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Molecular characterisation of the nucleic acids recovered from aged forensic samples

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Abstract The molecular composition of the genetic substrate recovered from seven aged forensic samples has been extensively investigated. A simple enzymatic test based on *DNAseI* incubation of the extracts showed that the UV-fluorescent material from the forensic specimens is composed of nucleic acids, with the DNA fraction representing at least 90% of the total amount. Since spectrophotometric determinations of the extracts showed unreliable results due to anomalous OD_{260}/OD_{280} ratios, quantification of the nuclease-sensitive genetic material was performed by a slightly modified agarose plate method. The first quantitative data on exogenous contamination in aged forensic samples are provided by slot-blot hybridisation of the extracts to human, bacterial and fungal probes. Only limited amounts of human and contaminant DNA were detected in the samples. The molecular integrity of the primary structure of these aged DNA samples was analysed by reversed-phase HPLC/MS. The data show a good correlation between the degree of chemical damage and the ability to hybridise to molecular probes. The ability to achieve specific genetic profiles was assessed by multiplex PCR amplification of STR loci. Our data show that accurate determination of the molecular composition of the DNA recovered from forensic samples can be extremely useful for a reliable evaluation of the PCR typing results.

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Introduction

PCR analysis of DNA samples recovered from post-mortem tissues is widely used in molecular palaeontology/anthropology and forensic medicine [1, 2, 3, 4]. DNA extracted from such particular specimens has been shown to be damaged, with the amount of template and its chemical integrity playing a major role in the fidelity of the PCR reaction [5].

DNA quantification of the extracts recovered from forensic casework material is needed to optimise the concentration of the DNA to be added to the PCR reactions; however, it is a common experience that spectrophotometric determination of these DNA extracts provides unreliable results, reflected by anomalous OD_{260}/OD_{280} ratios. In addition, the amount of the genetic material is sometimes so low that spectrophotometric determination cannot be performed. For these reasons, ethidium bromide (EtBr) staining and slot-blot hybridisation procedures seem to be more reliable [6, 7, 8]. Recently, even a competitive PCR method was developed for the analysis of complex biological samples [9].

The EtBr staining method is based on the fluorescence emitted by the dye intercalated between neighbouring base-pairs along the DNA chain [10]. This method is quite sensitive but does not identify the origin of the DNA (human, bacterial, fungal etc.) and the different proportions. In addition, it is possible that other substances co-extracted with the DNA, may emit fluorescence in the presence of UV radiation.

The use of human-specific probes in slot-blot hybridisation experiments is a more sensitive and specific approach, which has shown that one of the most typical features of the genetic material of aged forensic samples is the low amount of DNA specifically hybridised to human probes [3, 11, 12, 13, 14, 15, 16, 17]. A generally accepted explanation for this observation is that these DNA

samples may contain large amounts of exogenous contamination arising from co-extracted micro-organisms that colonised the forensic specimens; moreover, it is possible that a proportion of the DNA from forensic samples may be so damaged in its primary structure that it is unable to hybridise to human probes. This occurrence could even make the genetic typing of the sample impossible and/or cause ambiguous results to be obtained. This particular topic is a crucial point for a more complete evaluation of the genetic results in forensic medicine, but so far has not received the attention it deserves. For this reason we investigated the molecular composition of the DNA recovered from seven forensic samples representing a wide variety of biological specimens. Enzymatic tests and slot-blot hybridisation experiments were performed in order to positively demonstrate the presence of DNA and provide quantitative data on the degree of exogenous contamination (bacterial and fungal) in aged forensic samples. A correlation between the molecular damage of the primary structure of the DNA from the forensic samples and the ability to achieve specific genetic profiles was investigated.

Material and methods

Control DNA samples

Control DNA samples were represented by human DNA (cell line K562, Promega Madison, Wis., catalogue number DD2011), *E. coli* DNA (strain B, Sigma, catalogue number D2001) and *S. cerevisiae* DNA (Promega, catalogue number G3101). As prokaryotic controls, DNA from the following bacterial species was analysed: *Borrelia burgdorferi*, *Clamydia psittaci*, *Streptococcus faecalis*, *Streptococcus salivarius*, *Salmonella typhimurium*, *Staphylococcus intestinalis*, *Bacillus subtilis*, *Bacillus anthracis*, *Legionella pneumophila*, *Mycoplasma hominis*, *Thermophilus acidophilus* and *Microcystis aeruginosa*. The fungal controls were represented by the following species: *Candida albicans, Candida glabrata, Aspergillus nidulans, Trichophyton violaceum* and *Actynomyces viscosus* and DNA recovered from the following animals was also analysed: dog, cattle, chicken, salmon, rabbit, horse, pigeon, cow, eel and *Drosophila melanogaster*. DNA from carrots (*Daucus carota*) was employed as plant DNA control.

DNA was extracted from all the samples using the Wizard Genomic DNA purification kit (Promega, Madison, Wis.) following the manufacturer's specifications.

All the control samples were quantified by spectrophotometric determination at OD_{260}/OD_{280}.

Forensic DNA samples

Seven forensic samples previously analysed [18] were selected for this study and are listed in Table 1. DNA was isolated from a 400 cm^2 bloodstain (sample B), from ≈80 mg of tissue (samples D, 578, PI 1 and PI 4) and from ≈1 g of bone marrow (samples PI 3 and 602/P) by overnight extraction at 37°C followed by phenol/chloroform purification as described elsewhere [19]. In order to prevent contamination, DNA extractions were carried out in a UV-radiated working area with operators wearing disposable laboratory coats, sterile surgical masks and gloves. Disposable plastic tubes, tips and autoclaved solutions dedicated to DNA extraction were used and blank controls were always included.

DNA identification of forensic samples

About 1/50 of each forensic sample and 100 ng human control DNA was incubated in a final volume of 20 µl in the presence of 10 U *DNAseI* (Promega) for 6 h at 37°C. After incubation, the samples were run on 1% agarose gels in TBE buffer containing 0.5 µg/ml of EtBr for 45 min at 70 V. The same amount of undigested forensic and control samples was run simultaneously. The gels were exposed to a UV source (302 nm) and digital images were then acquired using a KODAK EDAS 120 system. Since samples B, D and 578 exhibited a residual fluorescence after incubation with *DNAseI*, these samples were additionally digested with 5 U *DNAseI* and 10 U *RNAseI* (Roche). Incubation conditions and gel electrophoresis parameters were as given.

DNA quantification of forensic samples by the agarose plate method

Spectrophotometric determination data, as reported in a previous study [18], are shown in Table 1. The concentration of the forensic aged DNA samples was estimated by a modified agarose plate method [6]. A series of two-fold dilutions of human control DNA in 0.5 µl containing 100–1.56 ng DNA were mixed with an equal

Table 1 The forensic specimens analysed in this study (*DNA source* refers to the biological tissue from which DNA was recovered, *BS* bloodstain, *M* muscle, *SC* subcutaneous tissue, *B* bone marrow. *Age* refers to the presumptive age in months of the samples before extraction, *UV* spectrophotometric OD_{260}/OD_{280} ratios, amount of *h* human, *b* bacterial and *f* fungal DNA in nanograms identified by slot blot hybridisation, data expressed as mean values calculated from densitometric measures obtained from each set of three filters; standard deviations are given in the brackets; *n.d.* not detected,lower limit of sensitivity <390 pg control DNA, *uDNAb* is the relative amount of undamaged DNA bases measured by HPLC analysis, relative standard deviation <0.01%. The value of the human control was assessed by pooling data from 19 DNA samples)

Sample	DNA source	Age	UV[a]	Human (h)	Bacterial (b)	Fungal (f)	Total (h+b+f)	uDNAb[a]
B	BS	150	1.53	n.d.	3.04 (0.82)	n.d.	3.04	0.545
D	M	0.5	1.66	2.34 (0.30)	2.65 (0.28)	n.d.	4.99	0.793
578	SC	0.5	2.12	n.d.	n.d.	n.d.	n.d.	0.657
PI1	M	1	1.86	17.40 (1.11)	n.d.	0.71(0.09)	18.11	0.930
PI3	B	0.3	1.93	21.35 (1.23)	n.d.	0.20 (0.03)	21.55	0.907
PI4	M	0.25	1.93	8.90 (0.335)	n.d.	n.d.	8.90	0.819
602/P	B	12	1.22	n.d.	3.78 (1.03)	2.44 (0.50)	8.22	0.826
K562	–	–	1.79	100 (3.59)	n.d.	≈2	–	0.972

[a]Data from *18*.

volume of 1% low melting point agarose and spotted onto 1% agarose plates containing EtBr (0.5 μg/ml). Each series was replicated 50 times. After 15 min, digital images of the UV-induced fluorescence were then acquired using a KODAK EDAS 120 system and analysed by Quantity One software (BioRad). A good linear regression (r>0.9) was found only in the range 50–12.5 ng control DNA. Two-fold dilution steps of each forensic sample were then compared to the control DNA dilution series in the linear range, and then quantified. All these tests were replicated at least 10 times. The relative standard deviation value (standard deviation/means×100%) calculated for these quantitative determinations was less than 10%.

The forensic DNA samples incubated with *DNAseI* and *DNAseI* plus *RNAseI* were spotted onto agarose plates, following the same procedures.

Slot-blot hybridisation

From each bacterial species, human, *Saccaromyces cerevisiae*, animal and plant control DNA, 100 ng of DNA was incubated in 0.3 N NaOH at 60°C for 15 min in a final volume of 80 μl. After the addition of a volume of 12×SSC, the samples were spotted onto nylon Hybond XL membranes (Amersham) using a slot-blot apparatus (BRL). Similarly, another set of filters was set up by spotting 100 ng DNA of each fungal species and the same amount of human, *E. coli*, animal and plant control DNA.

A serial dilution of the human cell line K562 DNA (100–0.02 ng) and 100 ng of genetic material from each forensic sample were tested using the slot-blot method following the protocol given. A second and third set of filters were set up using serial dilutions (100–0.02 ng) of *E. coli* bacterial control DNA and *S. cerevisiae* fungal control DNA and the same amount (100 ng) of the seven forensic samples. For each set of filters, 100 ng of animal and plant control DNA was always included and set up in triplicate.

A 262-bp-long *Alu* consensus fragment was synthesised in vitro by PCR amplification of a human DNA sample, using the following pair of primers:

- Forward primer: 5'gcctgtaatcccagcacttt3'
- Reverse primer: 5'gagacagggtctcgctctg3'.

The PCR product was purified and used as a human-specific probe by labelling with α^{32}dCTP (Megaprime labelling system, Amersham). Hybridisation was performed at 65°C in SDS-phosphate buffer, the washes were carried out at final stringency conditions of 0.1×SSC at 65°C.

A cDNA probe from *E. coli* 16S-23S rRNA (Roche) was used as bacterial-specific probe [19, 20]. Avian reverse transcriptase (Promega) was employed to synthesise cDNA in the presence of α^{32}dCTP. Hybridisation and washes were performed using the same experimental conditions.

To detect the presence of fungal DNA, a 926-bp PCR fragment from the 18S rDNA sequence of *S. cerevisiae* [19, 21] was used. The PCR product was purified and employed as fungal-specific probe by labelling with α^{32}dCTP. Hybridisation was performed at 65°C in SDS-phosphate buffer and the washes carried out at the final stringency conditions of 0.1×SSC at 67°C.

The multi-locus probe λ33.15 [22] was used to check the presence of hybridisable DNA on the filters. Hybridisation was performed at 58°C in SDS-phosphate buffer and washes carried out at the final stringency conditions of 1×SSC at 58°C.

The filters were exposed to phosphor screens and analysed by densitometry, using a Molecular Dynamics phosphorimager (Molecular Dynamics, Amersham Biosciences, Calif.), and ImageQuant software.

PCR amplification

PCR amplification was set up in a UV-irradiated area following all the precautions to avoid "carry-out" and "carry-over" contamination [23]. From the forensic samples 100 ng of the extracts was amplified by PCR, using the commercial kit AmpF*l*STR Profiler Plus PCR amplification kit (PE Applied Biosystems). This product amplifies in a multiplex PCR reaction the 9 short tandem repeat (STR) loci D3S1358, vWA, FGA, D8S1179, D21S11, D18S51, D5S818, D13S317, D7S820 and the sex determination marker Amelogenin. The samples were amplified through 30 PCR cycles, according to the manufacturer's specifications and 1 μl of each amplified sample was analysed by capillary electrophoresis using an ABI Prism 310 Genetic Analyser (PE Applied Biosystems, Foster City, Calif.); alleles were scored using the Genotyper Software ver 2.1 (PE Applied Biosystems).

Results

Degraded UV-fluorescent material from all the forensic samples analysed was recovered and visualised on agarose gels. Since it is possible that unknown contaminants could contribute to the fluorescence observed in the extracts, aliquots of the forensic samples were incubated in the presence of the endonuclease *DNAseI*. This enzyme completely eliminated the bulk of UV-fluorescent material in samples PI1, PI3, PI4 and 602P (see Fig. 1) but not in samples B, D and 578 where a residual amount of fluorescence (less than 10%) was observed. However, the addition of extra *DNAseI* and the presence of *RNAseI* completely eliminated these residues. The UV-fluorescent material extracted from the forensic specimens was thus positively identified as nucleic acids with the DNA fraction representing at least 90% of the total amount.

DNA determination by UV absorbance showed anomalous OD_{260}/OD_{280} ratios (see Table 1) for most of the ex-

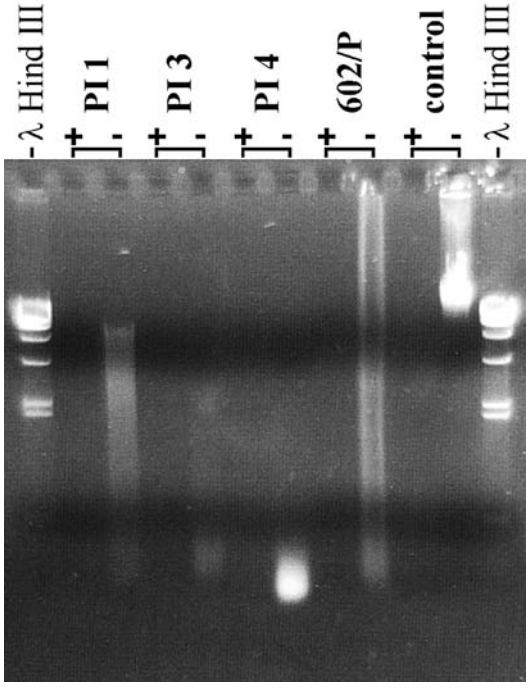

Fig. 1 EtBr stained agarose gel electrophoresis of extracts from four forensic specimens and a high molecular weight control DNA sample, incubated in the presence of *DNAseI* (+) and run together with the same amount of undigested DNA (-). λDNA cleaved with Hind III is the molecular size marker

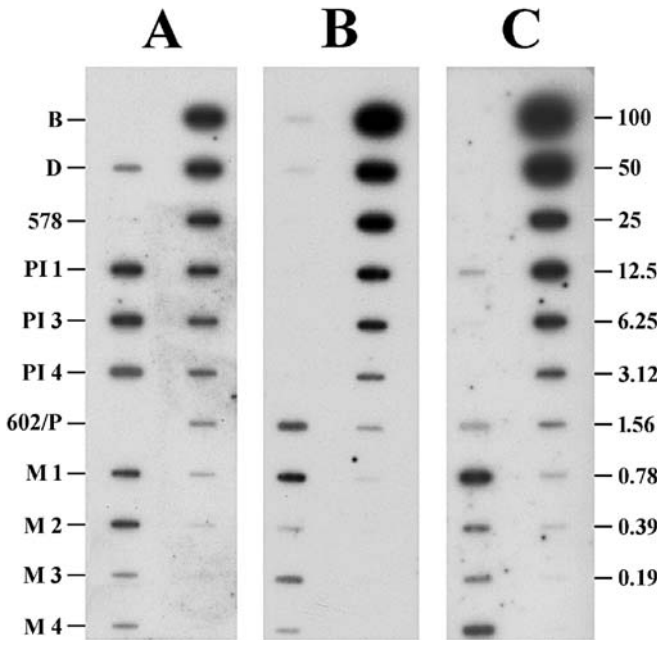

Fig. 2a–c Identification and quantification of the DNA recovered from the forensic specimens. From each sample, 100 ng UV-fluorescent material was spotted in the left side of the filters and hybridised with **A** human *Alu* probe , **B** 16S-23S rRNA bacterial probe and **C** 18S rDNA fungal probe. On the right side of filters **A**, **B** and **C**, serial dilutions (from 100 ng to 0.19 ng) of human (K562 cell line), bacterial (*E. coli* strain CB767) and fungal (*Saccharomyces cerevisiae* strain FY1679/G1307) control DNA samples were slot-blotted and hybridised in parallel with the different probes. *M1–M4* are internal control DNA samples set up by mixing human, bacterial and fungal DNA in the following proportions: *M1* 5 ng human DNA, 5 ng bacterial DNA, 5 ng fungal DNA, *M2* 5 ng human DNA, 1.25 ng bacterial DNA, 1.25 ng fungal DNA, *M3* 1.25 ng human DNA, 5 ng bacterial DNA, 1.25 ng fungal DNA, *M4* 1.25 ng human DNA, 1.25 ng bacterial DNA, 5 ng fungal DNA

tracts and consequently was not considered reliable. The nuclease-sensitive fluorescent material recovered from the forensic samples was therefore quantified by a slightly modified agarose plate method. To prevent anomalous diffusion of the samples onto the agarose plates, we mixed low melting point agarose with the DNA extracts and spotted 1 µl onto the plates. Control DNA dilution series showed that less than 3.12 ng DNA could be detected. In addition, a good linear regression was observed, in the reference samples, in the range 50–12.5 ng DNA (correlation coefficient r>0.9). The forensic samples were thus quantified by comparison with the linear range of dilutions of the control DNA.

The ability of the 16S-23S bacterial cDNA probe to identify prokaryotic DNA was tested by hybridising it to the bacterial species previously selected. All the bacterial species were recognised and no cross-hybridisation was seen with any other control DNA. Similarly, the 18S fungal probe hybridised to all the fungal control DNAs but showed minimal cross-hybridisation (about 2%) with human control DNA while a higher value (up to 5%), was observed with animal and plant control DNA. The lower limit to the sensitivity of the probes was estimated to be 0.39 ng DNA.

From each forensic sample, 100 ng of nuclease-sensitive fluorescent material was spotted onto nylon filters, hybridised to human, bacterial and fungal probes and finally quantified by comparison with known amounts of specific DNA controls as shown in Fig. 2. The relative amounts of human, bacterial and fungal DNA identified in each forensic extract are given in Table 1.

It is noteworthy that the human-specific probe identified only limited amounts of DNA in 100 ng of nuclease-sensitive fluorescent material and in addition, neither bacterial nor fungal contamination seemed to be remarkably

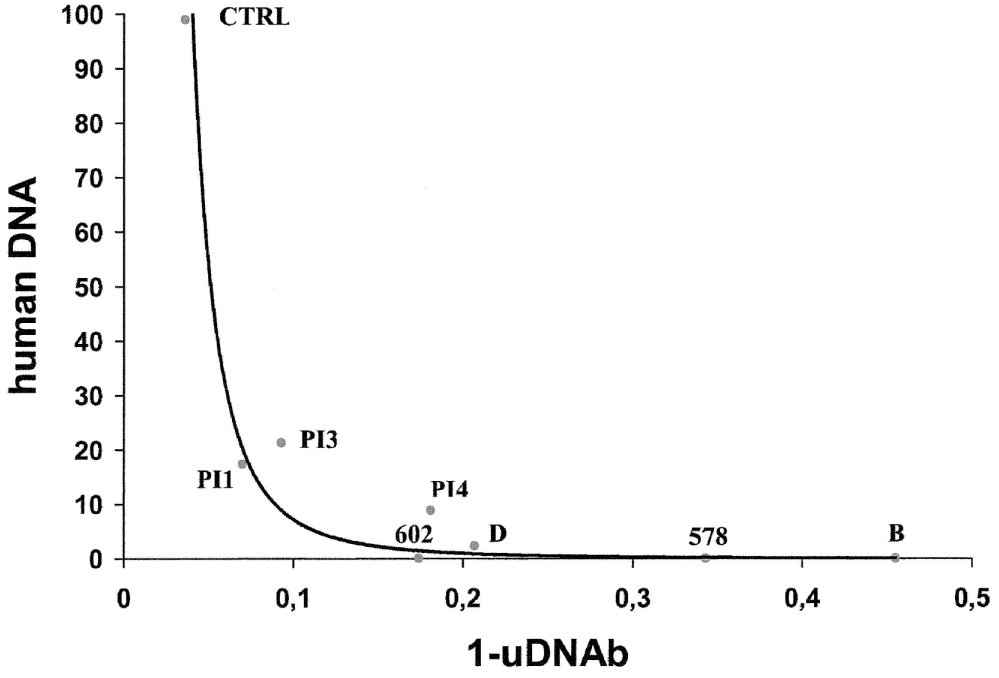

Fig. 3 Correlation between the ability to hybridise to the *Alu* probe and the relative values of uDNAb. *X axis* 1-uDNAb (see Table 1), *Y axis* nanograms of human DNA detected in 100 ng of nuclease sensitive material. No hybridisation signal was obtained in samples B, 578 and 602/P (lower limit of sensitivity <0.39 ng human control DNA)

high. This finding was confirmed when the forensic extracts were tested with the minisatellite probe λ33.15 which did not detect larger amounts of hybridisable DNA (data not shown).

Previous data on reversed-phase HPLC analysis [18, 19] showed that the degree of chemical damage in these aged samples was quite high compared to the controls, as indicated by the lower relative values of undamaged DNA bases (uDNAb) (see Table 1).

The amount of DNA identified with the human-specific probe and the relative amount of uDNAb were then compared in each forensic sample and a good correlation observed (see Fig. 3).

From the forensic samples, 100 ng of the extracts was amplified by PCR using a commercial kit containing 9 STR loci and the sex determination marker Amelogenin. Specific profiles were seen for samples PI1, PI3, PI4 and 602/P while no specific peak for any STR locus was observed for samples B and 578; sample D showed a complete genetic profile only when diluted, probably because of unknown contaminants that inhibited the TaqI polymerase activity. No peak was seen in the blank controls.

Discussion

The molecular characterisation of genetic material recovered from post-mortem specimens is an important, but neglected, subject in forensic medicine. Identification and quantification of human DNA in the extracts from the biological samples and the degree of chemical decay of its primary structure should be considered for a reliable evaluation of the PCR typing results. Previous studies [19, 24] showed a high frequency of sequencing artefacts and unreliable genetic characterisations in aged forensic samples.

Seven forensic samples representing a wide variety of biological specimens were selected for an extensive investigation on the molecular composition of the aged genetic material.

A simple enzymatic test demonstrated that the UV-fluorescent material recovered from the samples was mainly represented by DNA (more that 90%), since it is degraded by DNAse*I*. The residual amount of fluorescent *RNAse*I-sensitive material in samples B, D and 578 could represent RNA or, more likely, the presence of apurinic-apyrimidinic sites along the DNA molecule [25]. No unknown fluorescent substances were identified in the forensic extracts.

Quantification of the nuclease-sensitive genetic material was performed by a slightly modified agarose plate method. The good reliability and reproducibility of the method allowed the DNA concentration (with a relative standard deviation less than 10%) of the aged samples to be assessed. From each forensic sample 100 ng DNA was thus analysed by slot-blot hybridisation using human, bacterial and fungal-specific probes. Our results show that the amount of DNA specifically hybridised to the human-specific probe was very low, varying from 20 to 2 ng, an amount several-fold lower than the amount spotted. No human DNA was detected in samples B, 578 and 602/P (lower sensitivity value <0.39 ng control DNA). In addition, only very limited amounts of bacterial and fungal DNA were detected and quantified in the forensic samples. Our data reveal a significant discrepancy between the amount of genetic material spotted onto filters and the amount of DNA identified by the different probes; sample 578, for example, seemed not to contain DNA as no hybridisation signal was seen for any of the probes used (see Table 1). The most likely explanation for this finding is that the DNA was severely damaged, especially in its human fraction, due to endogenous hydrolytic processes.

Reversed-phased HPLC/MS analysis of the forensic extracts showed, in fact, a wide range of chemical damage to the primary structure of the DNA, with a significant reduction of the four canonical bases; a correlation between the relative amount of undamaged DNA bases and the signal obtained with the human-specific probe can be observed in Fig. 3. It is, however, well known that chemical modifications tend to decrease the ability of the DNA to hybridise to molecular probes [26, 27]. No correlation between the age of the samples and the degree of chemical damage was observed.

PCR analysis of samples PI1, PI3, PI4 and 602/P showed specific genetic profiles for all the STR loci amplified; the high levels of damaged DNA bases found in the extracts could explain the negative results obtained for samples B and 578. It is noteworthy that a specific genetic profile could be obtained from PCR amplification of sample 602/P in spite of the lack of human DNA, as assessed by slot-blot hybridisation.

In conclusion, our data show that none of the methods commonly used allows accurate quantification of the DNA recovered from aged forensic sample. Slot-blot hybridisation analysis of samples B, 578 and 602/P, for example, did not identify human DNA and only small traces of contaminant DNA were seen; nevertheless, both the *DNAseI* assay and reversed-phase HPLC/MS positively confirmed the presence of genetic substrate. Moreover, chemical modification of the primary structure of the DNA seems to play an important role as it reduced the possibility of obtaining genetic profiles. The protocol of molecular analysis we set up could be used in a future study, to correlate DNA damage and PCR fidelity. In fact, PCR artifacts mainly represented by preferential amplification of one allele, extra bands and multiple allelic profiles, are occasionally observed during PCR amplification of aged samples [28, 29, 30]. These artifacts arise from in vitro amplification of low amounts of damaged DNA, and may potentially produce ambiguous genetic results [31, 32]. Any relevant information on the molecular composition of the DNA recovered from forensic samples can thus be extremely useful for the evaluation of the PCR typing results.

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