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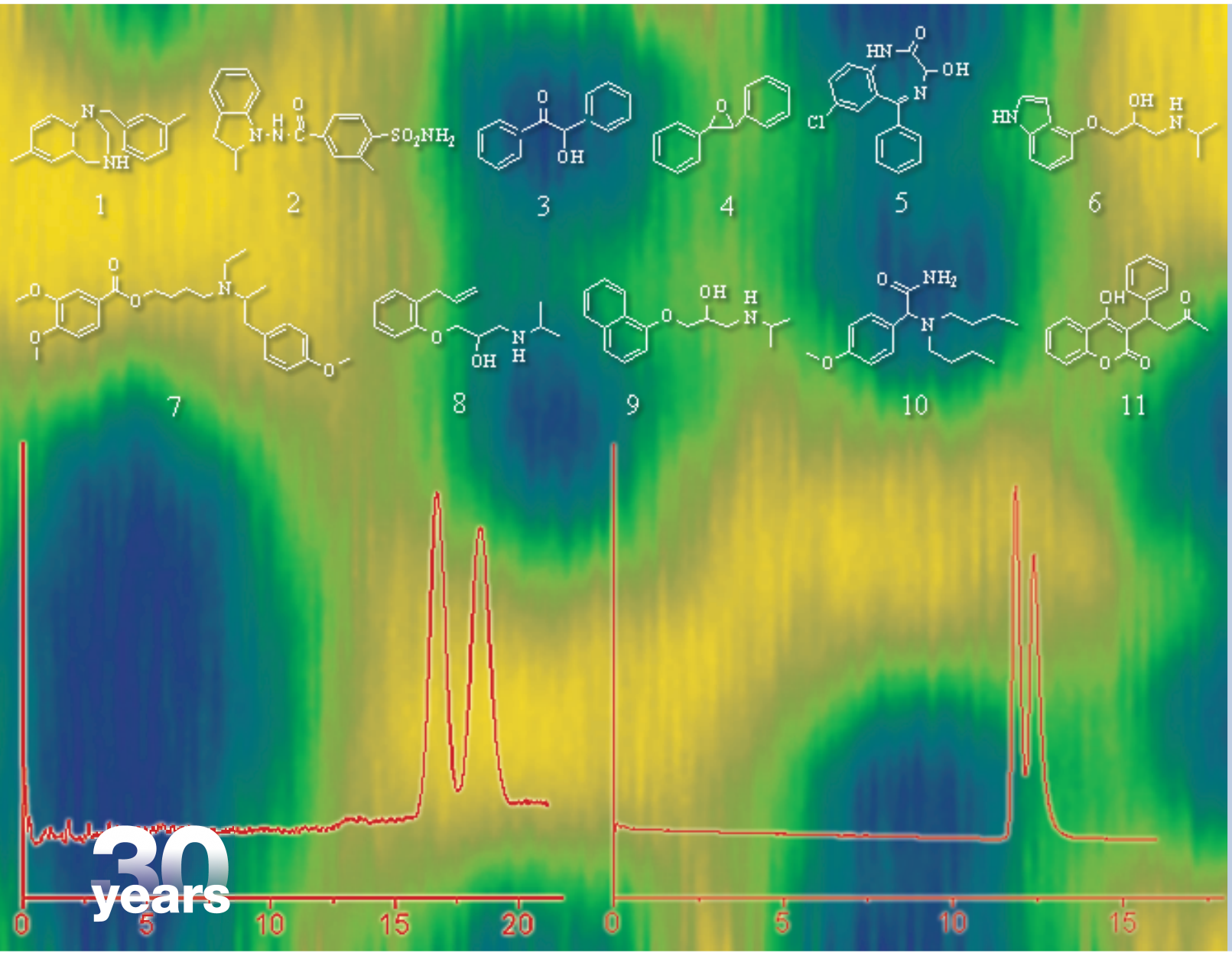

Special Issue

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CE and CEC Innovations

Editor: Ziad El Rassi

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Received April 30, 2009
Revised August 31, 2009
Accepted August 31, 2009

Research Article

Estimating the integrity of aged DNA samples by CE

A CE/UV method was developed to separate by a micellar system the four DNA bases and other five purinic–pyrimidinic compounds (5-methyl-cytosine, uracil, xanthyne, hypoxanthyne and 5-bromo-uracil). Selectivity, precision, accuracy and sensitivity were assessed and proved to be suitable for the analysis of the primary structure of DNA. This method was adopted to study 16 aged samples including two Egyptian mummies, formaldehyde-fixed paraffin-embedded tissues and other forensic specimens. Lower relative values of the four canonical unmodified DNA bases (uDNAb) and more complex pherograms were found in the aged samples when compared with the modern controls. The results of the CE analysis, together with those obtained by classical molecular methods (agarose gel electrophoresis, DN*ase I* and RN*ase A* assays, and UV spectrophotometry), were finally evaluated for assessing the reliability of STR typing. Since samples with low uDNAb showed no amplification or unreliable STR profiles, the uDNAb value is discussed as a further quality criterion in the evaluation of the genetic data obtained from aged samples.

Keywords:
Ancient DNA / CE / DNA damage / PCR fidelity / STR typing
DOI 10.1002/elps.200900283

1 Introduction

Genetic typing is usually performed by PCR, an error-prone *in vitro* DNA amplification technique [1]. PCR has been adopted since the beginning of the 1990s, and it represents a common method to acquire genetic results from a variety of different biological samples. In particular, PCR is the only technique by which genetic data can be gathered from aged biological samples such as museum specimens, tissues archives, and forensic samples [2, 3]. However, PCR fidelity is influenced by several factors (source of the polymerase, buffer composition, primers design, *etc.*) with the amount and the integrity of the genetic template playing the major role [1, 4, 5]. In addition, since a high number of PCR cycles (35–40 cycles) is usually necessary to amplify low amounts of DNA, PCR artefacts and contaminations can sometimes be originated. These undesired amplification products may represent important sources of mistyping potentially giving false profiles, as it is impossible to distinguish them from authentic replicates of the original DNA.

These critical issues and the strong need for authenticity criteria on the reliability of genetic results are well recognised in most biomedical application fields but emphasised especially in ancient DNA (aDNA) studies [2, 3, 6–10].

The investigation of *post mortem* tissues shows that DNA decay spontaneously occurs [11] and it inevitably accumulates [12–17]. As further matter of complication, chemicals used for sample preservation such as formaldehyde, for instance, are well-defined agents that cause DNA modifications [18]. The molecular composition of aged DNA samples has been already studied by different methods, including GC/MS [19] and reversed-phase HPLC/MS [17, 20]. Although the adoption of MS-based techniques provides highly reliable results, they are still not applicable in routine practice of DNA analysis either because of their overall complexity, or due to the limited amounts of genetic material that can be recovered from aged samples.

In the past years, CE has been successfully used to solve crucial concerns in fields such as urinary cancer biomarkers

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Abbreviations: A, adenine; **aDNA,** ancient DNA; **5-Br-U,** 5-bromo-uracil; **C,** cytosine; **FFPE,** formaldehyde-fixed paraffin embedded; **G,** Guanine; **H,** hypoxanthyne; **IPC,** internal positive control; **mAU,** milli-absorbance units; **4MBS,** 4 modified bases solution, working standard solution 500 μmol/L of U, X, H and 5-met-C; **MSM,** master standard mix; **mtDNA,** mitochondrial DNA; **5-met-C,** 5-methyl-cytosine; **MW,** molecular weight; **Na_2EDTA,** EDTA di-Sodium salt; **T,** thymine; **TSS,** Test Standard Solution; **U,** uracil; **uDNAb,** unmodified DNA bases; **UV/RT,** ratio between the total DNA concentration assessed by spectrophotometry at 260 nm and the human DNA amount estimated by Real-Time PCR; **X,** xanthyne

determination [21], DNA adducts analysis [22], and inherited disorders [23–32]. For this reason, we evaluated a possible application in the forensic field, setting up a CE-based method to investigate the integrity of the primary structure of DNA recovered from aged biological samples.

The aim of the present study is to evaluate if the CE analysis of the DNA primary structure, together with other tests routinely applied in forensic DNA investigations, could bring to trustful decisions on the reliability of STR profiles obtained from aged biological samples.

2 Materials and methods

2.1 Chemicals

Cytosine (C), Guanine (G), Thymine (T), Adenine (A), Uracil (U), 5-methyl-cytosine (5-met-C), xanthyne (X), hypoxanthyne (H) and 5-bromo-uracil (5-Br-U) were purchased from Sigma (Sigma-Aldrich, MO, USA) and were of 98% minimum certified purity. Concentrated HCl (37%, w/v), NaOH pearls, sodium tetraborate, sodium phosphate monobasic, SDS, and water were analytical-grade reagents obtained from Fluka (Sigma-Aldrich).

2.2 Standard reference solutions

Aliquots of each pure substance (C, G, T, A, U, 5-met-C, X, H, and 5-Br-U) were dissolved in 1% HCl (v/v) in order to obtain the concentration of 200 μmol/L each (solution named master standard mix (MSM)).

A test standard solution (TSS) at the level of 200 μmol/L of C, G, T, and A was prepared dissolving the corresponding pure substances in 1% HCl (solution named TSS200). Lower level reference solutions were obtained by diluting TSS200 with 1% HCl at 100, 50, 25, and 12 μmol/L (Table 1).

A working standard solution at the level of 500 μmol/L of U, X, H and 5-met-C (4MBS) was prepared in aqueous 1% HCl (v/v) for qualitative analysis of aged samples. All these solutions were stored at –20°C in the dark until use.

2.3 CE/UV apparatus and analytical conditions

A MDQ (Beckman Coulter, CA, USA) apparatus was employed for this work. The CE system was operated at 254 nm detection wavelength, using an uncoated fused silica capillary (length 57 cm; 50 μm internal diameter; window at 50 cm) run at 20 KV for 35 min at 35°C in 0.05 M sodium tetraborate, 0.1 M sodium phosphate monobasic and 0.3 M SDS buffer adjusted to pH 8.4 by the addition of 1N NaOH drops. The buffer was filtered through a 0.2 μm nylon GD/X Syringe Filter (Whatman International, UK) and stored at room temperature until use. Under these working conditions, a current of up to 188 μA is generated. Before each injection, the capillary was washed for 3 min with water, 3 min with 0.1N NaOH and conditioned for 3 min with the running buffer at the pressure of 20.0 psi. All samples were injected at 0.5 psi for 15 s (corresponding to an injection volume of 18 nL). Raw data of the analyses were processed by the Beckman Karat (version 5.0) software after 8 min of run.

The precision of the present procedure was investigated by injecting the scalar dilutions of TSS200 reported above. The choice of 5-Br-U as internal standard allowed identifying all injection problems potentially originated by pressure failure or capillary obstruction.

The sensitivity of the method (S/N 3:1) was tested up to 1.5 μmol/L by analysing scalar dilutions of TSS200.

In order to monitor the analytical performance, at the beginning of every session MSM was tested. Analytical precision of peak areas and migration times under these working conditions were verified also by testing both TSS200 and TSS25 on within and between replicate run analyses.

2.4 Samples

Samples were selected based on the availability of enough specimens to ensure that all experiments could be repeated at least in duplicate tests. The 16 samples analysed in this study are listed in Table 2 together with the biological sources (tissues) and their estimated dating. In particular, seven forensic samples (*f*-samples, aged from 0.1 to 20 years) and five formaldehyde-fixed paraffin-embedded (FFPE) tissues (aged from 5 to 12 years) were considered. Out of the forensic samples, two were previously studied by HPLC/MS (*f*-PI1 and *f*-PI3) [17]. Muscle specimens collected from two Egyptian mummies (samples MD and MF, dated about 2100 and 2600 years, respectively) were chosen as ancient samples. In addition, DNA was extracted from buffy coat prepared from a large blood donation unsuitable for clinical purposes (sample C13) stored at room temperature for 2 wk; the same DNA was then stored at +4°C with prolonged expositions at room temperature for more than one year in order to obtain a naturally spoiled sample (sample C13X). Ten fresh blood samples collected from different healthy volunteers were used as control.

2.5 Sample processing

2.5.1 DNA extraction

Before DNA extraction, FFPE samples were sliced into 20 μm sections and treated twice with xylene for 5 min followed by ethanol washing from 100 to 70%. The bone samples were incubated in 0.5 M Na_2EDTA for 18 h at room temperature while the specimens from Egyptian mummies were re-hydrated in water for 18 h at room temperature.

DNA was recovered from all samples using a standard organic extraction protocol based on phenol/chloroform purification and ethanol precipitation [33]. All these DNA

Table 1. Calibration data, linearity range, sensitivity, accuracy, and intra-day precision of the CE method

TSS	Cytosine		Guanine		Thymine		Adenine	
Concentration	$C_{measured} \pm SD$	REC $\pm$ SD	$C_{measured} \pm SD$	REC $\pm$ SD	$C_{measured} \pm SD$	REC $\pm$ SD	$C_{measured} \pm SD$	$C_{measured} \pm SD$
(μmol/L)	(μmol/L)	(%)	(μmol/L)	(%)	(μmol/L)	(%)	(μmol/L)	(%)
200	200 $\pm$ 4	100 $\pm$ 2	200 $\pm$ 3	100 $\pm$ 2	199 $\pm$ 5	100 $\pm$ 3	200 $\pm$ 4	100 $\pm$ 2
100	99.6 $\pm$ 0.4	99.6 $\pm$ 0.4	100 $\pm$ 4	100 $\pm$ 4	101 $\pm$ 2	101 $\pm$ 2	100 $\pm$ 3	100 $\pm$ 3
50	49.9 $\pm$ 0.1	99.7 $\pm$ 0.2	51 $\pm$ 3	102 $\pm$ 5	51 $\pm$ 3	102 $\pm$ 5	50 $\pm$ 3	100 $\pm$ 6
25	24.6 $\pm$ 0.3	98 $\pm$ 1	24 $\pm$ 2	95 $\pm$ 10	26 $\pm$ 3	105 $\pm$ 10	24 $\pm$ 2	97 $\pm$ 8
12	12.7 $\pm$ 0.2	106 $\pm$ 2	12 $\pm$ 2	101 $\pm$ 21	10 $\pm$ 3	82 $\pm$ 22	12 $\pm$ 2	104 $\pm$ 18
LRE	$y = 63x+8$		$y = 145x–228$		$y = 75x–145$		$y = 157x–300$	
r^2	>0.999		>0.999		>0.999		>0.999	
LOQ (S/N $>$ 10)	12		12		12		12	
LOD (S/N $>$ 3)	2.0		1.8		2.9		1.6	

For every single analyte are shown the concentrations measured together with their standard deviation (Cmeasured $\pm$ SD) and the corresponding recoveries and relative standard deviation percent (REC $\pm$ SD). LRE: linear regression equation calculated on peak areas (y) *versus* analyte concentrations (*x*) in the range of 200–12 μmol/L. r^2: square value of the correlation coefficient obtained from the linear correlation of peak areas and analyte concentrations. All data presented are calculated as mean of at least three measurements.

samples were further purified through Ultracel 100 K Amicon Ultra columns (Millipore, MA; USA). All the recommended precautions to avoid contamination were followed. Blank controls were introduced alongside the purification steps. No ultrafiltration has been carried out on the fresh control blood samples and on sample C13.

The molecular weight (MW) of the extracted DNAs was visualised on a 1.2% agarose gel by electrophoresis by comparison with two MW markers (λ/*Hind*III and Easy Ladder purchased from Bioline, UK). In order to show that the UV fluorescence of the samples could be ascribed to the presence of nucleic acids, a small aliquot of each extract was incubated with 1U of *DNaseI* for 1 h at 37°C. After incubation, the *DNase*-treated samples were run through agarose gels side by side to the untreated samples. Positive and negative controls were performed. In addition, some aged sample was incubated with R*Nase A* (10 μg for each sample) for 1 h at 37°C and run in agarose gels.

2.5.2 DNA quantification

One microliter aliquots from each DNA sample were quantified using a NanoDrop ND-1000 (Thermo Fisher Scientific, MA, USA) spectrophotometer. Absorbance at OD_{260} and OD_{280} was determined for each sample at least in triplicate measurements.

In order to assess the amount of human amplifiable DNA, 40–50 ng of each sample were used to carry out a Real-Time PCR quantitative assay using the Quantifiler™ Human DNA Quantification kit (Applied Biosystems, Foster City, CA, USA) [http://www3.appliedbiosystems.com/cms/groups/applied_markets_support/documents/generaldocuments/cms_041395.pdf]. This kit allows the simultaneous amplification of a 62 bp human-specific sequence within the telomerase reverse transcriptase gene (hTERT) and a synthetic internal positive control (IPC) probe (IPC of amplification) included to determine the presence of inhibitors in the amplification reactions. The Real-Time assays were performed on an ABI PRISM® 7700 instrument (Applied Biosystems) in two replicate tests. Negative PCR controls were included together with blank extraction controls.

2.5.3 Hydrolysis

DNA can be hydrolysed either by enzymatic or heat-acid procedures [34]. Aged DNA samples can be scarcely sensitive to the enzymatic activity of the DN*aseI* [33, 35], therefore a heat-acid protocol has to be adopted for these samples. Although this protocol itself causes a further, albeit low, DNA damage [34], all the experiments were performed in formic acid (90%, v/v) at 170°C for 30 min as lower temperatures (140, 110, and 80°C) gave unsatisfactory yields of hydrolysis especially concerning the pyrimidimic compounds [36, 37].

To establish the amount of DNA needed for CE analysis, scalar amounts of human DNA ranging from 10 μg to 50 ng were analysed. Therefore, it was chosen to hydrolyse DNA amounts ranging from 2 to 5 μg for each sample in a final volume of 500–800 μL 90% (v/v) formic acid in screw-capped glass tubes. Blank hydrolysis controls were introduced in every batch of reactions. Negative extraction controls were also tested. After this treatment, the samples were lyophilised by a Concentrator 5301 (Eppendorf International, Germany) at 60°C.

2.6 CE/UV analysis of hydrolysed DNA samples

After hydrolysis and lyophilisation, the DNA samples were resuspended in 25 μL of 1% HCl with 200 μmol/L of dissolved 5-Br-U and analysed as reported above in replicate

Table 2. Samples studied and analytical results

Sample	Source	Age (years)	MW (Kb)	DN*asel*	260/280	UV/RT	[C+G]/4B	uDNAb	STR analysis
2A	FFPE	12	< 0.4	–*	1.89	–	0.752	0.672	–
3B	FFPE	12	< 0.4	–*	2.14	–	0.685	0.667	–
4B	FFPE	12	< 0.4	–*	2.07	–	0.707	0.686	–
MD	muscle	2100	< 0.2	+/–*	0.53	–	0.658	0.329	–
MF	muscle	2600	< 0.2	+/–*	1.12	–	0.626	0.631	–
f-PI1	muscle	0.1	< 23	+	1.64	1.2	0.623	0.876	*f.p.*
f-PI3	muscle	0.1	< 23	+	1.79	1.7	0.442	0.986	*f.p.*
f-E3	ligament	1	< 2	+/--*	1.47	6985	0.557	0.862	*n.p.*
f-E4	liver	0.1	< 2	+	1.54	4.8	0.442	0.959	*f.p.*
f-E5	bone	15	< 9	+	2.02	364	0.560	0.095	*u.p.*
f-E6	bone	15	< 9	+	2.19	3711	0.561	0.485	*u.p.*
f-E7	bone	20	< 23	+	2.22	3921	0.611	0.261	*u.p.*
E9	FFPE	5	< 2	+/--*	1.42	69.1	0.667	0.813	*p.p.*
E10	FFPE	8	< 6	+/--*	1.40	142.5	0.545	0.821	*n.p.*
C13	buffy coat	0.05	< 1.5	+	1.64	3.2	0.402	0.989	*f.p.*
C13X	DNA	1.6	< 0.4	+	1.59	72.3	0.405	0.974	*u.p*
CTRL	blood	–	>23	+					
Mean ± SD					1.84 ± 0.02	1.2 ± 0.8	0.40 ± 0.01	0.995 ± 0.007	*f.p.*
Max					1.86	3.2	0.41	0.999	
Min					1.80	0.5	0.38	0.975	
N					10	10	10	10	

Sample: laboratory coding of the samples analysed in this study; samples 3B and 4B originate from the same donor; MD and MF: specimens collected from two Egyptian mummies; *f*-samples: forensic samples
Ten human control samples (CTRL) from fresh blood were pooled together to provide tentative reference values (mean ± SD, maximum and minimum values observed, and number of samples tested, respectively). Source: biological source from which the DNA was recovered; 2A: spleen; 3B: thyroid; 4B, E9 and E10: liver. Sample C13: buffy-coat prepared from a large blood donation stored at +20°C for 2 weeks; sample C13X originates from the DNA preparation of C13 (see Section 2 for details). Age: presumptive age of the samples (in years) before DNA extraction. *FFPE tissues:* dating based on the time elapsed from the surgical/autopsy collection; *MD, MF*: dating obtained by archaeological methods; *f-PI1, f-PI3, f-E3, f-E4*: dating based on medico–legal parameters; *f-E5, f-E6, f-E7*: dating based on burial time before exhumation. MW: maximum MW of the samples as assessed by 1.2% agarose gel electrophoresis. DN*ase* I: sensitivity of the sample to the enzyme estimated as residual EtBr fluorescence;+: full digestion;+/– partial digestion;+/- - minimal residual after digestion; –: no digestion; * samples treated with RN*ase* A. 260/280: OD_{260}/OD_{280} ratio as assessed by NanoDrop analysis. UV/RT: ratio between the total DNA concentration assessed by spectrophotometry at 260 nm and the human DNA amount estimated by Real-Time PCR.; –: no human DNA identified. [C+G]/4B: ratio between the [C+G]/[C+G+T+A] concentrations as assessed by CE. uDNAb: relative area of the four canonical DNA bases as determined by CE. STR analysis: *f.p.*: full profile; *p.p.*: partial profile; *u.p.* unreliable profile; *n.p.*: no profile (see Section 3 for details).

runs. To determine the qualitative assessment of the analytical recovery, the aged samples were spiked with 1/10 (v/v) of 4MBS in six selected cases.

The amount of unmodified DNA bases (uDNAb) was calculated by the formula: (aT+aC+aG+aA)/[aTot–a(5–Br–U)], where a is the peak area of each substance as measured by the Karat software and aTot is the total area obtained by the sum of the areas of all peaks detected at 254 nm after 8 min of run. The method accuracy was evaluated calculating the analytical recoveries of the released bases obtained after the hydrolysis of known amounts of modern control DNAs.

2.7 STR typing

The STR analysis was carried on eleven samples wherein the presence of amplifiable human DNA was assessed in the reliability range of Quantifiler™ Human DNA Quantification kit.

After quantification by Real-Time PCR, 500 pg of each DNA sample were initially amplified using the AmpF/STR Identifiler™ kit (Applied Biosystems) for 30 cycles of PCR, in duplicate experiments. A second round of STR typing PCRs was set up on amounts of DNA increased to 2 ng, only for those samples with sufficient genetic material. Samples E3, E6, E7 resulted to contain DNA amounts lower than 500 pg, therefore were split in two aliquots each one analysed in a different replicate amplification.

Positive controls (500 pg of high MW DNA) were simultaneously amplified as well as negative controls (no DNA). One microliter of each amplified sample was run on an ABI Prism 310 Genetic analyser (Applied Biosystems), according to the manufacturer's specifications. Electropherograms were analysed using a Gene Mapper ID v3.2 (Applied Biosystems) software for allelic sizing. The cut-off for an unambiguous allele call was set up to >100 relative fluorescence units.

3 Results and discussion

3.1 CE

Previous data [3, 19, 20, 38] described the presence of many different molecular products of bases modification in DNA damaged samples. A number of transformations have been shown to lead from the four bases to products of known and still unknown molecular structure [38]. The characterisation of these compounds and the study of their chemical origin have been undertaken since years, but it is still the subject of thorough investigations [39, 40]. For these reasons, it is compelling to set up efficient methods to separate simultaneously as many bases analogues as possible in a single run when analysing potentially damaged samples. Since preliminary experiments following published protocols [23–32, 36] showed unsatisfactory separation of the nine purinic–pyrimidinic compounds of the MSM, we optimised the present protocol which is based upon micellar conditions at high concentration of SDS (0.3 M) at the pH of 8.4 while the other pH conditions tested (8.1 and 9.0) were discarded because of poor selectivity; the analytes at pH 8.1 eluted too early while at pH 9.0 they eluted at higher retention times but were poorly resolved. In addition, since MDQ apparatus allows currents up to 300 μA, a voltage of 20 KV assured the base-to-base separation of the sharp peaks shown in Fig. 1. Moreover, as shown in Fig. 1, the CE analysis of the blank extraction controls evidences the presence of the internal standard (5-Br-U) and of a system peak at about 9.6–10.4 min, always preceding U (if present) of about 0.3 min.

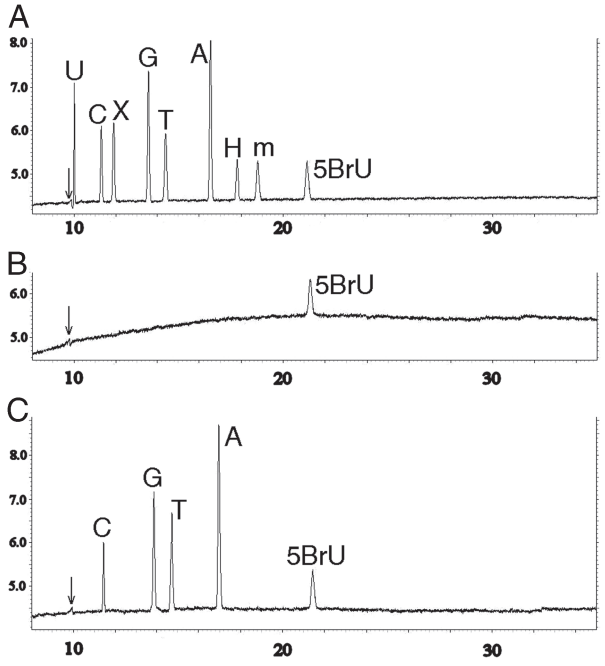

Figure 1. CE analysis of reference solutions MSM (A), blank extraction control (B), and control DNA (C). Peak *m* is 5-methyl-Cytosine. X-axes: retention times in minutes. Y-axes: milli-absorbance units (mAU). A system peak is shown by the arrows.

The order of the elution of the analytes is reported in Table 3 and shows the precision of the qualitative analysis obtained in 44 runs of a single prolonged session. The intermediate precision (RSD%) of inter-session experiments increased up to 10–15% because of factors such as the change of the capillary with time (and the need to equilibrate it by several runs) and the stability of the buffer (no more than 2 wk at room temperature).

The precision of the measurement of the concentrations of the TSS in intra day test is reported in Table 1 while CV % was <10 for each analyte in inter days tests. Replicate analyses of the TSS had to be performed during each working session, since the absorbance of the DNA bases could be influenced by several factors such as the analytical wavelength, the running buffer composition, and the clearness of the capillary window.

The LOQ was assessed at 12 μmol/L for every base. The LODs are reported in Table 1 and are in agreement with those previously described in other biomedical studies, which used CE for the analysis of DNA-related products [23–32, 36].

The complete hydrolysis of 1 μg of human DNA is calculated to give, in a final working volume of 25 μL, a solution where C, G, A, and T are about 24.7, 24.7, 35.5, and 35.5 μmol/L, respectively. Therefore, the LOQ of 12 μmol/L is suitable for determining the levels of bases expected from DNA samples having a nominal concentration of less than 1 μg. The presented protocol, however, tested for sensitivity as shown in Table 1, allowed the detection of the bases after the hydrolysis of about 100 ng of human control DNAs. Nevertheless, since modified products of the DNA bases may occur at levels much lower than those of the parent compounds, and thus possibly could not be detectable, higher amounts of genetic material (2–5 μg) were employed in this study.

As mentioned above, the human DNA is characterised by a content of about 41% in terms of C and G (or C+G%) [41]. In the present study the C+G% found in fresh control samples (Table 2) was in excellent agreement with this reference data. This observation evidences that the proposed procedure is indeed a trustful mean for evaluating the primary structure composition of DNAs.

This was further confirmed by the estimation of the method accuracy after hydrolysing known amounts of control DNAs. Based on the calculations detailed above, the recovery of DNA resulted 0.79 ± 0.07 ($N = 10$) with uDNAb > 0.99 (see trace c of Fig. 1).

Since reliable results were obtained from the controls, CE analysis was applied to the 16 aged samples. All these, except C13 and the derived sample C13X, exhibited more complex pherograms (see Figs. 2 and 3 compared with Fig. 1) providing with several data on their chemical composition (see Tables 2 and 4). All these 14 samples, in fact, exhibited lower relative amounts of the four canonical DNA bases (uDNAb) and several additional peaks among which U, deriving from the deamination of C, was identified in eleven DNA samples. H and X were found in only two

Table 3. Analytes elution order, average retention times and corresponding system precision, as evaluated by the repeatability of measurements in a single prolonged analytical session

Analyte	rt ± SD (min)	Repeatability (RSD(%))	Number of measurements
Uracil	9.7 ± 0.1	1	22
Cytosine	11.0 ± 0.3	3	44
Xanthine	11.5 ± 0.2	1	13
Guanine	13.3 ± 0.5	4	44
Thymine	14.0 ± 0.6	4	44
Adenine	16.2 ± 0.8	5	44
Hypoxanthine	17.0 ± 0.5	3	13
5-Methyl-cytosine	18.1 ± 0.4	2	8
5-Bromo-uracil	21 ± 1	5	19

rt, average retention time calculated based on measurements on both reference solutions and authentic samples.

and, respectively, five samples providing further evidences that these intermediates are instable along the catabolic pathway of the purine compounds [3]. Regrettably, the base analogues commercially available as reference compounds are only a few, and no identification of the remaining peaks is possible. However, they are most likely products of the bases modification.

Samples *f*-PI1 and *f*-PI3, previously analysed by HPLC/MS [33], showed a good correspondence in terms of uDNAb (at that time, the values were 0.930 for *f*-PI1 and 0.907 for *f*-PI3). This observation should be considered as a further demonstration of the present procedure accuracy as two different techniques like HPLC/MS and CE/UV provided consistent data on the same samples analysed in different laboratories and times.

CE, however, as well as GC/MS and HPLC/MS, provides information on the *N*-rings still present along the DNA backbones recovered by ethanol precipitation. All these chemical methods are unable to distinguish human DNA from exogenous (bacterial/fungal) contamination and to detect the presence of apurinic–apyrimidinic sites which are well-documented changes even *in vivo* physiological conditions [11]. This last observation implies that the actual damage in aged samples could be higher than what assessed by the methods mentioned above.

3.2 Identification and quantification of the nucleic acids

Degraded UV-fluorescent material was recovered from each of the 16 aged samples as visualised after agarose gels electrophoresis in presence of Ethidium Bromide (see Fig. 4). The degree of degradation varied among samples. Six out of them showed a substantial reduction of the MW below 400 bp (see Table 2). In order to exclude that the observed fluorescence could arise from unknown contaminants, aliquots of each sample were incubated in presence of the endonuclease *DNase I,* as already described [33]. This enzyme completely digested the UV-fluorescent material recovered from eight samples while a consistent reduction of the signal was observed for five samples (see Table 2). It is noteworthy that three samples from FFPE tissues (2A, 3B and 4B) were resistant to the *DNase I* treatment (see Fig. 4) but were completely digested when *RNAse A* was added. Similarly, the addition of *RNase A* eliminated the residual

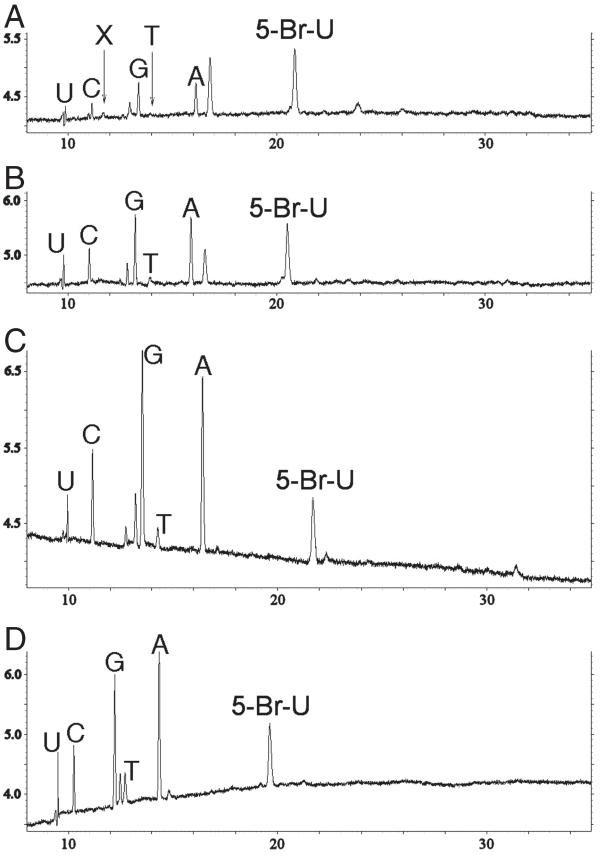

Figure 2. CE analysis of four aged samples analysed in this study. (A): sample MD; (B): sample MF; (C): sample *f*-E3; (D): sample E9. These samples always provided more complex pherograms as compared with the controls (see trace c of Fig. 1 and text). *U* is a common finding in the samples (see Table 4). 5-Br-U: internal standard. X-axes: retention times in minutes. Y-axes: mAU.

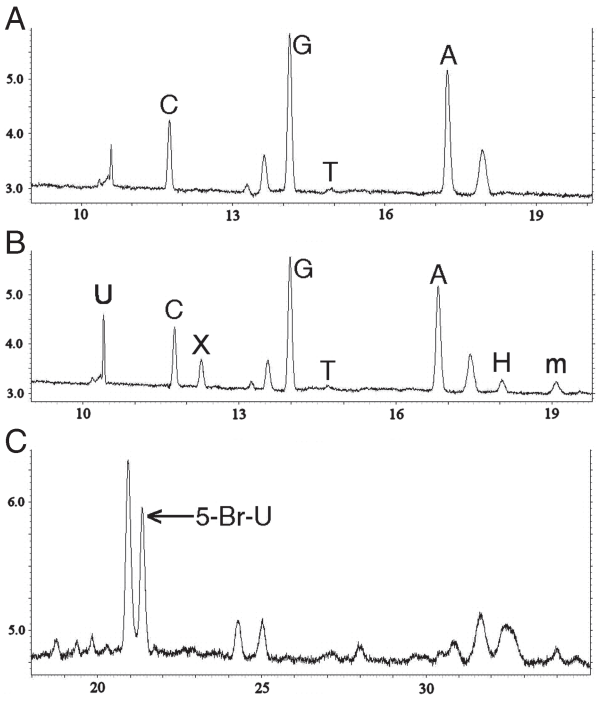

Figure 3. CE analysis of sample 4B from 8 to 20 min (A). Trace b shows the same sample spiked with 1/10 (v/v) of 4MBS. The addition of this standard confirmed only the presence of *U*. m: 5-methyl-Cytosine. Trace c shows the analysis of sample *f*-E7 from 18 to 35 min. Several unknown late eluting substances are observed. X-axes: retention times in minutes. Y-axes: mAU.

amount of fluorescence showed by the five samples only partially digested by *DNAseI*. This fluorescent *RNAse A* sensitive material could correspond to RNA or, more likely, reflect a damaged structure of the DNA [42]. These results showed that the UV-fluorescent material recovered from the aged samples were indeed nucleic acids.

All the aged samples were thus quantified by spectrophotometry at OD_{260}, which provided values ranging from 36 ng/μL to 3.7 μg/μL. As reported in Table 2, the OD_{260}/OD_{280} ratios were always anomalous if compared with the controls. Since the spectrophotometric determination does not discriminate the human DNA from exogenous contaminants such as bacterial and fungal DNA, 40–50 ng of each extract were then quantified by a Real-Time PCR procedure. This assay, targeting a 62 bp human DNA single copy sequence, identified human DNA in 11 out of 16 samples with a concentration varying from very low (*f*-E3: 7 pg/μL) to quite relevant amounts (*f*-PI1: 2.5 μg/μL). No co-extracted inhibitors of the amplification process were identified in the aged samples as assessed by the cycle threshold values (<29 cycles) of the IPC probe. No amplification signals were observed in blank extraction control samples.

The ratios between the concentration assessed by spectrophotometry at OD_{260} and the human amount estimated by Real-Time PCR for each sample are shown in Table 2 where they are listed in the UV/RT column. UV/RT ratios within the range of the controls (minimum 0.5, maximum 3.2, mean ± SD 1.2 ± 08) were found only for samples *f*-PI1,

Table 4. Results of the CE analysis

Sample	C	G	T	A	OIA
2A	0.118	0.313	0.005	0.236	U
3B	0.072	0.305	< LOQ	0.290	U
4B	0.105	0.294	0.007	0.280	U
MD	0.045	0.130	< LOQ	0.154	U, X
MF	0.094	0.233	0.043	0.261	U
f-PI1	0.127	0.326	0.062	0.361	U, X
f-PI3	0.105	0.258	0.190	0.433	–
f-E3	0.115	0.279	0.088	0.380	U
f-E4	0.105	0.249	0.197	0.408	H, X
f-E5	0.021	0.023	0.022	0.029	–
f-E6	0.091	0.145	0.102	0.147	U
f-E7	0.058	0.081	0.049	0.073	U, X
E9	0.119	0.333	0.035	0.326	U
E10	0.131	0.284	0.099	0.438	U, H, X
C13	0.107	0.217	0.229	0.436	–
C13X	0.111	0.203	0.223	0.437	–
CTRL					–
Mean ± SD	0.114 ± 0.006	0.25 ± 0.02	0.21 ± 0.01	0.43 ± 0.01	
Max	0.124	0.27	0.23	0.45	
Min	0.105	0.19	0.19	0.42	
N	10	10	10	10	

Relative area of the each DNA base (C, G, T and A) found in the samples, calculated as ratio between the area of the individual compound to the sum of the areas of the peaks of the four bases

Anomalous values with lower relative amounts of T are always present in aged samples. OIA: other identified analytes. CRTL: control samples.

f-PI3 and C13. In the remaining extracts, the human DNA fraction resulted to be only a minor component. For example, the human fraction in sample *f*-E3 was very limited, as assessed by the high value of the ratio (*i.e.* 6985). No templates were found in the two Egyptian mummies and in three FFPE samples (2A, 3B and 4B).

Lack of sensitivity to PCR amplification can be due to several reasons with the availability of preserved templates remaining the first [1, 2]. In the case of aged/forensic samples, exogenous (bacterial/fungal) contamination can be a relevant source of UV-absorbing substances. However, it has been already shown by hybridisation techniques [33] that such contamination provides only a partial explanation for the bulks of genetic material. In our samples, even though contamination could not be excluded at all, both fragmentation and DNA chemical decay provide more reasonable explanations for no or low sensitivity to the Real-Time PCR quantitative assay.

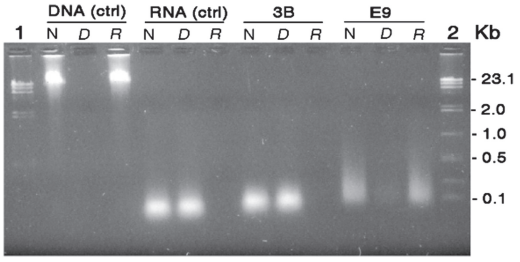

Figure 4. Agarose gel electrophoresis of samples 3B and E9. *N*: native sample; *D*: digestion with *DNase I*; *R*: digestion with *RNase A*. DNA (ctrl): high MW DNA from control sample; RNA (ctrl): 16S-3S rRNA; 1: λ/*Hind*III; 2: λ/*Hind*III plus Easy ladder. Sample 3B shows no sensitivity to *DNase I* while it is fully digested by *RNase A*. Sample E9 shows a minimal residual fluorescence only after incubation with DN*asel* (see also Table 2).

3.3 STR typing

STR typing in replicate tests was performed on the 11 samples wherein the presence of human amplifiable DNA was determined by the Real-Time PCR assay.

Full profiles, defined by the complete and reliable typing of the 16 genetic markers included in the kit, were obtained analysing samples *f*-PI1, *f*-PI3, *f*-E4 and C13 while no profile was the result of sample *f*-E3 and E10 typing. A partial profile, characterised by the amplification of only few STRs in the low MW range (D8S1179, D3S1358, D19D433, vWA, D5S818 and amelogenin loci), was seen amplifying sample E9. Finally, the

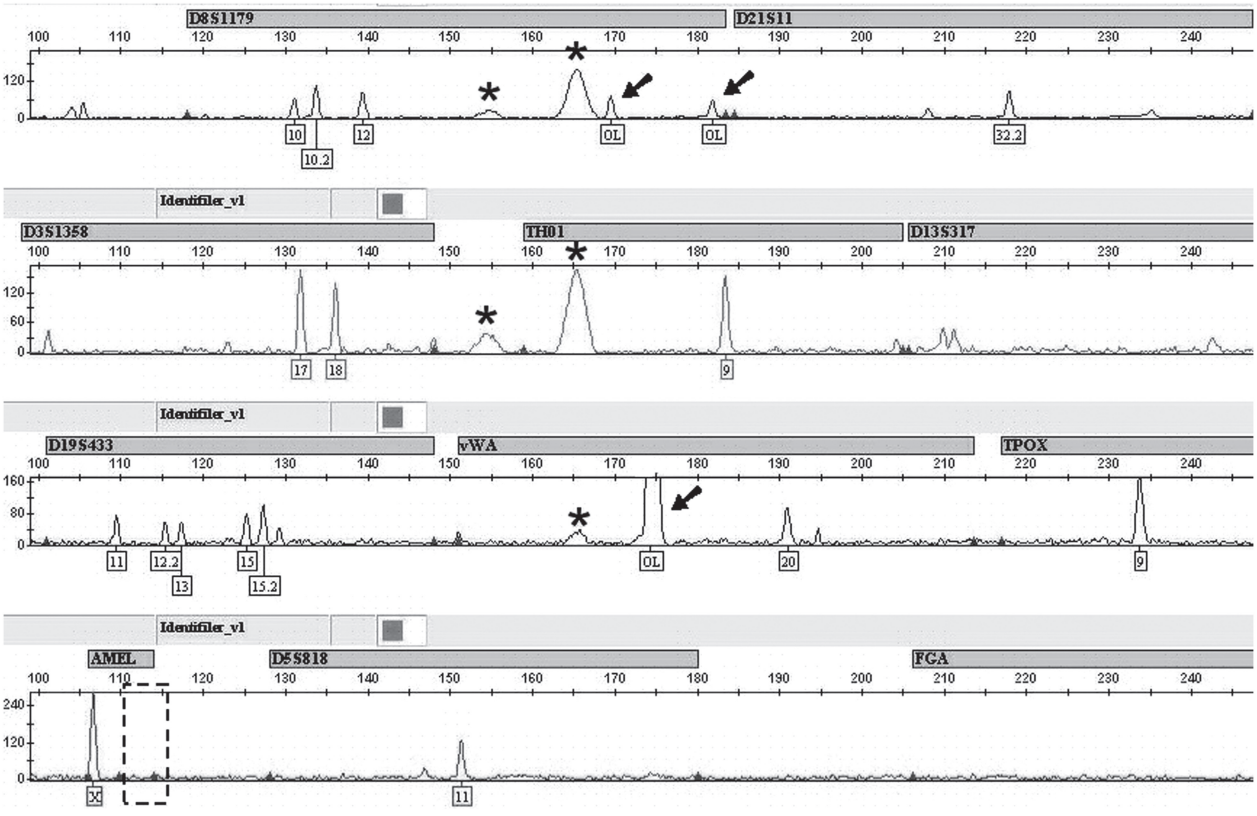

Figure 5. STR profile obtained from sample *f*-E7 (20 years old femur of a male individual). Several PCR artefacts peculiar of low copy number DNA can be scored: additional allelic peaks and off-ladder (OL) alleles (pointed by the arrows) were seen for D8S1179, D19S433 and vWA loci. The genetic typing of the sample provided only an AMEL (amelogenin) X-specific peak. This finding could be explained by a complete drop-out of the amelogenin Y peak (shown by the dotted box). An asterisk shows artificial peaks known as ''dye blobs''. The cut-off for an unambiguous allelic call was set up to > 100 relative fluorescence units (*Y* axes).

STR typing of samples *f*-E3, *f*-E6, *f*-E7 and C13X showed low-quality profiles typical of the LCN-DNA condition (low copy number) [43] with extra alleles, allele imbalance and allelic drop-out as shown in Fig. 5. These PCR artefacts affected the reproducibility of the genetic results whose quality was not improved even by increasing the DNA amount of template. The inability of assessing a consensus genetic profile from the replicate amplifications of these samples led us to define these results as unreliable profiles.

A common characteristic of all the samples we investigated is to exhibit chemical–physical features pointing to a damage of the DNA primary structure as compared with the controls. However, none of the data on the chemical–physical features of the nucleic acids (MW, sensitivity to nucleases, $OD_{260/280}$ and CE data) investigated here seems to be related to the presumptive dating of the samples. This provides further evidences supporting data, which indicate that environmental conditions play a major role in assuring DNA stability [35, 44].

The five samples which gave reliable STR profiles (full or partial) showed uDNAb values higher than 0.81. The four full profiles originated from four 0.05–0.1-year-old samples showing UV/RT ratios close to the range of the controls and uDNAb values higher than 0.876. The partial profile was obtained from a 5-year-old sample with uDNAb of 0.813 and UV/RT ratio about 60 times higher than the controls. All the remaining samples were 1–20 years old and gave no or unreliable STR profiles.

None of the features studied in Table 2 provides *a priori*, by itself and alone, indication on the successful and reliable outcome of the STR typing. In addition, neither the combined evaluation of two or more of these features helps in this task. For example, sample C13X gave unreliable profiles in spite of unquestionable values provided by CE analysis ([C+G]/4B: 0.405; uDNAb: 0.974) and a UV/RT ratio about 60-fold the reference value of the controls. A possible explanation for the STR failure of this sample can be likely found in its heavy fragmentation (MW < 0.4 kb). Similarly, sample E3 had a relatively high uDNAb value (0.862) and a MW < 2.0 kb but no STR profiles were ever gathered. In this case, the extremely low fraction of PCR-sensitive human DNA (as shown by a UV/RT ratio of 6985) could explain the STR typing failure.

On the contrary, a very clear finding is that samples having uDNAb values lower than 0.81 never showed full or partial profiles. This is in agreement with other data showing that the integrity of the template plays a fundamental role in the PCR success [1, 2, 4, 5] and supports a correlation between an unsuccessful and/or unreliable outcome of the STR typing and low uDNAb values.

4 Concluding remarks

This study reports on the use of CE as tool for studying the primary structure of the genetic material recovered from aged specimens. The data obtained by PCR, together with the results achieved from classical molecular assays such as agarose gel electrophoresis, enzymatic digestions and UV spectrophotometry, were evaluated in their consistency with the results of CE.

It is well known that the successful and reliable outcome of DNA amplification depends basically on the availability of complete templates, while severe molecular damage of the primary structure of the DNA can lead both to PCR failure and artefacts [1, 4, 5, 20, 45]. Moreover, DNA damage inevitably accumulates in *post mortem* tissues and thus the need of authenticity criteria in evaluating the genetic data obtained from aged samples is a relevant concern [3, 44].

The assessment of the amount of canonical DNA bases (uDNAb) still present alongside the DNA chain has been already described by HPLC/MS [17, 20, 45], but the CE method proposed here is simpler and inexpensive, providing reliable indication on the integrity of the basic moieties of the DNA samples.

The presented data suggest that uDNAb value is useful in the critical evaluation of the reliability of STR profiles, and can be considered as further quality criterion of the genetic testing.

First, full STR profiles arose only from samples whose decay was limited as indicated by a high uDNAb value. Secondly, all samples, which never gave genetic results, had low uDNAb values, with an aging ranging from 1 to 2600 years. However, no relation between the aging of the sample and its chemical decay has been found as yet [3, 17, 19].

Sample C13X seems to provide an example of conflicting data because an unreliable genetic profile was found in a rather recent specimen with a high uDNAb value. This could be explained assuming a fragmentation of the sample, as indicated by the low MW of the DNA extracted (< 0.4 kb), which caused the unsuccessful outcome of the PCR analysis owing to the absence of human-sensitive sequences with suitable MW [1, 3]. An additional explanation could be the presence of apurinic–apyrimidinic sites [1, 3] within the molecular frame, which is undetectable by the present method.

Hence, when a full STR profile is obtained from samples having low uDNAb, a recent contamination should be at least taken into account since a successful genetic outcome should not be expected from heavily damaged samples.

In conclusion, even if the number of samples studied here is limited, the uDNAb value measurement can be proposed as a scientific authenticity criterion in the evaluation of genetic data obtained from *post mortem* samples independently from their aging.

The major limit of the CE/UV method described here is the relatively high amount of sample required for one analysis, so that very limited specimens cannot be studied. As a future perspective, a larger number of aged samples will be considered to validate the criterion proposed here also evaluating a complete set of genetic typing including miniSTR, SNPs, mtDNA assays and clone sequencing.

The authors thank Dr. A. Dugulin (Musei Civici di Trieste, Italy), Dr. F. Costantinides and Dr. F. Tomasella (University of Trieste, Italy) for helpful support in sampling the two Egyptian mummies. A special thank to Dr. C. Guarnaccia (ICGEB, Padriciano, Trieste, Italy) for his invaluable support in the CE analysis.

The authors have declared no conflict of interest.

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