 cm (25.4 cm to the detector) × 100 μm I.D. UV absorbance detection at 254 nm. Run at 25°C at 100 V/cm. (A) Marker V; (B) marker VI; (C) 1-kb ladder.

trolyte, the dipeptide His–Gly (50 m*M*, pH = p*I* = 73). Two phenomena can be appreciated: the resolution is even better than in His buffers and the elution times are about 20% longer.

Fig. 3 compares the resolution of different DNA fragments in different polymers and in different buffers. Fig. 3A shows the separation of Marker V in the same 1.5% poly(DMA) sieving matrix but in 100 m*M* Tris–TAPS, pH 8.1. Although for the larger fragments (the set of 10 last peaks, from 184 to 570 bp) resolution and transit times are comparable to those obtained in isoelectric buffers (notably His–Gly, cf. Fig. 2A), the situation is dramatically changed in the case of smaller fragments (the two sets of three peaks to the left of the unresolved 123/124 bp doublet. Whereas the first (51, 57, 64 bp) and second (80, 89, 104 bp) triplets are well resolved in both His (Fig. 1A) and His–Gly (Fig. 2A), they tend to coalesce in Tris–TAPS. Fig. 3B shows the separation of Marker V in standard TBE buffer, pH 8.3, in presence of 0.7% HPC and at two different voltage gradients. Two phenomena can be

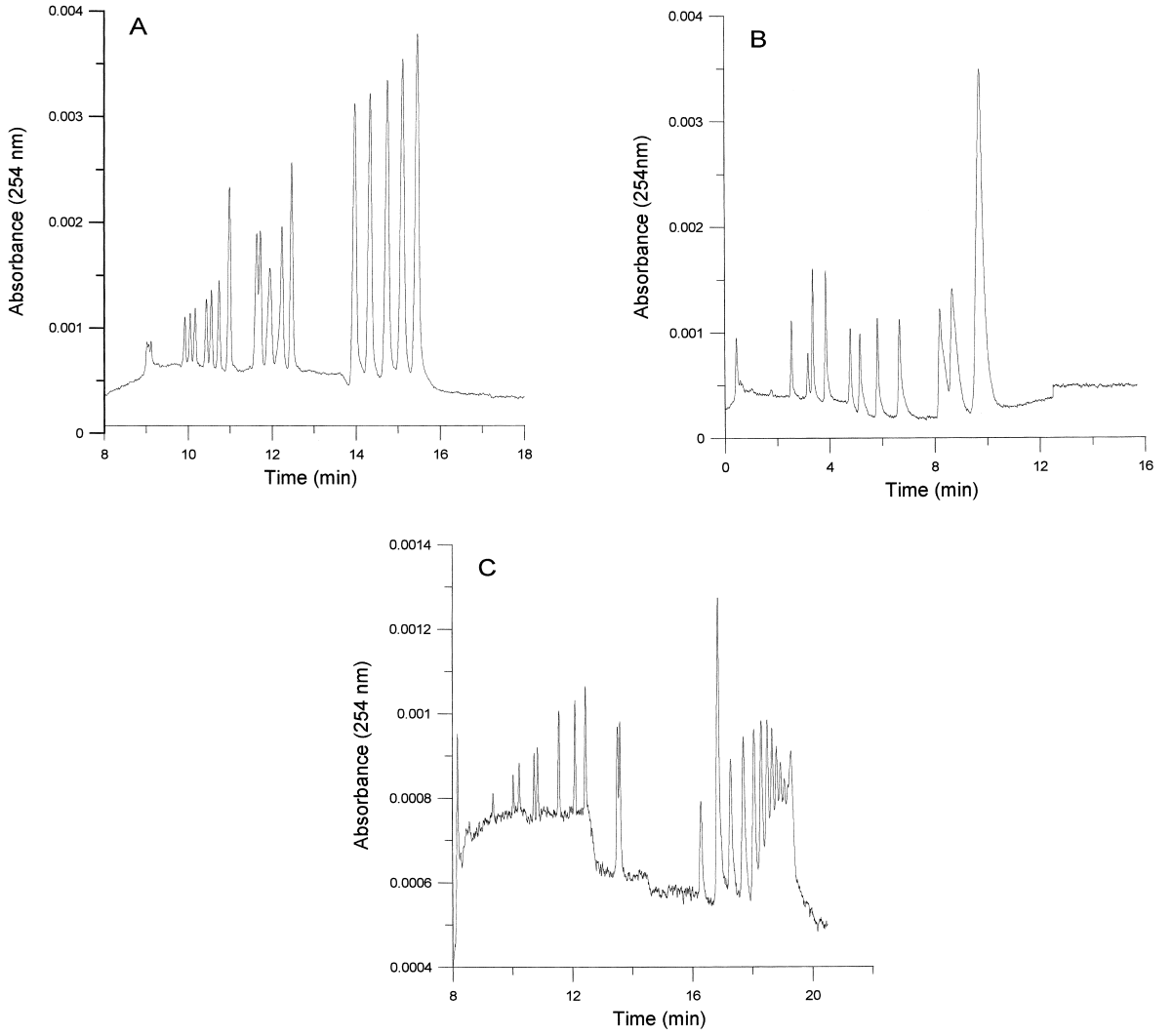

Fig. 2. CZE of different DNA markers in isoelectric buffers. Conditions: 50 m*M* His–Gly buffer, pH = p*I* = 7.3 in presence of 1.5% poly(DMA); poly(AAP) coated capillary, total length 30 cm (25.4 cm to the detector) × 100 μm I.D. UV absorbance detection at 254 nm. Run at 25°C at 100 V/cm. (A) Marker V; (B) marker VI; (C) 1-kb ladder.

appreciated: at lower voltages (100 V/cm) the resolution of the two sets of quintuplets (184–267 bp and 434–576 bp) is comparable to that in isoelectric buffers, but it is completely lost for the two triplets of six smaller peaks; at higher voltages (200 V/cm) resolution rapidly worsens for both small and large fragments. Fig. 3C shows the same separation in 0.7% HPC in 150 m*M* isoelectric His at three different voltage gradients: 50, 100 and 200 V. As compared with Fig. 3B, one can appreciate that not only the larger fragments are better resolved and more symmetric, but even the small ones are baseline resolved at all voltage gradients applied. Finally, Fig. 3D displays the separation of Marker V in 150 m*M* His but in yet another type of polymer, 6% polyacrylamide. This last electropherogram too confirms the excellent resolution obtainable in isoelectric buffers, in general of superior quality as compared to conventional background electrolytes. As a conclusion of this set of experiments, it would

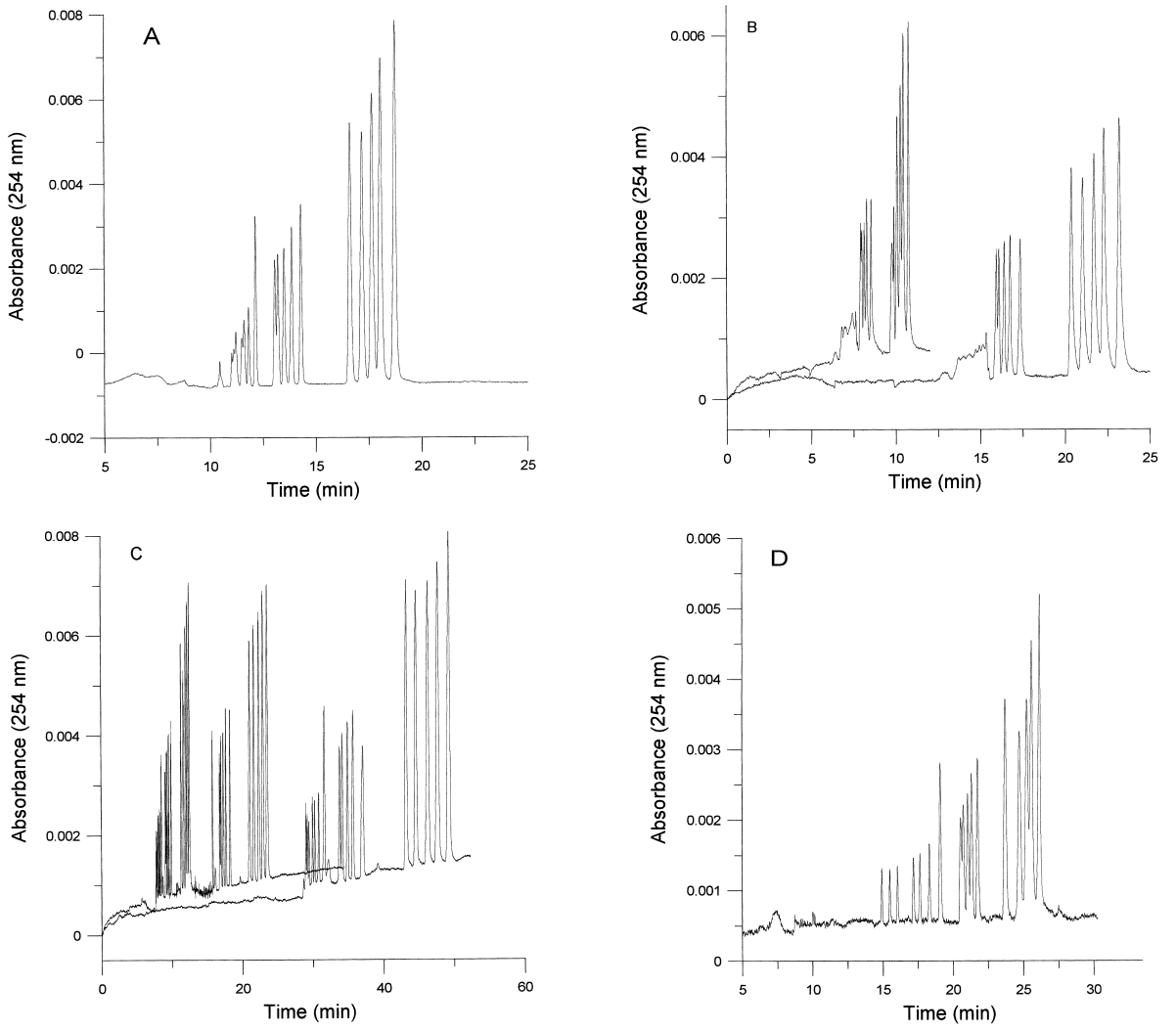

Fig. 3. CZE of DNA markers in different buffers. (A) Marker V DNA run in 100 m*M* Tris–TAPS, pH 8.1 in presence of 1.5% poly(DMA). (B) Marker V DNA, run in 87 m*M* TBE buffer, pH 8.3, in presence of 0.7% HPC at (from right to left) 100 and 200 V/cm. (C) Marker V DNA run in 150 m*M* His buffer in presence of 0.7% HPC at (from right to left) 50, 100 and 200 V/cm. (D) Same as C, but in presence of 6% polyacrylamide, 200 V/cm. In all cases, a poly(AAP) coated capillary, total length 30 cm (25.4 cm to the detector)×100 μm I.D. was used. UV absorbance detection at 254 nm; run at 25°C.

appear that isoelectric buffers offer superior performance in comparison with non-isoelectric ones and that resolution is largely independent from the type of sieving liquid polymer adopted and, for voltage gradients not greater than 200 V/cm, also from the voltage applied. Nevertheless, it was noted that, after a week of use, the performance of isoelectric His rapidly worsened, to the point that, after two weeks of continuous use, no discernible pattern of DNA fragments could be eluted by CZE of any of the DNA markers analysed. At this point, having excluded the effect of other parameters (type

of sieving polymer, voltage gradient adopted), it was reasonable to assume that some time-dependent phenomenon occurred to the buffer itself.

3.2. On the ageing of isoelectric buffers

A 150 m*M* isoelectric His solution was left ageing up to 35 days and its absorbance spectrum recorded. As shown in Fig. 4A, a fresh His solution shows a sharp peak at 206 and no chromophores in the 230–330 nm interval. However, upon ageing, it can be appreciated that the main peak keeps decreasing and a new species, centred at 278 nm, keeps increasing (as visible on the expanded scale of Fig. 4B). Fig. 5A shows the absorbance decrements for the 206 nm chromophore upon ageing of three different His preparations, while Fig. 5B gives the corresponding increments of the 278 nm peak; at the end of the 35-days observation period, the degradation of His could be as high as 20–25% (average of four different experiments). When the CZE runs of Fig. 1 were repeated in solutions of His aged one week and more, the electropherograms became progressively blurred, till no clear peak pattern could be distinguished (data not shown). In parallel with UV spectra, we have additionally recorded the conductivity and pH of the same His solutions upon ageing. As shown in Fig. 6, the conductivity of two different His solutions markedly increased, in some cases >2 orders of magnitude as compared to fresh solutions, when ageing was continued up to 75 days. The pH of the solutions keeps increasing too, although the overall increment is quite modest (from 7.4 up to pH 7.9).

We have attempted to observe the potential degradation product by CZE at both, quite acidic (pH 2–3) and quite basic (pH 9–10) pH values, but all attempts failed; we could only see, in both system, either a broadening of the main peak or a peak of odd shape, a spike followed by a plateau (resembling an isotachophoretic peak profile; data not shown). These CZE separations were thus discontinued. We finally resorted to mass spectrometry (MS) coupled to RP-HPLC, in an attempt at separating and elucidating the structure of the potential degradation product. As shown in Fig. 7, the total ion current

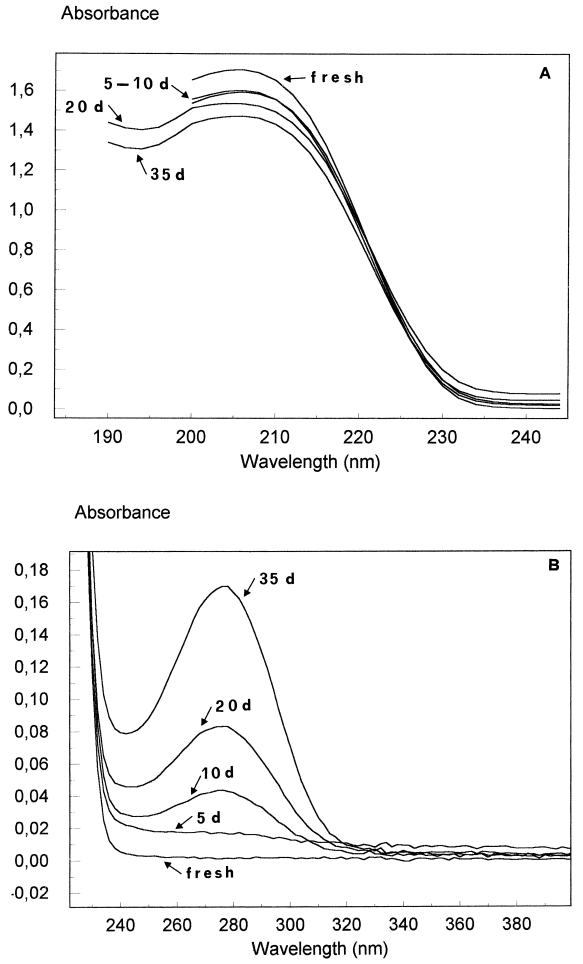

Fig. 4. UV absorbance spectrum of 0.3 m*M* isoelectric His. (A) Absorbance spectrum in the 180–320 nm range. (B) Expanded scale spectrum in the 240–400 nm interval. In addition to the spectrum of freshly dissolved His, the following aged His solutions have been analysed: 5-, 10-, 20- and 35-days old. Note, upon ageing, the appearance of a new chromophore centred at 278 nm.

(TIC) chromatogram of freshly dissolved His shows a single, rather symmetric peak (Fig. 7A). The injection of a fresh aqueous solution of His (0.5 mg/ml) into the LC–MS system gave the UV (A) and TIC (B) chromatograms of Fig. 7. The positive ES mass spectrum associated with the TIC peak centred at t_r=2.27 min contains a dominant species at m/z 156.1 $[His+H]^+$ and a minor peak at m/z

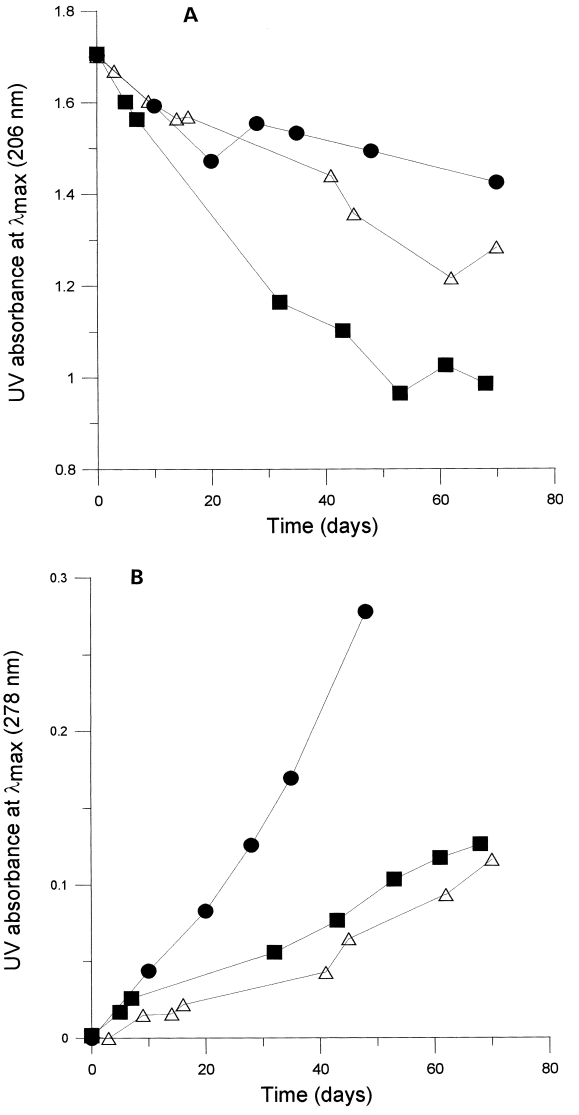

Fig. 5. Plots of the decrements (A) and increments (B) of absorbance with His ageing. (A) Decrements of the 206 nm chromophore upon ageing of three different His solutions, studied over a period of up to 70 days. (B) Increments of the 278 nm chromophore upon ageing of three different His solutions, studied over a period of up to 70 days.

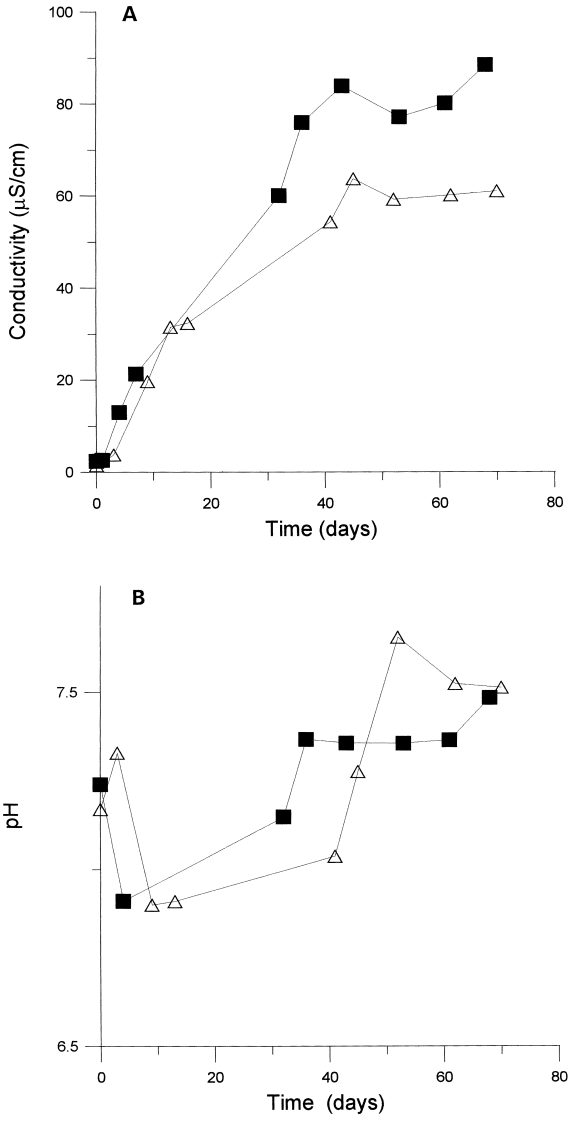

Fig. 6. Conductivity and pH increments upon ageing of His solutions, studied over a period of 70 days.

110.8, which could be attributed to the fragment $[His–CO_2]^+$. Repeating the same LC–MS measurement for an aged solution of His (ca. 30 days) resulted in the UV (A) and TIC (B) chromatograms in Fig. 8. The pronounced tailing in the latter peak (absent in Fig. 7) infers the presence of unresolved component(s). This deduction is supported by the positive ES mass spectra associated with the front (C) and the tail (D). The first spectrum is dominated by m/z 156.2 $[His+H]^+$, whereas the second spectrum has two significant signals at mass to charge ratios of 110.1 and 93.2.

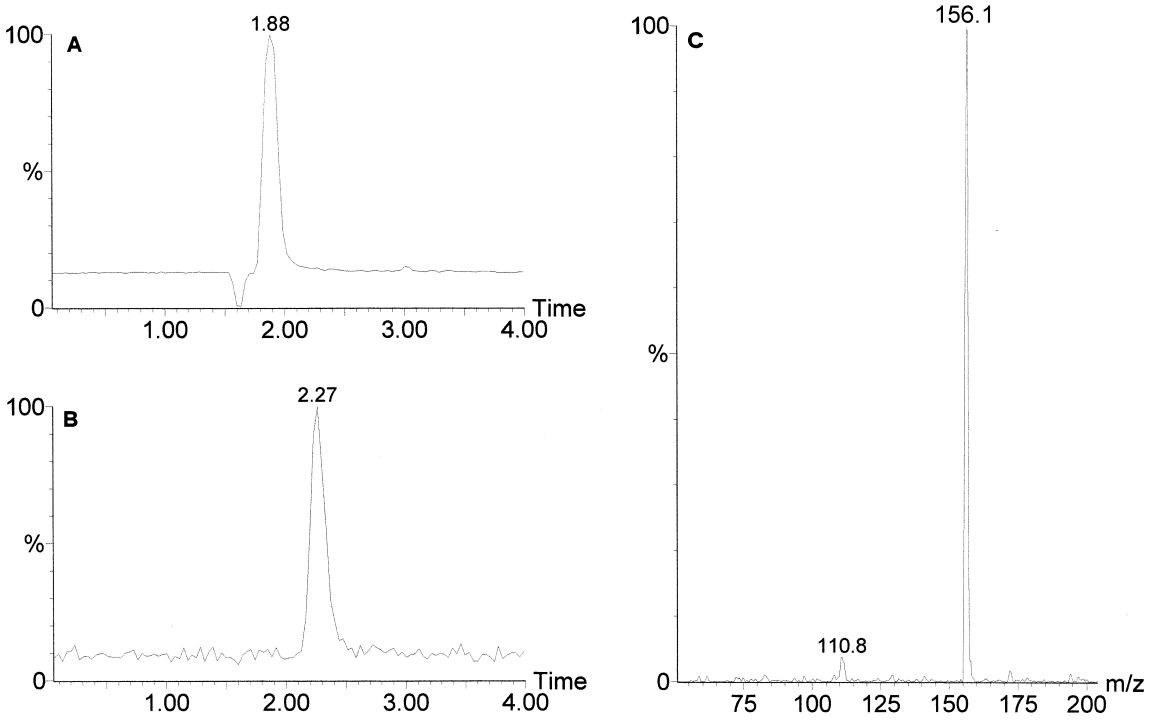

Fig. 7. UV (A) and total ion current (TIC) (B) chromatograms and positive electrospray mass spectrum (C) associated with the TIC peak centred at $t_r = 2.27$ min of freshly prepared His. The latter shows a single peak at m/z 156.1 $[His+H]^+$ with traces of a peak with m/z of 111.

4. Discussion

The present data confirm and considerably expand previous finding on the CZE separation of dsDNA in isoelectric buffers. The discussion that follows highlights the most prominent aspects of this research.

4.1. On the binding of isoelectric His to dsDNA

While our previous data had simply hypothesised such a binding, and given a model (see Fig. 7 in [9]) of His molecules massively coating the outside of the two helices and thus binding to any negatively charged oxygen atom in the phosphodiester bridge, the present data further corroborate the previous model. According to the model given in Fig. 7b in [9], the dsDNA fragments should display a higher mass, a large radius of gyration and a more stiff double strand configuration. If this were true, one should see the effects in the CZE profile. This is in fact dramatically evident in the elution patterns of Fig. 3A–D. In dilute polyacrylamides, such as in 1.5% poly(DMA) (Fig. 3A) or in high M_r celluloses (such as in 0.7% HPC, Fig. 3B) the smaller DNA fragments should be poorly sieved and thus poorly separated. This is precisely what happens in conventional buffers, such as Tris–TAPS and TBE, to the two sets of triplets in Marker V, spanning the 51 to 104 bp size range. This poor sieving had in fact already been previously reported by Blanch's group [15–17], who first proposed dilute hydroxyethylcelluloses (HECs) in separation of large DNA fragments. On the contrary, excellent resolution of these six fragments is obtained in the same, dilute HPC polymer, when utilising 150 m*M* His instead (Fig. 3C), as though these small fragments behaved as large particles, subjected to the frictional forces of the sieving polymer solution. This combined evidence, together with the data of Stellwagen et al.

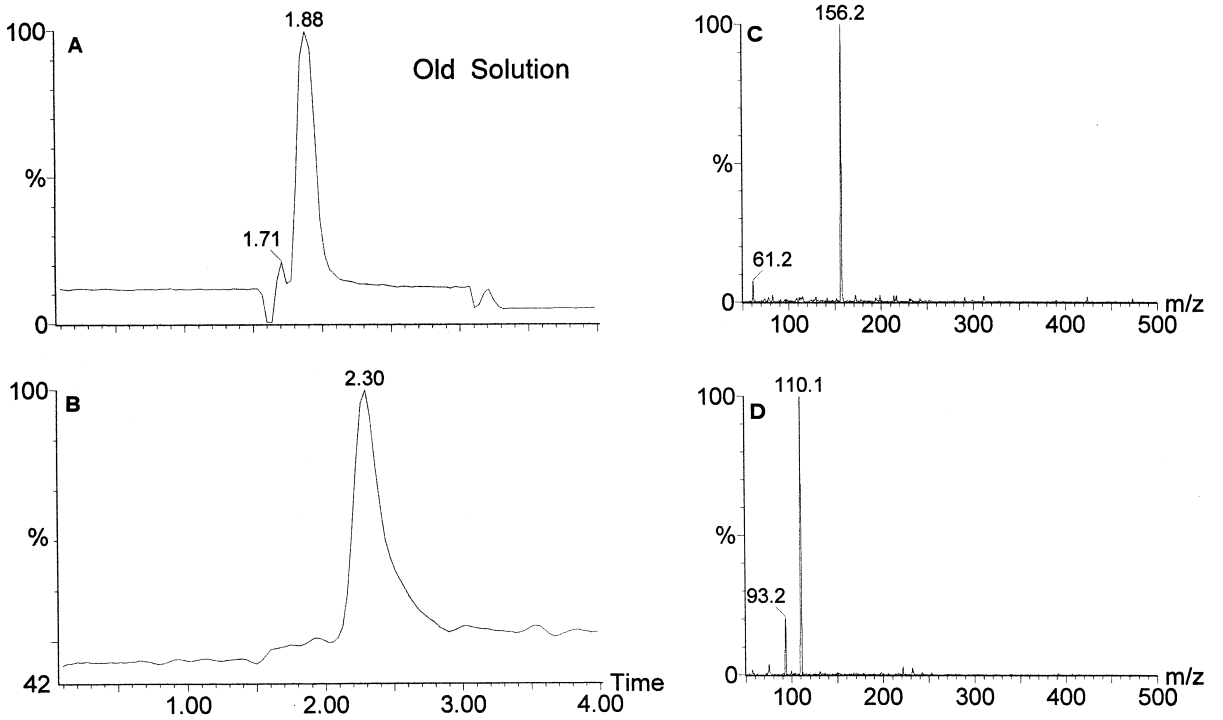

Fig. 8. UV (A) and total ion current (TIC) (B) chromatograms and positive electrospray mass spectra associated with the front (C) and tail (D) of the TIC peak centred at $t_r = 2.30$ min for an aged (ca. 30 days) His solution. Note in (D) two peaks at m/z 110.1 and m/z of 93.2.

[11], by CZE in free solution, points to a real mechanism of direct binding of His to dsDNA.

4.2. On the degradation of His and His–Gly upon ageing in solution

The present data clearly prove that the worsening of the CZE elution profile of dsDNA in isoelectric buffers, such as His and His–Gly, is directly correlated to the degradation of these buffers. This is well illustrated by RP-HPLC coupled to MS, which gave two possible degradation products present in aged His solutions, having m/z ratios of 110.1 and 93.2. Below, some explanations are offered on the possible structure of at least one of them. Since the electrospray ionization here used is known to produce protonated species, the two peaks should have an original neutral mass of 109 and 92, respectively. We can consider here two scenarios: biological (i.e. enzymatically driven) or chemical degradation. In the first case, enzymatically driven degradation is typically brought about by L-amino acid oxidase, an ubiquitous enzyme present in most organisms, including molds, bacteria and yeasts. If this were the degradation pathway, it would typically occur via oxidative deamidation of the compound to the corresponding α-keto acid, as follows:

$$RCH(NH_2)COOH + O_2 + H_2O \rightarrow$$
$$RCOCOOH + NH_3 + H_2O_2$$

In turn, the α-keto acid produced during the enzymatic reaction will be decarboxylated to the next lower carboxylic acid, as follows:

$$RCOCOOH + H_2O_2 \rightarrow RCOOH + CO_2 + H_2O$$

Note, in addition, that even the enzymatic oxidation of an α-amino acid proceeds via the initial formation of a transitory dehydroamino acid [RC(=NH)COOH] (a Schiff base, in fact), which, in virtue of its chemical lability, undergoes a non-enzymatic hydrolytic breakdown to the corre-

sponding α-keto acid (RCOCOOH), as depicted above [18]. However, were this the case (and we doubt it, since the His solution is prepared in a sterile way, although it is subsequently handled in a non-sterile fashion), there remains the fact that the mass of the final product (RCOOH) would be 125, in no way corresponding to that of the two main degradation products observed (110 and 92 u). That the initial degradation step would occur via a formation of a Schiff base is quite likely, though, since the dipeptide His–Gly (which lacks the α-amino group) does not show any degradation when stored in solution up to a month (MS data not shown).

In the second case (chemical degradation), the possible mechanism could be photolysis of His induced by adsorption of UV light (the buffer solutions are typically exposed to irradiation by standing on the bench for most of the working day). By irradiation, the most universally agreed upon degradation mechanism involves deamidation. For His, however, more ammonia evolved from its destruction by light than could be accounted for in terms of liberation of the α-amino group, a phenomenon explicable only if it assumed that the extra ammonia was derived from a nitrogen atom in the imidazole group. The possibility that irradiation of His might also involve a decarboxylation reaction was also suggested by the presence in the solution of products with histamine-like reaction [19]. Recently Cheng et al. [20] reported an oxidative degradation pathway of His, by which the C_2 in the imidazole ring is first oxidised to 2-oxo-His (or 2-keto-His); subsequently, after ring opening via further oxidation and additional breakdown of the imidazole moiety, it gives as a final product either asparagine (132 u) or its hydrolytic derivative, aspartic acid (133 u). Even these last two amino acids, though, do not display a mass corresponding to any of the fragments here detected. However, the major species in Fig. 8d (m/z 110.1) coincides with the protonated species derived from the formation of a Schiff base on the α-amino group and subsequent decarboxylation without transformation of the final Schiff base into a chetonic group (a histamine-like molecule terminating with an imino, rather than with an amino group). At present, though, the minor species at m/z 93.2 remains unexplained.

5. Conclusions

The present report clearly shows that CZE of dsDNA in isoelectric buffers (notably His and His–Gly) offers distinct advantages over conventional, non-amphoteric buffers in both continuous and pulsed electric fields. Nevertheless, due to the fact that these buffers are more prone to degradation, we recommend that His buffers should be used for no more than a week and then made fresh. His–Gly also degrades but at a slower rate, thus this last buffer can be used for a period of up to two weeks before refreshing.

Acknowledgements

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