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RESEARCH ARTICLE

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Genetic individual identification from dried urine spots: A complementary tool to drug monitoring and anti-doping testing

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[Correction added on May 20, 2022, after first online publication: CRUI funding statement has been added.]

Abstract

The collection of liquid biological matrices onto paper cards (dried matrix spots [DMS]) is becoming an alternative sampling strategy. The stability over time of molecules of interest for therapeutic, sport drug monitoring, and forensic toxicology on DMS has been recently investigated representing a reliable alternative to conventional analytical techniques. When a tampering of a urine sample in drug monitoring or doping control cases is suspected, it could be relevant to know whether genetic profiles useful for individual identification could be generated from urine samples spotted onto paper (dried urine spot [DUS]). To understand the influence of sex, storage conditions, and time on the quality and quantity of the DNA, five female and ten male urine samples were dispensed onto Whatman 903 paper and sampled after different storage conditions over time, from 1 to 12 weeks. Direct PCR was performed starting from 2-mm punches collected from each spot amplifying a panel of markers useful for individual identification. The female DUS stored in different conditions produced genetic profiles fully matching the reference samples. The same result was obtained for the male DUS but using urine 30X concentrated by centrifugation instead of the original samples. Our data show that this approach is valid for genetic individual identification of urine samples spotted onto paper cards up to 12 weeks after deposition and could be easily incorporated in anti-doping or drug screening protocols to help on the suspicion of evidence tampering or to solve questions on the reliability of samples collection.

KEYWORDS
drug monitoring, DUS, genetic identification, STR, urine manipulation

1 | INTRODUCTION

In the past 15 years, micro-sampling techniques using dried matrix spots (DMS) have undergone considerable development, with an increasing impact in the field of qualitative and quantitative laboratory analyses.[1–3] The most important, as well as the most studied and widely applied DMS,[4–7] is the dried blood spots (DBS) technique, described for the first time by Guthrie and Susi in 1963 and used in neonatal screening for the diagnosis of congenital phenylketonuria.[8] In the following years, the sampling of biological matrices on paper supports was used on multiple body fluids, such as plasma (DPS),[9,10] urine (DUS),[11,12] saliva,[13] tears,[14] breast milk,[15] synovial fluid,[16] cerebrospinal fluid (CSF),[17] and amniotic fluid.[18] This diffusion is well explained by highlighting the numerous advantages that the use of DMS for laboratory analysis entails: ease of sampling, especially in case of blood; simplicity in transport and storage, being suitable to be kept at room temperature even for long periods of time; improvement in the quality of life of subjects suffering from chronic diseases who must undergo periodical analyses; and suitability to a multitude of different analytical approaches.[1,3]

In the toxicological and genetic fields, the use of DUS, as a method of sample conservation, has become increasingly extensive thanks to the extreme versatility and advantages offered by micro-sampling on filter papers. The fields of application range from quantitative approaches (e.g., drug monitoring)[19,20] to qualitative screening, for example disease diagnosis,[21] anti-doping tests, or search for substances of abuse.[22]

In Italy, according to the current legislation, the search for substances of abuse in the urinary matrix is performed by the laboratories of pharmacotoxicology for clinical and medico-legal purposes and on specific categories of subjects such as drug addicts placed under the care of the Drug Addiction Service (SerT and SerD),[23] citizens followed by the license commissions in case of suspension for driving under the influence of alcohol or drugs,[24] workers who perform tasks involving particular safety issues,[25] athletes who are subject to doping controls,[26] clinical cases from the hospital emergency room or specific departments or, finally, subjects who apply for a firearm license.[27]

Furthermore, the analyses must be carried out in accordance with specific guidelines drawn up and approved by the Italian National Institute of Health (Istituto Superiore di Sanità [ISS])[28] and by the Group of Italian Forensic Toxicologists (Gruppo Tossicologi Forensi Italiani [GTFI]).[29,30]

The current Italian legislation requires that in case of a positive toxicological test result, the subject can ask for a counter-analysis on the same sample within 10 days, which must be performed within the following 180 days. In addition, the urine sample must be stored in the laboratory for a period of 3 months. The counter-analyses are usually requested because an analytical error made by the laboratory is suspected, requiring that the analyses have to be carried out in a different laboratory. Sometimes, in a limited number of cases, an accidental or fraudulent swap of biological samples can explain an unexpected result.

To date, few scientific studies have examined the possibility of obtaining genetic profiles from liquid urine mainly to determine the authenticity of the samples in doping control cases[31–34] and an even more limited number of papers reports on genetic profiles from urine samples stored on filter paper supports.[35,36] The aim of this work was therefore to evaluate the possibility of using urine samples, deposited on Whatman 903 filter cards (the same used by toxicology laboratories and cheaper than FTA filter cards), as a viable, stable over the time and therefore useful for the counter-analysis and economically convenient source of DNA for personal identification purposes. In addition, the feasibility of obtaining genetic profiles of sufficient quality by direct PCR was investigated, thus avoiding the time-consuming DNA extraction phase. The effect of the storage of the urine samples at +4°C for 5 days on the quality of the genetic profiles was evaluated, as well.

2 | EXPERIMENTAL

2.1 | Ethics statement

Fifteen subjects were recruited inside the Department of Public Health, Experimental and Forensic Medicine of the University of Pavia and signed the informed consent in agreement with the approval of the Ethical Committee of the A.U-O of Trieste (Italy). All samples were immediately anonymized.

2.2 | Samples

Urine samples of the first daily micturition and buccal swabs (reference samples) were collected from 10 male and 5 female healthy individuals. The swabs were stored at −20°C until DNA extraction. Two sets of urine samples were prepared. The first one (Set 1) consisted of urine samples, as collected the day of the micturition; the second one (Set 2) was represented by the same urine samples stored at +4°C for 5 days in a fridge. These two sets reproduced the two extremes of the possible preservation conditions before urine samples are submitted to the laboratories of pharmacotoxicology for the analytical procedures. Indeed, some samples are submitted the same day of the micturition to the laboratory, whereas others remain stored at +4°C for up to 5 days. Some sample of the second set showed urinary sediments precipitated after storage at +4°C. These samples were briefly resuspended by vortexing and then heated at 37°C until complete dissolution of the sediment.

From each set, three different samplings were set up: (A) 50 µl of the urine samples were dispensed in Eppendorf tubes for DNA extraction and quantification; (B) 50 µl of aliquots were spotted onto Whatman 903 filter papers (Sigma-Aldrich, USA, WHA10531018) and allowed to air dry overnight; (C) 1.5 ml of aliquots of the urine samples were dispensed in Eppendorf tubes and centrifuged at 10,000*g* for 10 min, in an Eppendorf centrifuge. The supernatant was discarded leaving a volume of approximately 50 µl. The pellet was then

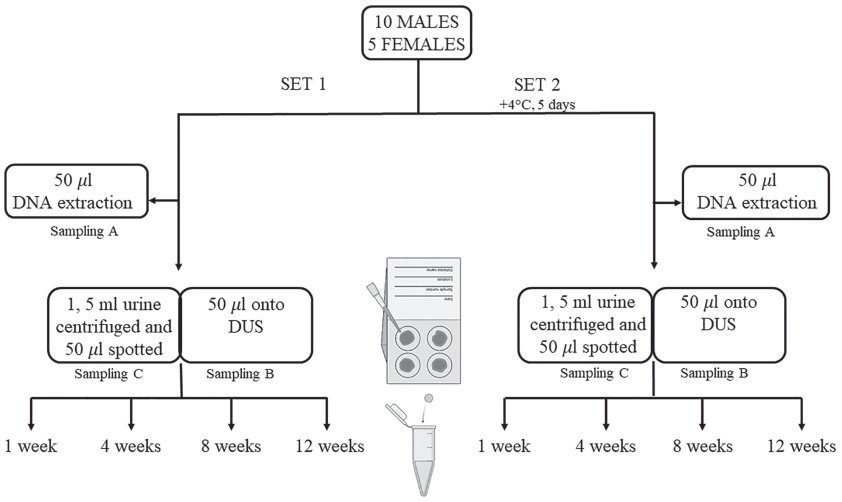

FIGURE 1 Flowchart describing the design of the experiments

resuspended and the sample spotted onto Whatman 903 filter papers. These samples represented approximately a 30X enrichment of the urinary cellular content. The Whatman 903 filter papers were then stored in the dark at room temperature. The flowchart describing the design of the experiments is shown in Figure 1.

From the two sampling sets (B) spotted onto Whatman filter papers, 2-mm punches were collected along the concentric circle passing through the points corresponding to the half radius of the circular spots, using a Harris Uni-Core cutting device (GE Healthcare Life Sciences, Whatman) following a scheduled timeline corresponding to 1, 4, 8, and finally 12 weeks. The device was cleaned in between each sampling. According to the outcome of the genetic testing, 2-mm punches were collected from the two sampling sets (C) of 30X enriched urine samples, following the same approach. The punches were used for direct PCR amplification of autosomal STR markers, as described below.

2.3 | DNA extraction

2.3.1 | Urine specimens

DNA was extracted from 50 µl of urine samples (sampling A) collected the day of the micturition (Set 1) and after storage at +4°C for 5 days (Set 2), in order to quantify the DNA. The DNA was extracted from the urine samples using the DNA IQ kit (Promega, USA), following the manufacturer's instructions. Briefly, two volumes of Lysis buffer were added to the urine samples and 7 µl of magnetic beads were dispensed; after three washes, the DNA was recovered in 50 µl of Elution buffer. Blank controls were introduced alongside the purification steps.

2.3.2 | Buccal swabs

The DNA was extracted from the swabs collected from the participating subjects using the QIAamp DNA Mini kit (Qiagen, Germany) following the manufacturer's recommendations. The DNA was eluted in 100 µl.

2.4 | DNA quantification

Two-microliter aliquots from each extracted DNA sample were quantified by Real-Time PCR using the Quantifiler Duo DNA Quantification kit (Thermo Fisher Scientific, USA) in duplicate assays, following the manufacturer's recommendations, on a 7500 Real-Time PCR System (Thermo Fisher Scientific, USA). Calibration was performed in duplicate using DNA standards at different concentrations (from 23 pg/µl to 50 ng/µl). Positive controls represented by commercially available DNAs with certified concentration (*2800M* Control DNA - cat. number D 7101, Promega, USA; AmpFℓSTR™ DNA Control 007 - cat. Number 100028107, Applied Biosystems, USA) were used. Blank controls of the DNA extraction procedure and of the qPCR assay (no template controls) were included in the experiments.

2.5 | DNA amplification—STR analysis

The urine samples spotted onto Whatman 903 were characterized in direct multiplex PCR amplifying the 16 autosomal STR markers, plus Amelogenin, included in the commercial kit *PowerPlex ESI 17 Fast* (Promega, USA). The 2-mm punches were placed in 0.2-ml PCR tubes and covered with 15 µl of PCR mix composed of 3-µl Promega ESI Fast Mastermix 5X, 1.5-µl Promega ESI Fast primer set solution 10X and 10.5-µl sterile H_20. Direct PCR is a technique to amplify genetic targets directly from biological samples omitting the DNA extraction/quantification steps.

Two hundred and fifty picograms of DNA extracted from the reference samples (buccal swabs) was amplified for the same set of markers, to obtain a reference genetic profile for the comparisons with the ones obtained from the urine spots.

PCR amplification was performed on an Eppendorf Mastercycler Nexus (Eppendorf, Germany) for 30 cycles, according to the *PowerPlex ESI Fast* amplification manual.

Positive (250-pg DNA of the 2800M control DNA, cell line) and negative controls (containing no DNA) were always included in each round of amplifications.

One microliter of each amplified PCR product was separated through capillary electrophoresis on an ABI-PRISM 310 (Thermo Fisher Scientific, USA) sequencer and the genotypes were analyzed with the software GeneMapper ver. 3.2.1 (Thermo Fisher Scientific, USA). The analytical threshold was set to 50 rfu.

2.6 | DNA data analysis and interpretation

The genetic profiles obtained from the urine spots were compared with those obtained from the corresponding reference samples (buccal swabs) to verify the concordance of the genotypes and if the profiles were complete (16 STR markers plus Amelogenin) or partial, with loss of genetic information (*locus dropouts* [LDO]), especially for the high molecular weight markers. In addition, PCR amplification artifacts potentially affecting the quality of the profiles, such as *allelic dropouts* (ADO), with loss of one allele of a heterozygous genotype, *allelic imbalance* of heterozygous genotypes with peak height ratio (PHR) values above 60%, and additional extra alleles that cannot attributed to the reference samples (*allelic dropins* [ADI]) were identified and scored in the urine spots.

2.7 | DNA profile quality score

To describe the quality of the genetic profiles obtained from the urine samples spotted onto the Whatman papers, two parameters were considered.

The first one is the number of markers matching those of the corresponding reference samples. A full 17 loci profile (16 autosomal STRs plus Amelogenin) was considered the best quality result. The number of locus dropouts (LDO) originating partial profiles was recorded as well as the presence of amplification artifacts such as allelic dropouts (ADO), allelic dropins (ADI), and allelic imbalances (PHR). All these artifacts affect the quality of the genetic profiles as they decrease the power of discrimination and consequently the random match probability (RMP), which is the probability that two randomly selected individuals have identical genotypes by chance.[37] To calculate this value, the allelic frequencies of the Italian population published by the ISFG Italian Speaking Group GeFI were considered.[38] The reciprocal of the RMP is the likelihood ratio (LR) that represents how many times is more likely the evidence if it belongs to the reference sample than if it comes from an unrelated person. To check the impact of the artifacts on the LR and on the power of discrimination of the profiles, a normalized percentage decrease of the LR was calculated for each spot assigning 100% value to the LR calculated for the complete profile of each single sample.

The second parameter is an index of the DNA degradation/fragmentation calculated considering the allelic peak heights of the genotypes. To this aim, we used the regression coefficient generated by the software Euroformix,[39,40] which compared the average fragment lengths versus the sum of the peak heights at each observed locus. Peak heights were then converted into natural logarithm and the regression equation provided a degradation slope varying from 0 (completely degraded DNA) to 1 (high molecular weight DNA). Ten positive control DNA profiles (2800M cell line) were checked to calculate a reference degradation slope for a high molecular weight DNA profile.

2.8 | Statistical analysis of the data

Quantitative variables were described as with means and standard deviations. Degradation slopes were analyzed by means of a repeated measures analysis of variance (ANOVA) between-storage conditions (Sets 1 and 2), within the different timeframes of the experiment (from 1 to 12 weeks) and considering the storage-timeframe interaction (i.e., differential temporal trends between groups). The Huyn–Feldt correction of the repeated measures ANOVA's *F* test was adopted. Post hoc Mann–Whitney *U* tests were used to compare degradation slopes by treatment at each time. Interpretation was graphically confirmed.

3 | RESULTS AND DISCUSSION

3.1 | Urine samples DNA quantification

DNA concentration in human urine is known to be variable as it can be affected by many factors among which physiological/pathological conditions, sex, hydration status, time of the day, and lifestyle.[41,42] The amount of DNA recovered from the 50-μl aliquots of urine samples belonging to the two sets was determined using the commercial kit Quantifiler Duo, in duplicate amplifications. The results of the molecular quantifications, according to the autosomal probe, are shown in Table 1. The DNA amount in female samples collected the day of the micturition (Set 1) is larger than the corresponding set in males, confirming the same results described in other studies.[41–43] This finding was explained by anatomical/physiological differences between sexes.[42,43] A 20-fold variability in the DNA concentration was observed for the female samples (from 42 to 919 pg/μl) with a median value of 109 pg/μl, whereas most of the male samples (7 out of 10) provided DNA concentration values for both duplicate amplifications below the lowest point of the quantification curve of the assay (23 pg/μl). For the remaining three male urine samples, a 20-fold DNA amount variation (from 23 to 480 pg/μl) was recorded. The amount of DNA extracted from the female urine samples stored at +4°C for 5 days (Set 2) was significantly lower, varying from 36 to 759 pg/μl, with a median value of 81 pg/μl. The DNA amounts of the two sets of female and male samples showing DNA amounts above the lowest limit of the quantification curve (23 pg/μl) were then compared using the Wilcoxon signed rank test, which highlighted a statistically significant difference ($p = 0.04$), thus confirming a DNA amount reduction for the samples stored for 5 days at +4°C.

TABLE 1 Mean values (pg/μl) of the urine samples DNA quantifications

Samples	Set 1 (fresh)	Set 2 (5 days at 4°C)
F1	263	221
	min = 233; max = 294	min = 192; max = 249
F2	56	41
	min = 50; max = 62	min = 30; max = 52
F3	109	81
	min = 107; max = 111	min = 76; max 87
F4	42	36
	min = 38; max = 46	min = 27; max = 45
F5	919	759
	min = 899; max = 939	min = 748; max = 770
M1	*5*	*1*
M2	*4*	*undetermined*
M3	*5*	*5*
M4	480	436
	min = 445; max = 515	min = 410; max = 461
M5	*10*	24
		min = 21; max = 26
M6	*undetermined*	*5*
M7	151	142
	min = 127; max = 174	min = 134; max = 150
M8	23	24
	min = 14; max = 32	min = 19; max = 29
M9	*4*	*2*
M10	*10*	*undetermined*

Note: M and F: male and female samples, respectively. Set 1: urine samples collected and extracted the day of the micturition. Set 2: urine samples extracted after a 5-day storage at +4°C. Limit of detection (LOD) range = from 50 ng/μl to 23 pg/μl. Min and max values of the quantifications in duplicate are reported if at least one of the two values was in the range of the LOD.

3.2 | Genetic typing, dried urine spots, and female samples

3.2.1 | Set 1—Sampling B

The molecular analysis of the DUS originated from the female urine samples belonging to Set 1 provided complete genetic profiles up to 12 weeks after deposition onto Whatman 903. The LR values ranged from 10^{+21} to 10^{+25}. Each genetic profile was compared with the corresponding reference sample originated from buccal swabs showing a perfect match. Only few allelic imbalances (from 1 to 4) were recorded at different times for four out of five samples (see Figure 2) with the unbalanced heterozygous genotypes, which however perfectly fitted the heterozygous genotypes of the reference samples. No allelic or locus dropouts or allelic dropins were ever found comparing the DUS profiles to the reference samples. Some of the genetic profiles showed off-scale peaks due to signal saturation and were diluted in order to be visualized within the limit of linearity (LOL) of the automatic DNA sequencer detector.

3.2.2 | Set 2—Sampling B

Three out of five DUS samples belonging to Set 2 (F1–F3) showed the same trend of Set 1; complete 17 markers good quality profiles perfectly matching the corresponding reference samples were scored. Two DUS samples (F4 and F5) showed single allelic dropouts after 8 and/or 12 weeks (see Figure 2) with a minimal LR relative percentage loss ranging from 2.5% to 5% of the full profile. Two thirds of the genetic profiles originated from Set 2 DUS exhibited from 1 to 5 allelic imbalances. As for Set 1 samples, all the unbalanced heterozygous genotypes presented both alleles as matching the corresponding reference sample genotypes.

In conclusion, the loss of genetic informativeness of the female profiles for individual identification was very limited, for both sets, corresponding to at most 5% of the LR of a female complete profile. For this reason, there was no need to use any of the 30X concentrated DUS set up for both sets of female urine samples.

3.3 | Genetic typing, dried urine spots, and male samples

3.3.1 | Set 1—Samplings B and C

After 1 week, the punches originated from samples M1, M2, M4, M8, and M10 generated complete profiles with a number of loci, varying from 1 to 7, affected by allelic imbalances but with alleles always matching the ones of the reference samples (see Figure 2). Only a single locus dropout was seen for sample M7, with a minimal loss of genetic informativeness corresponding to 5% of the full LR.

These results were obtained, especially for samples M1, M2, and M10, despite the limited amount of DNA scored by the molecular quantification, which provided values far below the lowest quantification point of the calibration curve (see Table 1). This finding could be explained on the basis of a lower sensitivity of the Real-Time PCR assay compared with the one of the recently developed and very efficient STR multiplex typing kits[44,45] or on the basis of inefficient DNA extraction effects, with loss of DNA, more remarkable for the male urine samples characterized by a lower amount of genomic DNA compared with the female ones.

The punches collected from the urine spots of samples M1 and M4 showed complete profiles after 12 weeks with only a single allelic dropout (M1 week 8) producing a 10% reduction of the LR. At most, two allelic imbalances were seen through the weeks for these spots, with alleles always corresponding to the ones of the reference samples.

The genetic profiles of the remaining DUS (M3, M5, M6, and M9) showed, after 1 week, at least two amplification artifacts belonging to the category of locus or allelic dropouts (LDO or ADO) or allelic

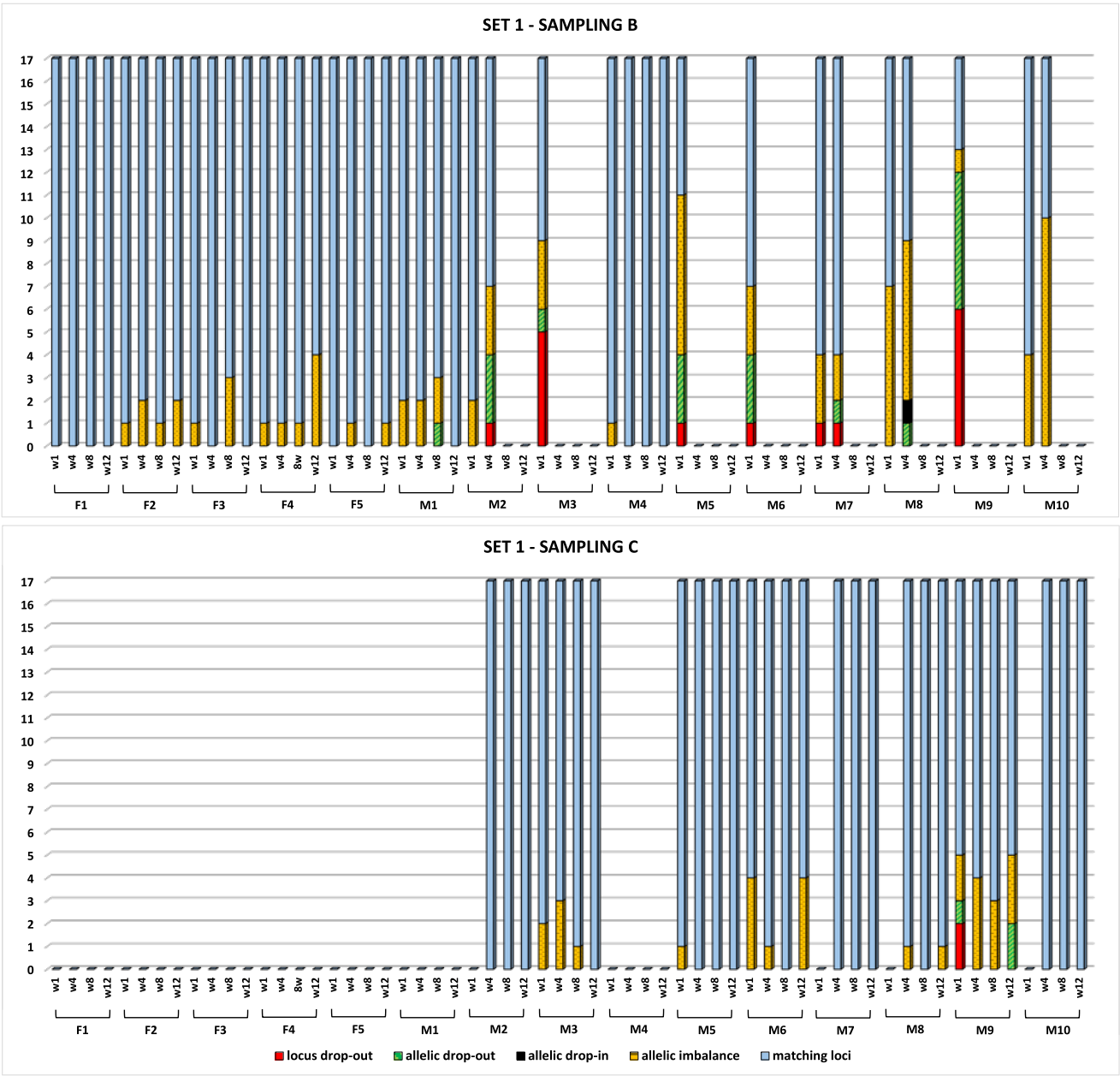

FIGURE 2 Quality of the genetic profiles obtained from the analysis of Set 1 DUS samples, according to the number of markers giving genotypes matching the ones of the reference samples and to the amplification artifacts identified in the profiles (locus dropouts [LDO]; allelic dropouts [ADO]; allelic dropins [ADI]; allelic imbalances) (for each category, see the figure legend below the bar chart). Sampling B: DUS spotted from urine samples, as collected the day of the micturition. Sampling C: 30X concentrated DUS spotted from urine samples, as collected the day of the micturition. F1–F5: female urine spots from 1 to 12 weeks; M1–M10: male urine spots from 1 to 12 weeks. *Y* axis: number of markers composing the genetic profiles (16 autosomal STR and Amelogenin) [Colour figure can be viewed at wileyonlinelibrary.com]

dropins (ADI), influencing the discrimination power of the profiles, or a substantial instability of the profiles attested by more than 50% of the markers affected by allelic imbalances (see Figure 2). These conditions of instability of the genetic profiles led us to perform the genetic amplification of the spotted urine samples using the 30X concentrated DUS to improve the amount of amplifiable DNA, thus counteracting the reduction of the LRs (from 30% to 73% for the four above mentioned samples) recorded for the urine samples spotted as such.

The 30X concentrated DUS of samples M3, M5, and M6 provided complete genetic profiles from 1 to 12 weeks showing only from 1 to 4 allelic imbalances with genotypes, which however perfectly matched the ones of the reference samples.

The genetic analysis of sample M9 30X DUS highlighted a fluctuating trend in the percentage reduction of the LR overtime; after 1 week, two LDO and one ADO were scored in the genetic profile with a 13% loss of the LR, which however did not substantially impact

on the genetic informativeness of the profile for individual identification (LR 10^{+18}). The DUS stored respectively for 4 and 8 weeks displayed full profiles with unbalanced alleles corresponding to those peculiar of the reference samples while, after 12 weeks, two ADO were detected with a relative loss of the LR corresponding to 9% (see Figure 2).

This trend focused on the possibility of variations in the quality of the genetic profiles originated by the position where the punches were collected over the surface of the DUS. Even if we were careful in collecting the punches along the same concentric circles of the spots, in our experience even slight shifts toward the inner or outer part of the DUS could originate genetic profiles with a different quality. Our data showed that, in general, better profiles could be achieved in the center of the DUS where it is more likely that most of the cells remained entangled in the paper texture when spotted, whereas a fewer number of cells are apparently dragged to the outer part of the DUS.

After 4 weeks, the genetic profiles obtained from the urine spots of samples M2, M7, M8, and M10 showed a number of amplification

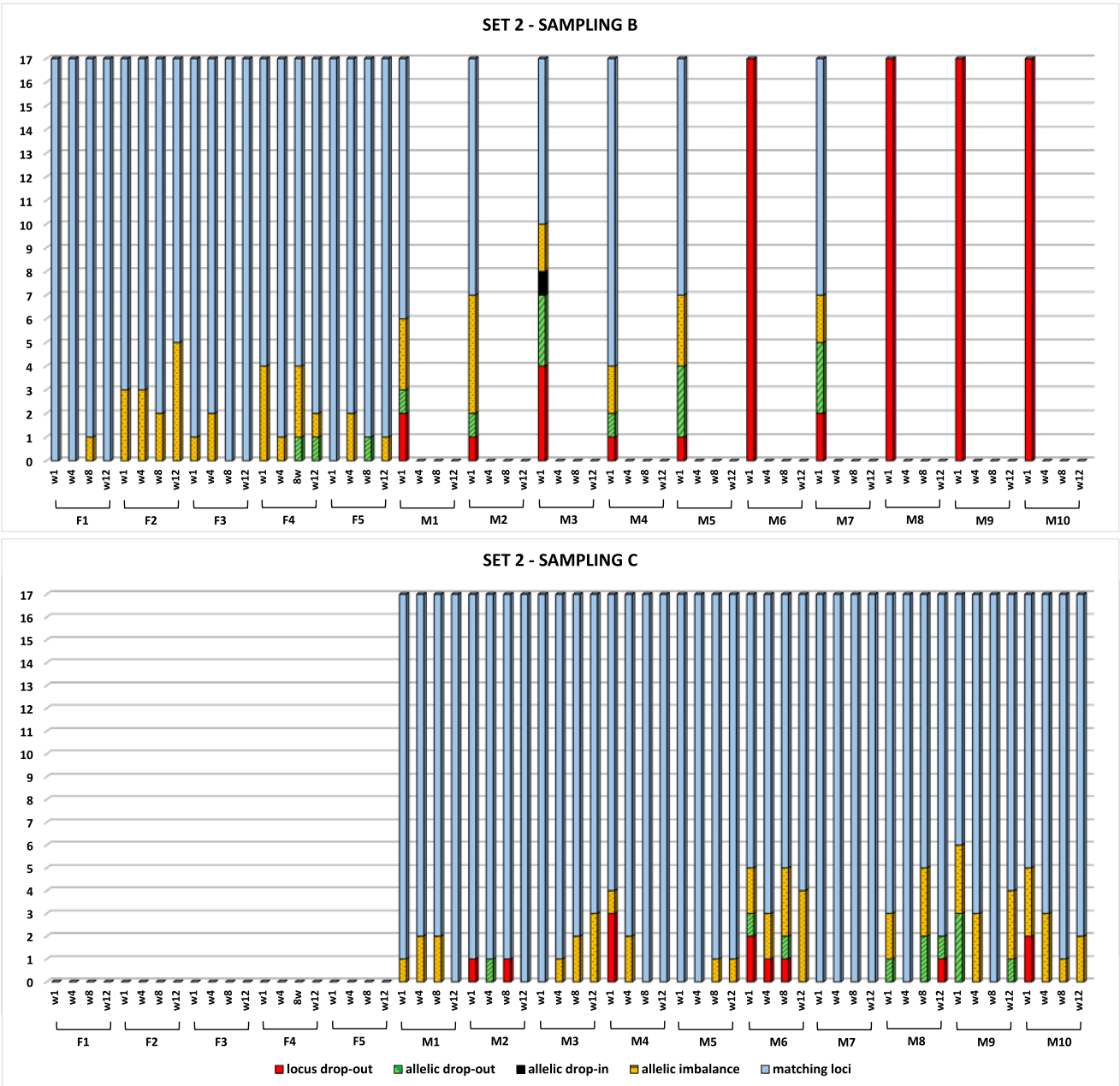

FIGURE 3 Quality of the genetic profiles obtained from the analysis of Set 2 DUS samples, according to the number of markers giving genotypes matching the ones of the reference samples and to the amplification artifacts identified in the profiles (locus dropouts [LDO]; allelic dropouts [ADO]; allelic dropins [ADI]; allelic imbalances) (for each category, see the figure legend below the bar chart). Sampling B: DUS spotted from urine samples stored at +4°C for 5 days. Sampling C: 30X concentrated DUS spotted from urine samples stored at +4°C for 5 days. F1–F5: female urine spots from 1 to 12 weeks; M1–M10: male urine spots from 1 to 12 weeks. *Y* axis: number of markers composing the genetic profiles (16 autosomal STR and Amelogenin) [Colour figure can be viewed at wileyonlinelibrary.com]

artifacts or allelic imbalances such to fulfill the criterion for the switch to the 30X concentrated DUS, which were amplified producing full profiles until the end of the test (12 weeks).

In conclusion, the direct PCR amplifications of the 2-mm punches originated from 9 out of 10 male urine samples spotted onto Whatman 903 generated full 17 markers profile after a 12-week storage, using the 30X concentrated DUS. Only a single sample (M9), after 12 weeks, showed two allelic dropouts that produced a 9% relative reduction of the LR, whose value (10^{+19}) could however easily support a personal identification. The full LR values for the 10 male samples varied from 10^{+21} to 10^{+25}.

3.3.2 | Set 2—Samplings B and C

The genetic profiles obtained from the punches originated from the male urine samples stored at +4°C for 5 days, after 1 week, were all characterized by at least two artifacts such as ADO, LDO or ADI, or by more than 50% unbalanced heterozygous genotypes (see Figure 3). In detail, no loci could be amplified for four samples (M6, M8, M9, and M10) and consequently the corresponding genetic profiles were completely missing while the loss of relative LR for the other six samples varied from 17% to 50%. This situation led us to perform the genetic analysis of all the 10 male samples on the 30X concentrated DUS immediately after 1 week.

The punches collected from the 30X DUS after 1 week showed, for four samples (M1, M3, M5, and M7), full profiles, with very few unbalanced loci, till the end of the test. The remaining six samples presented, in different timeframes, a loss of the LR from 5% to 22% due to the presence of one to three amplification artifacts with a reduction of the LR value to 10^{17}, however fully useful for individual identification. After 12 weeks, 8 out 10 of the 30X DUS were characterized by complete 17 loci profiles with a limited number of allelic imbalances with genotypes always matching the ones of the reference samples. Two samples after 12 weeks (M8 and M9) showed only a very limited number or amplification artifacts, with a percentage reduction of the LR corresponding to 10%.

In conclusion, the direct PCR amplification of the 2-mm punches originated from 8 out of 10 male urine samples spotted onto Whatman 903 generated full profiles with a limited number of unbalanced loci after a 12-week storage, using the 30X concentrated DUS. Two samples, after 12 weeks, showed one and two ADO, respectively, which produced at most a 10% relative reduction of the LR, whose value (10^{+19}) could however easily support a personal identification.

Most of the genetic profiles obtained from the 30Xs samples showed off-scale peaks due to signal saturation; for this reason, the amplified products were diluted.

3.4 | Slope trend, decaying degradation pattern

In order to evaluate the quality of the genetic profiles originated from each DUS composing the two sets of samples, according to the parameter degradation slope, calculations were performed using the software EuroforMix.[36,37] The mean and standard deviation values of ten 2800M control DNA samples were 0.95 ± 0.04 thus describing a reference value for a high molecular weight DNA. The degradation slopes were calculated for each genetic profile obtained from the two set of DUS samples whose genetic typing is represented in Figures 2 and 3. A two-way repeated measures ANOVA highlighted a statistically significant difference between the mean degradation slope of the two set of samples for both males ($F = 7.89$, $p = 0.0116$) and females ($F = 5.98$, $p = 0.040$). Besides, no significant differences were detected in the mean degradation slopes alongside the entire timeframe within the same set for male (0 vs. 4 vs. 8 vs. 12 weeks, $F = 1.26$, $p = 0.297$) and female samples (0 vs. 4 vs. 8 vs. 12 weeks, $F = 1.06$, $p = 0.382$); interaction term was not significant (see Figure S1).

These data supported the results already obtained from the DNA quantifications showing that the storage of the same set of urine samples at +4°C for 5 days affected the quality of the genetic profiles. On the other hand, no statistically significant qualitative differences were seen within the same set of urine samples, according to the parameter degradation slope.

4 | CONCLUSIONS

The data presented in the current paper showed that it is possible to achieve genetic profiles useful for individual identification from 2-mm punches collected from urine samples spotted onto Whatman 903 cards the day of the micturition and after a 5-day storage at +4°C, up to 12 weeks after the time of deposition. All the genetic profiles obtained from the DUS met the quality parameters described in GE.F.I[46] and perfectly matched the corresponding reference samples collected using saliva swabs.

For the male urine samples, which proved to contain a limited amount of DNA, these results were obtained mainly starting from a 30X concentrated DUS prepared by centrifuging 1.5 ml of urine in an Eppendorf tube, then discarding the supernatant until a final volume of approximately 50 µl is obtained, and finally spotting it onto the paper. This procedure is simple and does not need any particular skill, and it can be easily introduced in a routine collection of urine samples as it guarantees that a genetic profile useful for individual identification can be obtained especially if the punches are collected in the center of the DUS. Even if all the female samples analyzed in this study provided highly informative genetic profiles starting from the spots prepared with the original urine sample, for a standard routine use in drug monitoring for clinical and medico-legal purposes, we suggest to set up the 30X concentrated DUS to overcome the time-consuming process of separating male and female samples and to be quite confident on the positive outcome of the genetic typing. For the 30X concentrated female DUS, it will be likely necessary to dilute the amplified products, or to inject them for shorter times, in order to achieve "on scale" allelic peaks.

In addition, one of the most important advantages of the DMS, in general, is that the paper cards take up little space and can be easily stored at room temperature even for long periods of time.

The genetic profiles were achieved through direct PCR amplification of STR markers contained in a validated forensic commercial kit.[47] Direct PCR avoids the time-consuming and costly procedures of DNA extraction and molecular quantification; in addition, this procedure prevents the loss of the valuable DNA, which can be very limited in these kinds of stains.

Further studies on the stability over 12 weeks of molecules of interest for clinical/forensic toxicology and DNA on dried urine spots are currently underway and the stability of other molecules of interest for sport drug monitoring will be tested. Once the effect of long-term storage on DUS of the selected molecules will be investigated, the DNA protocol presented in this study could be considered as a complementary tool to drug monitoring and anti-doping screening test, in order to unambiguously assign the biological sample to a person when a tampering or a sample switching is suspected.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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