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Short Communication

PCR inhibitor removal using the NucleoSpin® DNA Clean-Up XS kit

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ABSTRACT

Forensic evidence samples are collected from an unlimited variety of substrates, which may contain compounds known to inhibit the polymerase chain reaction (PCR). These PCR inhibitors are co-extracted with the DNA sample and can negatively affect the DNA typing results, which can range from partial to complete inhibition of the short tandem repeat (STR) PCR. One potential solution is to remove the PCR inhibitors from the extracts prior to the STR PCR with the NucleoSpin® DNA Clean-Up XS kit. The kit contains a NucleoSpin® XS silica column that has a special funnel design of thrust rings along with a very small silica membrane, which allows for sample elution in a small volume that is appropriate for use with current STR typing kits. The NucleoSpin® DNA Clean-Up XS kit was optimized for the best possible DNA recovery and then evaluated for its ability to remove eight commonly encountered PCR inhibitors including: bile salt, collagen, hematin, humic acid, indigo, melanin, tannic acid and urea. Each of these PCR inhibitors was effectively removed by the NucleoSpin® DNA Clean-Up XS kit as demonstrated by generating more complete STR profiles from the cleaned up inhibitor samples than from the raw inhibitor samples.

1. Introduction

Biological evidence that is received for processing in a forensic laboratory comes in all forms on various substrates, which may present many challenges that include biological fluids deposited on complex substrates, limited sample size, and possible degradation. Subsequent sampling and extraction of the evidence for short tandem repeat (STR) typing can lead to inherent problems if the sample/substrate contains compounds known as polymerase chain reaction (PCR) inhibitors. These PCR inhibitors are co-extracted with the DNA samples and inhibit PCR [1]. One possible way to remove these PCR inhibitors is by using a silica-binding system [2–5]. Specifically, we describe the use of the NucleoSpin® DNA Clean-Up XS kit (MACHERY-NAGEL, Düren, Germany) for post extraction DNA concentration and clean-up of the aqueous extracts from the phenol/chloroform extraction or extracts from non-silica based centrifugation devices. The NucleoSpin® DNA Clean-Up XS kit was initially evaluated as an alternative to microdialysis centrifugation devices with blood, semen and saliva samples on a variety of substrates [6].

Here we report the evaluation of the NucleoSpin® DNA Clean-Up XS kit's ability to remove eight common PCR inhibitors from genomic DNA samples. The kit was first optimized for the highest possible DNA recovery by varying the load and elution buffer volumes during utilization of the kit. The kit was evaluated with mock DNA extracts prepared by mixing HL-60 (*Human promyelocytic leukemia cells*) DNA (ATCC, Manassas, VA) with varying concentrations of known PCR inhibitors: bile salt, collagen, hematin, humic acid, indigo, melanin, tannic acid and urea [7–12]. The ability of the NucleoSpin® DNA Clean-Up XS kit to remove these PCR inhibitors was assessed by comparing the STR results from the NucleoSpin® DNA Clean-Up XS cleaned up inhibitor samples to the STR results from the raw inhibitor samples.

2. Materials and methods

2.1. Samples

Pre-quantified, pooled, high molecular weight human genomic male DNA (Promega, Madison, WI) was used as the quantification standard for all experiments and in the optimization experiments. Pre-quantified HL-60 DNA was used in the inhibition experiments.

2.2. Optimized NucleoSpin® DNA Clean-Up XS procedure

In order to optimize the kit for the best DNA recovery, increased loading volumes (TE^{-4} buffer and binding buffer (NT)) and elution buffer (BE) volumes were evaluated as follows. The same male

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Table 1
Summary of average yield results from the optimization of the (a) binding buffer (NT) and (b) elution buffer (BE).

(a)

Binding buffer (NT)[a]	Average yield (ng)[b]	Abbreviated as
400 μL TE with 100 μL binding buffer loaded in a single step	0.61 ± 0.24	1×
800 μL TE with 200 μL binding buffer loaded in two steps	0.58 ± 0.23	2×
1200 μL TE with 300 μL binding buffer loaded in three steps	0.34 ± 0.14	3×
1600 μL TE with 400 μL binding buffer loaded in four steps	0.4 ± 0.03	4×

(b)

Elution buffer (BE)[a]	Average yield (ng)[c]	Abbreviated as
Elute DNA in 10 μL BE buffer, twice	1.07 ± 0.28	10 μL
Elute DNA in 15 μL BE buffer, twice	0.70 ± 0.12	15 μL
Elute DNA in 20 μL BE buffer, twice	0.66 ± 0.07	20 μL
Elute DNA in 25 μL BE buffer, twice	0.50 ± 0.05	25 μL

Shown in bold are the "optimal" parameters chosen for all downstream analysis.
[a] All samples brought to 12 μL following extraction.
[b] Starting concentration approximately 1 ng/μL.
[c] Starting concentration approximately 1.2 ng/μL.

genomic DNA used as a qPCR quantification standard was diluted with TE^{-4} to yield 0.0025, 0.00125, 0.00083 and 0.00063 ng/μL working solutions that were mixed with appropriate volumes of binding buffer as indicated in Table 1 prior to loading one or more 500 μL aliquots onto the NucleoSpin® XS columns by centrifuging at 11,000 × *g* for 30 s. Each NucleoSpin® column was washed with 100 μL wash buffer (B5) with 2 min centrifugation at 11,000 × *g*. The elution buffer volumes were varied from 10 to 25 μL, using the optimal "2×" loading volume, and centrifuged at 11,000 × *g* for 1 min, twice. The extracts were then heated for 8–38 min, depending on the elution buffer volume, at 90 °C in a dry bath to evaporate residual ethanol and to further concentrate the samples. The final extract volumes were normalized to 12 μL with the addition of TE^{-4} buffer.

2.3. Inhibitor preparation

Stock inhibitor solutions were prepared as follows: bile salt (Sigma, St. Louis, MO, #B3426), 40,000 ng/μL in sterile water; collagen solution (Sigma, #C8919) 0.1% in 0.1 N acetic acid; hematin (Sigma, #H3281), 2000 μM in 1 N sodium hydroxide (Sigma, #S5881) in sterile water; humic acid (Alfa Aesar, Ward Hill, MA, #41747), 700 ng/μL in sterile water; indigo (Alfa Aesar, Ward Hill, MA, #22996), 500 mM in 0.2% Triton X-100 (Sigma, #X100); melanin (Sigma, #M0418), 100 mg in 1 mL 1 N sodium hydroxide with subsequent dilution in 500 mL sterile water; tannic acid (Sigma, #403040), 6 ng/μL in sterile water; urea (Sigma, #U1250), 2,500,000 μM in sterile water.

2.4. Inhibitor concentrations

The working inhibitor solutions were prepared according to Table 2. Briefly, the stock inhibitor solutions were diluted in TE^{-4} buffer for each concentration, diluted with sterile water and spiked with HL-60 to achieve a total genomic DNA concentration of 0.1 ng/μL. Samples, prepared at 1×, 1/2× and 1/4× the target concentration for each inhibitor, were amplified/typed in triple replicates with the AmpF*l*STR® Identifiler® PCR Amplification kit (Applied Biosystems, Carlsbad, CA).

2.5. Inhibitor removal with NucleoSpin® DNA Clean-Up XS kit

The inhibitor concentrations used to evaluate the NucleoSpin® DNA Clean-up XS kit are indicated in Table 2 and are each four times (4×) the concentration that was observed to significantly inhibit the AmpF*l*STR Identifiler typing, which was defined as less than 10% of alleles detected. Specifically, the HL-60 DNA samples were considered to be significantly inhibited when fewer than 3 of the 25 alleles were detected with the AmpF*l*STR Identifiler kit. Therefore, these samples were prepared by spiking HL-60 DNA into the inhibitor solutions to achieve a final concentration of 0.00125 ng/μL or a total of 1 ng of HL-60 DNA in 800 μL. These samples (triplicates) were cleaned-up using the modified NucleoSpin® DNA Clean-up process as follows. Specifically, 800 μL of each working inhibitor solution was mixed with 200 μL binding buffer (guanidinium thiocyanate solution, pH 5), loaded in 500 μL aliquots and centrifuged at 11,000 × *g* for 30 s. Each NucleoSpin® XS column was washed with 100 μL wash buffer (buffered 80% ethanol solution) with 2 min centrifugation at 11,000 × *g*. For each column, 15 μL elution buffer (5 mM Tris HCl pH 8.5) was added and centrifuged at 11,000 × *g* for 1 min, and repeated once. The extracts were then heated for 8 min at 90 °C in a dry bath. The final volumes were normalized to 22 μL with the addition of TE^{-4} buffer. Another set of samples (triplicates) for each inhibitor was

Table 2
Summary of stock and working inhibitor solution concentrations.

Inhibitor	Bile salt (ng/μL)	Collagen (ng/μL)	Hematin (μM)	Humic acid (ng/μL)	Indigo (mM)	Melanin (ng/μL)	Tannic acid[b] (ng/μL)	Urea (μM)
Stock concentration	40,000	1000	2000	700	125	200	6	2,500,000
1/2× Working concentration	1684	63.5	45.0	22.5	6.2	100	**3**	85,763
1× Working concentration	**3368**	**127**	**90**	**45**	**12.4**	**200**	**6**	**171,526**
1/4× Working concentration	842	31.75	22.5	11.25	3.1	50	**1.5**	42,881.5
Concentration used to evaluate NucleoSpin® XS columns for inhibitor removal[a]	13,472	508	360	180	49.6	800	6	686,104

Shown in bold is the concentration in which significant inhibition was seen (less than ten percent of alleles above threshold).
[a] Concentration is four times (4×) that of the concentration in which significant inhibition was seen.
[b] Tannic acid – all three concentrations showed significant inhibition.

not cleaned-up using the NucleoSpin® XS columns but instead taken directly to DNA quantification.

2.6. DNA quantification using real-time PCR (qPCR)

All samples were quantified with the nuTH01-nuSRY-nuCSFIPC quadruplex qPCR assay [13] using an 7500 Real Time PCR system (Applied Biosystems) sequence detection system (7500 SDS software v 1.3) to estimate the quantity of amplifiable human DNA in each sample. All samples were run in comparison to seven known quantities of male genomic DNA (Promega, Male #G1471) ranging from 32 to 0.044 ng. An internal PCR control (IPC) was used to assay for detection of PCR inhibition.

2.7. STR genotyping

The AmpF*l*STR Identifiler PCR Amplification kit was used for STR genotyping. When available, PCR amplifications of 1 ng of autosomal DNA in a 25 mL reaction volume were performed according to vendor instructions on a GeneAmp 9700 PCR thermal cycler (Applied Biosystems). STRs were resolved and detected on a 3130 Genetic Analyzer (Applied Biosystems) according to vendor instructions. Electrophoresis data were analyzed using GeneMapper™ ID 3.2 (Applied Biosystems using 50 RFU peak amplitude thresholds.

3. Results and discussion

3.1. Optimized NucleoSpin® DNA Clean-Up XS procedure

The results from the optimization of the loading volume and elution buffer volume experiments are shown in Table 1a and b and Fig. 1a and b as an average yield for each variation of the buffers with associated standard deviations. The 2× loading volume was deemed to be optimal because the 1× and 2× average yields (ng) were not significantly different and previous work with semen extracts has shown some sample types benefit from an increased loading volume [6]. Likewise, the 15 μL elution buffer volume was selected as similar recoveries were obtained from 10, 15 and 20 μL elution volumes and as a slightly higher elution volume should yield a more desirable final extract volume that will not require normalization with the addition of TE^{-4}.

3.2. Inhibitor concentrations

The AmpF*l*STR Identifiler STR results are presented in Fig. 2a for the 1×, 1/2× and 1/4× inhibitor concentrations. These results were used to determine the concentration of each inhibitor that significantly inhibited the AmpF*l*STR Identifiler PCR. Samples were evaluated by the percent of alleles above the threshold for each inhibitor at varying concentrations. For most of the samples, the concentration which saw significant inhibition was the highest concentration tested. This concentration was quadrupled (4×) in order to evaluate the NucleoSpin® column's ability to remove common PCR inhibitors. The concentrations were quadrupled in an attempt to determine if the NucleoSpin® cleanup circumvents inhibition more effectively than the traditional method of diluting sample extracts containing PCR inhibitors. Sample dilution is a simple approach used in laboratories to dilute out the PCR inhibitor in the hope that there is enough remaining DNA in the sample to still develop a full STR profile. However, samples containing a total of 1 ng of template DNA and 4× the significant concentration of PCR inhibitor are not expected to yield STR typing results via the dilution method as a 1:4 dilution would contain sub-optimal quantities of template DNA and a significant concentration of PCR

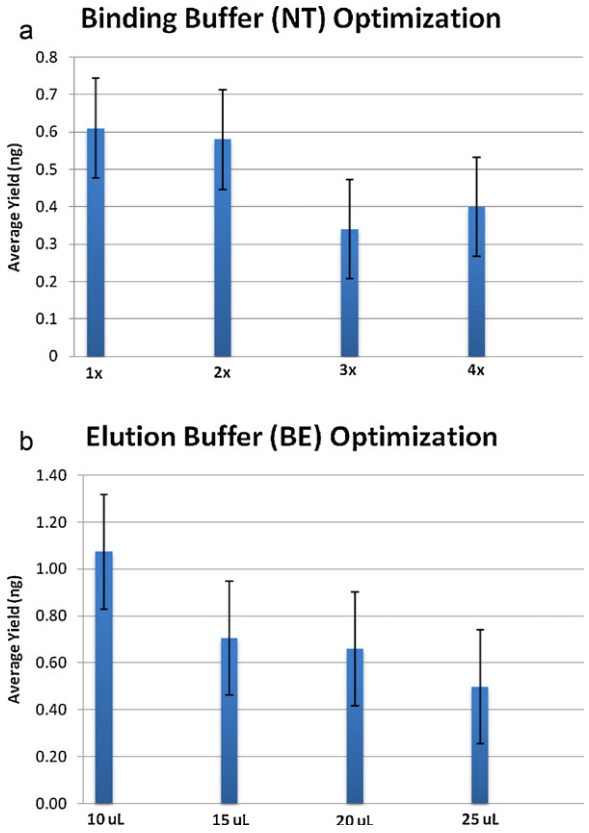

Fig. 1. (a) Summary of the average yields (ng) and standard deviations for the optimization study of binding buffer (NT). (b) Summary of the average yields (ng) and standard deviations for the optimization study of elution buffer (BE).

inhibitor remaining. For example, the 360 μM hematin concentration of the 4× hematin solution is sixteen times the 22 μM hematin concentration that has been shown to completely inhibit the Identifiler® reaction [14]. Therefore, a 1:30 dilution would be required to reduce the hematin concentration to the 12 μM level that has been shown to yield partial profiles with the optimal template quantities; this would result in the amplification of 0.03 ng of template DNA, which is below the template level expected to yield full profiles under optimal conditions [14]. Alternatively, the use of newer, more robust STR kits (i.e. PowerPlex® 16 HS, ESX, ESI, Identifiler® Plus, MiniFiler™, etc.) may also be another option for sample extracts containing co-extracted PCR inhibitors. However, this may not completely resolve the difficulties presented by these types of samples as they also prevent accurate quantitation by PCR based qPCR assays.

3.3. Inhibitor removal with the NucleoSpin® DNA Clean-Up XS kit

The NucleoSpin® XS columns appear to effectively remove most of the PCR inhibitors to yield more information than was previously seen at the lower concentrations of each inhibitor. Fig. 2a and b compare the STR results for the "raw" 1×, 1/2× and 1/4× inhibitor concentration samples to the 4× inhibitor solutions that were cleaned up with NucleoSpin® XS columns. This figure illustrates that running the samples through the NucleoSpin ®XS columns generally provides a greater amount of information in the STR profile as significantly more alleles were called in the cleaned-up samples. Compare, for example, the nearly null STR results for the raw 1× samples to the allele call rates for the cleaned up 4× samples. Similarly, the samples at the 4× inhibitor concentration that were not cleaned-up with the NucleoSpin® XS columns did not yield any detectable STR data. The columns worked especially

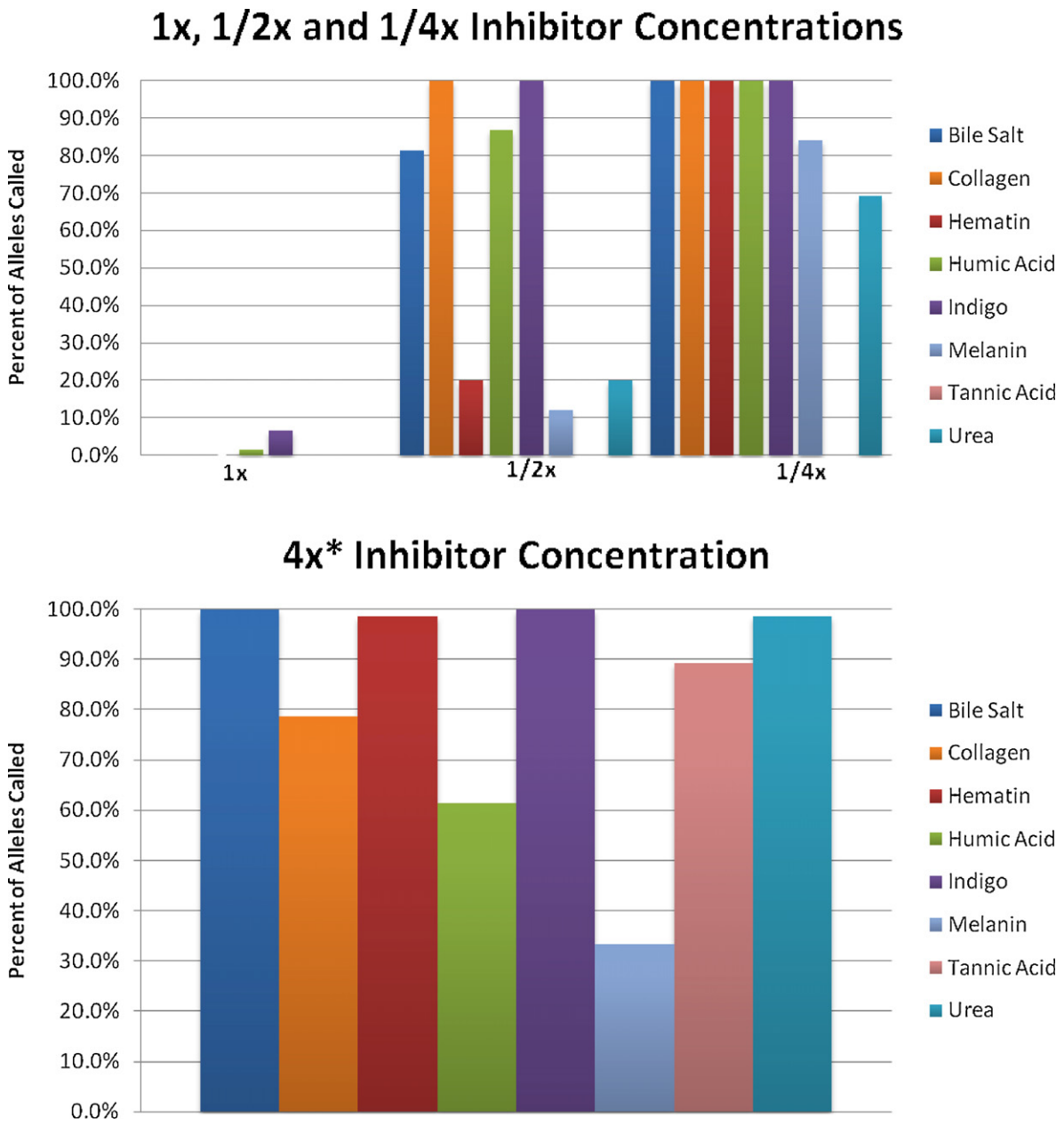

Fig. 2. (a) AmpF*l*STR® Identifiler® STR results for the 1×, 1/2× and 1/4× raw inhibitor concentrations not cleaned up with NucleoSpin® XS columns. (b) AmpF*l*STR® Identifiler® STR results from the 4× inhibitor samples (four times the concentration with significant inhibition) that were cleaned up with NucleoSpin® XS columns; samples at 4× concentration that were not cleaned up with NucleoSpin® XS columns (raw samples) resulted in no detectable STR data (no alleles called). *All concentrations are 4× with the exception of tannic acid which is 1×.

well to remove bile salt, hematin, indigo and urea. The average yields from the samples that were run through the NucleoSpin® XS columns are shown in Fig. 3a. Fig. 3b represents the associated average IPC (Internal PCR Control) Ct delay for each set of inhibitor samples. Higher recoveries were observed in some inhibitors compared to others; this may be due to the way in which some compounds are proposed to inhibit the PCR and/or as a result of low-level inhibitors affecting one or more of the quadruplex qPCR assay targets (67 bp nuCSF, 77 bp IPC, 137 bp nuSRY) more than the ~180 bp nuTH01 target, which is typically used to set up the Identifiler reactions. Specifically, a slight preferential amplification of the ~180 bp nuTH01 target may result from a low-level of inhibition of one or more of the other targets in the quadruplex qPCR assay [13]. There are three potential mechanisms by which the inhibitors are believed to interfere with PCR: (i) binding of the inhibitor to the polymerase [15,16]; (ii) interaction of the inhibitor with the DNA; and (iii) interaction with the polymerase during primer extension [1]. Melanin and humic acid are both believed to inhibit PCR by sequence specific binding to the DNA molecule preventing the DNA polymerase from extending along the DNA during primer extension; it is not surprising that these inhibitor samples yielded the least amount of information in the STR profile. Collagen had the third lowest percentage of alleles called and is also believed to bind to the DNA but does not alter the availability of DNA template; instead the binding appears to affect Taq processivity [1]. The amount of DNA present in each sample should have yielded a full STR profile if no inhibition was suspected. This suggests that the NucleoSpin® DNA Clean-Up XS kit may not

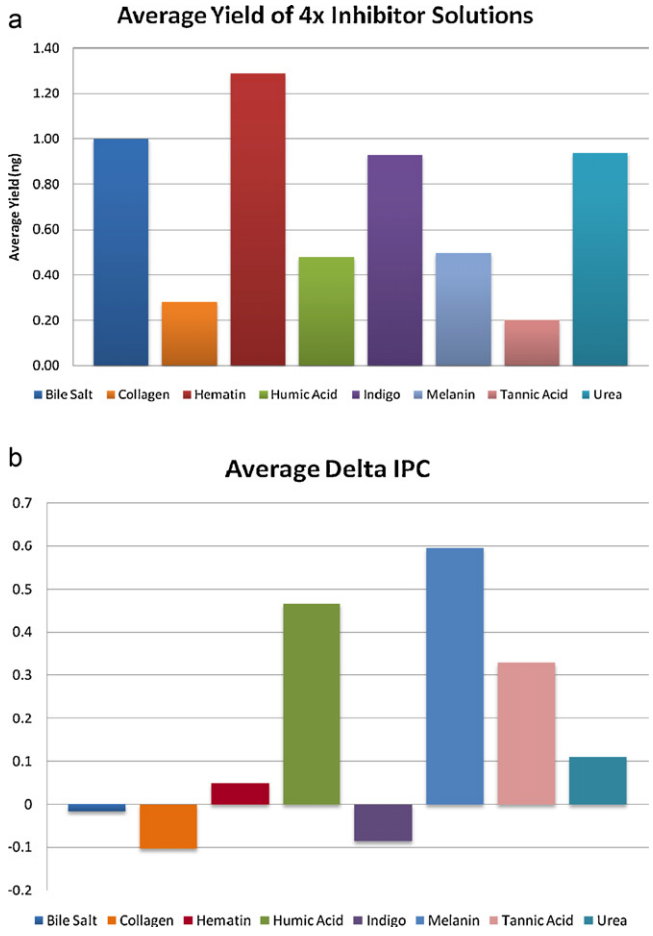

Fig. 3. (a) Quantification results represented as an average yield (ng) from each inhibitor sample at the 4× concentration that were run through NucleoSpin® XS columns. *Note*: The starting quantity of DNA loaded onto each NucleoSpin® XS column was 1 ng. (b) Average IPC (Internal PCR control) Ct delay, which is calculated as (Samples IPC Ct value) − (Average IPC Ct for standards less than 15 ng) for each set of inhibitor samples that were run through the NucleoSpin® XS columns.

remove PCR inhibitors that are bound to the DNA and some of the PCR inhibitor may remain in the samples preventing the STR reaction from occurring.

4. Conclusions

Our results indicate that the NucleoSpin® DNA Clean-Up XS kit can be used to effectively remove known PCR inhibitors that are routinely found in DNA extracts from forensic samples. The NucleoSpin® DNA Clean-Up XS kit was especially effective at removing five of the eight inhibitors tested (bile salts, indigo, hematin, tannic acid and urea) with greater than 80% of the alleles called in samples containing these inhibitors and moderately effective with the remaining three inhibitors (collagen, humic acid and melanin) with approximately 30–80% of the alleles called in samples containing these inhibitors. Conversely, when these raw PCR inhibitor samples were taken directly to qPCR quantification and STR typing, there were no alleles detected in any of the samples. Similar trends were observed in DNA recovery with bile salts, hematin, indigo and urea yielding 80% or greater recoveries with collagen, humic acid, melanin, and tannic acid yielding approximately 20–50% recoveries. Collectively, the STR typing results and DNA recoveries from melanin, humic acid and collagen are not surprising as all three are believed to inhibit the PCR as a result of their binding to the DNA [1] and may not be completely removed from the samples by the NucleoSpin® DNA Clean-Up XS kit. However, the NucleoSpin® DNA Clean-Up XS kit may provide an effective means for the removal of most PCR inhibitors with the expectation of a high recovery of DNA.

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