A comparison of five DNA extraction methods from degraded human skeletal remains

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ABSTRACT

Extracting DNA from degraded human remains poses a challenge for any forensic genetics laboratory, as it requires efficient high-throughput methods. While little research has compared different techniques, silica in suspension has been identified in the literature as the best method for recovering small fragments, which are often present in these types of samples. In this study, we tested five DNA extraction protocols on 25 different degraded skeletal remains. Including the humerus, ulna, tibia, femur, and petrous bone. The five protocols were organic extraction by phenol/chloroform/isoamyl alcohol, silica in suspension, High Pure Nucleic Acid Large Volume silica columns (Roche), InnoXtract™ Bone (InnoGenomics), and PrepFiler™ BTA with AutoMate™ Express robot (ThermoFisher). We analysed five DNA quantification parameters (small human target quantity, large human target quantity, human male target quantity, degradation index, and internal PCR control threshold), and five DNA profile parameters (number of alleles with peak height higher than analytic and stochastic threshold, average relative fluorescence units (RFU), heterozygous balance, and number of reportable loci) were analysed. Our results suggest that organic extraction by phenol/chloroform/isoamyl alcohol was the best performing method in terms of both quantification and DNA profile results. However, Roche silica columns were found to be the most efficient method.

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1. Introduction

Forensic DNA typing plays a key role in the identification of human remains in various contexts such as terrorism, organized crime, mass fatality incidents or mass graves. The Regional Government of Andalusia (Spain) has been making efforts since 2017 to identify the victims of the Spanish Civil War (1936–1939) and post-war period, estimating 45,569 victims in 709 mass graves [1]. The laboratory's mission is to perform the DNA analyses from both victims and family members, so far 2335 skeletal remains (mostly femur and tooth) and 1776 family members buccal swabs up to date. Conventional approaches are insufficient to handle this high number of samples, so high-throughput methods are required in such scenarios [2].

The DNA extraction protocol is a crucial element that can be chosen and modified by the analyst to obtain the best possible quality and quantity of extracted DNA. Since critical skeletal remains DNA is likely to be damaged, forensic DNA extraction protocols have to be adapted to recover the most short fragments [3]. Total demineralization was proposed as the best method for degraded human remains DNA analysis [4], while silica extraction was concurrently stated as the best method for ancient DNA analyses [5]. Since both extraction protocols are arduous, automated DNA extraction systems have been proposed for old skeletal remains analysis with accurate results [6].

In general terms, the literature has pointed out better yields with total demineralization protocols. Still, silica is especially efficient for short DNA fragments recovery, yet there are many factors to consider, such as: lysis buffer composition, the amount of bone powder input, the lysis buffer and bone powder ratio, or incubation temperature and duration [7]. A meta-analysis [8] found that magnetic beads methods (EZ1® DNA Investigator® and PrepFiler™ BTA) were the most widely used methods by Forensic DNA laboratories (ancient DNA studies were excluded), followed by organic extraction and silica. Higher DNA profiling success was observed with magnetic beads/resin based methods, as well as in those methods that incorporated a demineralization step.

Not many comparisons of DNA extraction methods for critical human remains have been published in the literature. Silica adsorption have been pointed out as the best method in Pleistocene

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bones [9], as well as in human petrous bones from 5 to 11th centuries AD and 20th century AD [10]. Other study did not found any difference between silica adsorption and organic extraction by phenol/chloroform/isoamyl alcohol, founding the two of them as the best methods compared to total demineralization or QIAGEN Blood Maxi Kit [11]. Total demineralization methods have been observed as worst for small size DNA recovery [12], correlating less lysis times with better DNA yields [13]. On this wise silica seems to obtain more suitable extracts for massive parallel sequencing [14]. Finally, total demineralization and organic extraction seems to show less DNA yield if human remains were buried [15].

The aim of this research is to identify the best DNA extraction protocol for this kind of skeletal remains. To achieve that, 25 different skeletal remains DNA were extracted all of them with five different bone DNA extraction protocols.

2. Material and methods

The following procedures were performed in an ancient DNA facility following the ancient DNA methods standards [16,17], including room UV light, HEPA filtered air positive pressure, negative extraction controls, negative and positive PCR controls, sterilized labware, and laboratory personnel DNA profiling, as contamination preserve and detection measures. Degradation indexes and 'ski-slope' profiles were used as critical DNA presence indicators.

2.1. Samples

Samples have been buried during 70–80 years in the South-West region of Andalusia, no in situ data about the place of exhumation has been reported to the laboratory, however, this part of Andalusia is characterized by a slightly acid pH [18], with the highest temperatures reached in Summer (28 ºC on average, reaching maximum temperatures of 45 ºC), more than 2800 h of insolation annually (5 kW/h/m2) and minimum temperatures of 12–14 ºC in Winter, being the average annual precipitation about 400–600 mm rain gauge, with few rainy days [19].

25 critical skeletal remain samples (see Table 1) from mass graves in Andalusia (Spain), buried from Spanish Civil War (1936–1939) and the post-war period were sanded and cut with a Dremel® rotatory tool. Bone fragments were pulverized in a TissueLyser II (QIAGEN, Hilden, Germany).

2.2. DNA extraction

Samples were extracted using five different DNA extraction protocols, which are summarised in Table 2: 1) organic extraction by phenol/chloroform/isoamyl alcohol (Ph-Chl-IA), 2) silica adsorption, 3) silica in columns by High Pure Viral Nucleic Acid Large Volume Kit (Roche, Basel, Switzerland), 4) a manual magnetic particles protocol by InnoXtract™ Bone (InnoGenomics, New Orleans, LA, USA), and 5) an automated magnetic particles protocol by AutoMate Express™ Nucleic Acid Extraction System and PrepFiler™ BTA Forensic DNA Extraction kit (ThermoFisher Scientific, Waltham, MA, USA).

2.2.1. Organic extraction

DNA was extracted from 1.0 g bone powder using an in-house protocol based on the classic organic extraction by phenol/chloroform/isoamyl alcohol method [20]. The bone powder was digested overnight at 56ºC with 5 ml lysis buffer (4125 µL EDTA 0.5 M, 300 µL SDS 10%, 375 µL proteinase K 10 mg/ml and 200 µL DTT 1 M). The lysate was purified with 4 ml phenol/chloroform/isoamyl alcohol 25:24:1 (Sigma-Aldrich, Saint Louis, MO, USA), and concentrated using an Amicon Ultra-4 Centrifugal Filter Unit 30 kDa (Merck KGaA, Darmstadt, Germany). Concentrated extract was purified with MinElute PCR Purification Kit (QIAGEN) to a final volume of 75 µL.

2.2.2. Silica adsorption

DNA was extracted from 500 mg bone powder using standard ancient DNA silica protocol [21]. The bone powder was digested two days with 2 ml lysis buffer (0.5 M EDTA, 0.14 mg/ml proteinase K, 1:1000 phenol red) at 37ºC. The samples were centrifuged, and the supernatant was mixed and incubated during 1 h with 40 ml binding buffer (500 ml Buffer PB (QIAGEN), 24.88 mM NaCL, 87.6 mM sodium acetate and 1:1000 phenol red) and 100 µL of silica in suspension, with pH adjusted to 4–5 with 37% HCl. The samples were centrifuged and silica pellet was resuspended in 80% cold ethanol, and washed twice with that ethanol [10]. Elution was performed with Buffer EB (QIAGEN), and the samples were then purified using the MinElute PCR Purification Kit (QIAGEN) to a final volume of 75 µL.

2.2.3. Silica in column

DNA was extracted from 150 mg bone powder using the High Pure Viral Nucleic Acid Large Volume Kit (Roche) [22]. For this, 1 ml extraction buffer (45 ml 0.5 M EDTA, 3.75 ml DNase and RNase free water, 25 µL Tween-20) and 25 µL Roche Proteinase K (10 mg/ml) were added and the samples were incubated 24 h at 37ºC and another 24 h at 56ºC. The lysate was centrifuged and 10 ml Buffer PB (QIAGEN) and 400 µL 3 M sodium acetate were added and incubated for 2 min. Mixture was centrifuged using High Pure Extenders and washed two times with PE buffer (Roche). The samples were eluted twice with EB Buffer (QIAGEN) to a final volume of 60 µL.

2.2.4. InnoXtract™ Bone

DNA was extracted from three 40 mg aliquots using the InnoXtract™ Bone kit (InnoGenomics). For this, 563 µL Bone Digest Buffer and 37 µL 20 mg/ml proteinase K were added to each sample, and they were incubated overnight at 56ºC. Then, 750 µL Bone DNA Binding Buffer and 20 µL Magnetic Bead Suspension were added to the lysate in a DynaMag™ 2 (ThermoFisher). Two washes were performed with Wash Buffer, and two more with 80% ethanol. Elution was performed with InnoXtract Elution Buffer, ant the aliquots were mixed and concentrated in a vacuum centrifugue to a final volume of 40 µL.

2.2.5. PrepFiler™ BTA forensic DNA extraction kit in AutoMate ™ Express

DNA was extracted from three 200 mg aliquots using the PrepFiler™ BTA Forensic DNA Extraction Kit in an AutoMate Express™ (ThermoFisher) following an in-house protocol. For each sample: 540 µL Lysis Buffer, 42 µL 20 mg/ml proteinase K and 18 µL DTT 1 M were added to each sample and incubated overnight at 56ºC. An eluate volume of 20 µL was set and the three aliquots were combined to a final volume of 60 µL.

2.3. DNA quantification

DNA extracts were quantified using the Quantifiler™ Trio DNA Quantification Kit (ThermoFisher) following manufacturer's protocol [23] on a QuantStudio™ 5 Real-Time PCR System.

Five parameters were analysed: the quantity small target (80 bp) DNA quantity, large target (214 bp), human male target (75 bp) DNA, degradation index (small target/large target ratio), and Internal PCR Control (IPC) threshold.

Table 1
Human skeletal remain samples.

Sample type	Humerus	Ulna	Tibia	Femur	Petrous	Total
n	1	1	1	10	12	25

Table 2
Summary of the different protocols tested.

Protocol	Based on	Skeletal quantity (mg)	Lysis (h)	Automated
Ph-Chl-IA	Organic extraction	1000	Overnight	No
Silica (suspension)	Silica	500	48	No
Silica (column)	Silica	150	48	No
InnoXtract™	Magnetic particles	3 × 40	Overnight	No
PrepFiler™ BTA	Magnetic particles	3 × 200	Overnight	Yes

2.4. DNA amplification and data visualization

DNA amplification was performed using GlobalFiler™ PCR Amplification Kit according to the manufacturer' specifications [24]. 15 µL DNA input was added from each extract from each DNA extraction method. Electrophoresis was carried out on an ABI 3500 Genetic Analyzer following manufacturer's recommended conditions, and the data were analysed using GeneMapper™ IDX 1.4.

Five variables were studied: 1) the number of alleles above analytical threshold (50 RFU based on internal validation), 2) the number of alleles above stochastic threshold (365 RFU based on internal validation), 3) the average RFU, 4) the heterozygous balance (calculated as small allele/large allele height), and 5) the number of reportable markers (homozygous alleles higher than 365 RFU or heterozygous alleles higher than analytical threshold and > 0.6 peak height ratio). Non autosomal markers information was discarded, leaving 21 autosomal STR markers.

Statistical analyses were performed using jamovi 2.2.5 [25].

3. Results and discussion

3.1. Real-time PCR results

DNA quantification results are showed in Table 3, including the average ($\bar{x}$) and standard deviation (SD) of each studied parameter, as well as the values normalized by input skeletal quantity ($\bar{x}$/100 mg).

In general, larger amounts of bone powder input resulted in larger quantities of DNA. On average, the Ph-Chl-IA extraction protocol achieved the highest small human DNA yield, followed by InnoXtract™ silica in suspension, PrepFiler™ BTA and silica in column. Hence, the Ph-Chl-IA protocol obtained four times more DNA than the least efficient method. The same trend was observed for large human DNA yield, with Ph-Chl-IA performing the best, followed by InnoXtract™, and silica in suspension, PrepFiler™ BTA and silica in column. Degradation indexes were the highest by InnoXtract™ and lowest by Ph-Chl-IA, with silica in suspension

Table 3
Results of DNA quantification by DNA extraction protocol.

|	Parameter	$\bar{x}$	SD	$\bar{x}$/100 mg	SD
Ph-Chl-IA	*Small target (ng/µL)*	0.469802	0.548940	0.046980	0.054894
	Large target (ng/µL)	0.031656	0.042412	0.003039	0.004200
	Male target (ng/µL)	0.367421	0.505054	0.033803	0.049414
	Degradation Index (DI)	28	71	-	-
	IPC (Ct)	27.53	0.23		
Silica (suspension)	*Small target (ng/µL)*	0.124917	0.152789	0.024983	0.030558
	Large target (ng/µL)	0.009166	0.014518	0.001833	0.002904
	Male target (ng/µL)	0.107788	0.154530	0.021558	0.030906
	Degradation Index (DI)	53	135	-	-
	IPC (Ct)	27.33	0.15		
Silica (column)	*Small target (ng/µL)*	0.099855	0.141144	0.063907	0.093072
	Large target (ng/µL)	0.006872	0.008106	0.004032	0.005278
	Male target (ng/µL)	0.067249	0.089702	0.044833	0.059801
	Degradation Index (DI)	36	105	-	-
	IPC (Ct)	27.48	0.34		
InnoXtract™	*Small target (ng/µL)*	0.219482	0.246216	0.182901	0.205180
	Large target (ng/µL)	0.013760	0.017418	0.011467	0.014515
	Male target (ng/µL)	0.159749	0.203042	0.133124	0.169202
	Degradation Index (DI)	91	265	-	-
	IPC (Ct)	27.74	0.16		
PrepFiler™ BTA	*Small target (ng/µL)*	0.112145	0.127529	0.018691	0.021255
	Large target (ng/µL)	0.007391	0.007278	0.001129	0.001210
	Male target (ng/µL)	0.085819	0.105887	0.013707	0.017505
	Degradation Index (DI)	34	72	-	-
	IPC (Ct)	27.43	0.23		
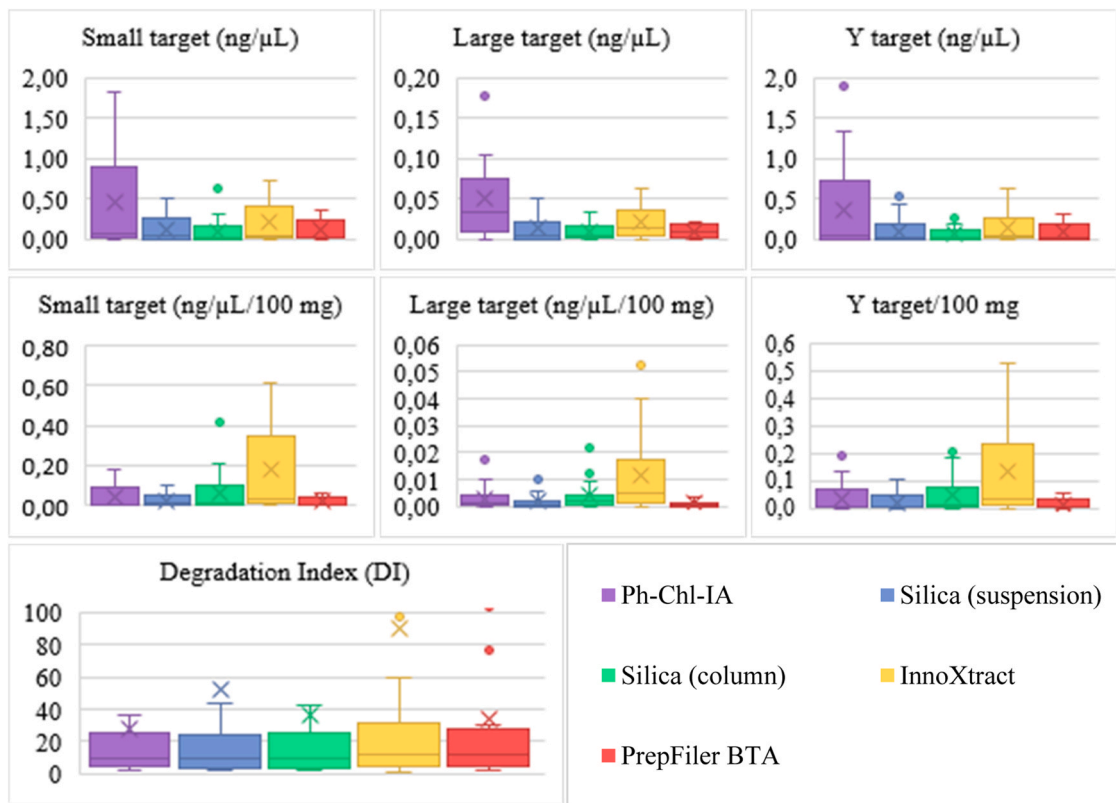
Fig. 1. Boxplots of DNA quantification, both raw data (above) and normalized data (below).

producing intermediate values. The degradation index, which is the ratio between small and large human DNA targets, provides information about the degree of dispersion between both targets, indicating that with the Ph-Chl-IA protocol recovers both fragments equally, while the InnoXtract™ kit extracts smaller fragments more efficiently than larger fragments.

Interestingly, the situation changes when considering the efficiency of each protocol in relation to the amount of bone powder input (see Fig. 1). InnoXtract™ was the kit with the highest small human DNA yield, followed by silica in column, Ph-Chl-IA, silica in suspension and PrepFiler™ BTA. The same ranking was observed for large human DNA yield, with InnoGenomics kit performing better than the other protocols, yielding 10 times more small target and as much as 100 times more large target DNA than the least efficient protocol, despite requiring the smallest amount of bone powder input.

One way ANOVA (Welch's) with DNA extraction protocol as the grouping variable showed statistically significant differences in all quantification variables except to degradation index. The Tukey post-hoc test reveals statistically significant differences among the different extraction protocols in small target (between Ph-Chl-IA and the other kits, p-value < 0.001), small target per bone powder milligram (between InnoXtract™ and the rest of kits, p-value < 0.001), large target (between Ph-Chl-IA and PrepFiler™ BTA, silica in column and silica in suspension, p-value < 0.003), and large target per bone powder milligram (among InnoXtract™ and the other kits, p-value < 0.001). No differences were founded, once again, by degradation index (DI). This makes sense since the same samples have been analyzed by the different extraction protocol so the observed DI should be the same. However, it has been showed that there are differences among protocols when it comes to small/large target retention (as seen with InnoXtract™).

Finally, no inhibition was detected in this stage for any of the tested method, so all of them were suitable for removing or reducing common PCR inhibitors found in soil and similar samples. However, literature has pointed out that Ph-Chl-IA is less efficient than commercial kits in removing this type of substances [26].

The study by Rohland & Hofreiter with cave bear bones quantified DNA extracts and found higher yields with DNA IQ™ system (magnetic particles commercial kit), followed by silica in suspension, with Ph-Chl-IA being the least efficient method. Silica in suspension was better than the magnetic particles protocol when exhaustively compared [9]. Another study conducted with 5th-11th century human remains founded silica in suspension with the highest DNA quantities, followed by silica in column, with Ph-Chl-IA being the less efficient method [10]. Comparing organic extraction and PrepFiler™ BTA with tooth samples, a study found higher yields with Ph-Chl-IA. However, PrepFiler™ BTA gave better yields when data is normalized [27]. In addition, another comparison with fresh and casework bones finds PrepFiler™ BTA better than silica in suspension when it comes to DNA quantification results [28]. A preliminar comparison among silica in suspension, PrepFiler™ BTA and InnoXtract™ placed the first two protocols as the best compared to the others in terms of quantification [29].

In general, literature finds magnetic particles commercial kits having better DNA yields than manual protocols, and among them, silica (suspension or column) captures more DNA than Ph-Chl-IA. On the one hand, our findings differ from literature considering only the obtained data, being Ph-Chl-IA the protocol that obtained the highest small and large DNA quantities. On the other hand, when this data was normalized with the bone powder input quantity, the same conclusions as above are obtained, with InnoXtract™ being the best method in terms of quantity. Interestingly, no normalized data showed silica in suspension as better than silica in column (as

Table 4
DNA profile results by extraction protocol.

	Parameter	x̄	SD	x̄/100 mg	SD
Ph-Chl-IA	*Alleles >AT*	29	12	2.91	1.16
	Alleles >ST	19	10	1.87	1.03
	RFU	3640	3299	364.04	329.86
	PHR	0.7	0.2	-	-
	Reportable loci	15	6	1.48	0.64
Silica (suspension)	*Alleles >AT*	24	10	4.83	2.05
	Alleles >ST	14	10	2.74	2.07
	RFU	2435	2777	487.07	555.47
	PHR	0.7	0.1	-	-
	Reportable loci	12	6	2.34	1.26
Silica (column)	*Alleles >AT*	24	11	15.84	7.35
	Alleles >ST	12	10	8.21	6.88
	RFU	1483	1625	988.64	1083.03
	PHR	0.7	0.1	-	-
	Reportable loci	11	7	7.47	4.47
InnoXtract™	*Alleles >AT*	13	12	10.63	10.22
	Alleles >ST	7	9	5.97	7.17
	RFU	1588	2281	1323.58	1900.46
	PHR	0.6	0.2	-	-
	Reportable loci	6	6	5.07	5.32
PrepFiler™ BTA	*Alleles >AT*	25	13	4.09	2.09
	Alleles >ST	14	10	2.27	1.75
	RFU	1358	1382	226.26	230.35
	PHR	0.7	0.1	-	-
	Reportable loci	12	7	1.99	1.17

expected). However, this was reversed with normalization, with silica in column being more efficiency than silica in suspension.

3.2. DNA profile results

The results of the DNA profiles results (number of alleles above analytical threshold (AT), number of alleles above stochastic threshold (ST), relative fluorescence units (RFU), peak height ratio (PHR), and reportable loci are shown in Table 4.

On average, Ph-Chl-IA detected the highest number of alleles (29 ± 12), followed by PrepFiler™ BTA (25 ± 13), silica in column and silica in suspension (24 ± 10), with InnoXtract™ detecting the lowest number of alleles (13 ± 12). Organic extraction resulted in the highest number of alleles above stochastic threshold (19 ± 10), with PrepFiler™ BTA and silica in suspension detecting 14 ± 10 each, followed by silica in column with 12 ± 10, and InnoXtract™ detecting only 7 ± 10 alleles. This indicates that 65% of the alleles detected by Ph-Chl-IA met stochastic threshold, while 56% of the alleles detected by PrepFiler™ BTA met this threshold (50% for the other protocols). Hence, Ph-Chl-IA resulted in the highest RFU (3640 ± 3299), followed silica in suspension with 2435 ± 2777, InnoXtract™ with 1588 ± 2281, silica in column with 1483 ± 1625, and PrepFiler™ BTA with 1358 ± 1382. Finally, Ph-Chl-IA was the most efficient method in terms of the highest number of reportable loci (15 ± 6), followed by silica in suspension and PrepFiler™ BTA (12 each), silica in column (11 ± 7), and InnoXtract™ (5 ± 6).

The same situation as with the quantification data arise here when data normalization by bone powder input is applied, and the previous ranks are inverted (see Fig. 2). Silica in column was the most efficient method in terms of detected alleles per 100

milligrams of bone powder (15.84 ± 7.35), followed by InnoXtract™ (10.63 ± 10.22), silica in suspension (4.83 ± 2.05), PrepFiler™ BTA (4.09 ± 2.09), and Ph-Chl-IA (2.91 ± 1.16). Silica in column was also the protocol with the highest number of alleles above stochastic threshold per 100 milligrams of bone powder (8.21 ± 6.88), followed by InnoXtract™ (5.97 ± 7.17), silica in suspension (2.74 ± 2.07), PrepFiler™ BTA (2.27 ± 1.75) and Ph-Chl-IA (1.87 ± 1.03). InnoXtract™ was the protocol with the highest RFU per 100 milligrams (1323.58 ± 1900.46), followed by silica in column (988.64 ± 1083.03), silica in suspension (487.07 ± 555.47), Ph-Chl-IA (364.04 ± 329.86), and PrepFiler™ BTA (226.26 ± 230.35). Finally, silica in column was on average and per bone powder quantity the protocol with the highest number of reportable loci (7.47 ± 4.47), followed by InnoXtract™ (5.07 ± 5.32), silica in suspension (2.34 ± 1.26), PrepFiler™ BTA (1.99 ± 1.17), and Ph-Chl-IA (1.48 ± 0.64). In both cases (non-normalized and normalized data) it should be noted that Ph-Chl-IA is the protocol with less variability and, therefore, the most consistent.

One way ANOVA (Welch's) with DNA extraction protocol as the grouping variable showed statistically significant differences in all detection variables except to peak height ratio, which makes sense since this is the only studied variable that relies directly on kit's primer efficiency. Tukey post-hoc tests showed statistically significant differences by detected alleles (between InnoXtract™ and the rest of methods, $p < 0.01$), by reportable alleles (between InnoXtract™ and Ph-Chl-IA, $p < 0.001$), by RFU (between InnoXtract™ and Ph-Chl-IA, p-value=0.023, and between Ph-Chl-IA and PrepFiler™ BTA and silica in column, $p < 0.05$), by detected alleles/bone powder milligram (between InnoXtract™ and phenol/chloroform, PrepFiler™ BTA, silica in column, and silica in suspension, $p < 0.05$, between phenol/chloroform and silica in
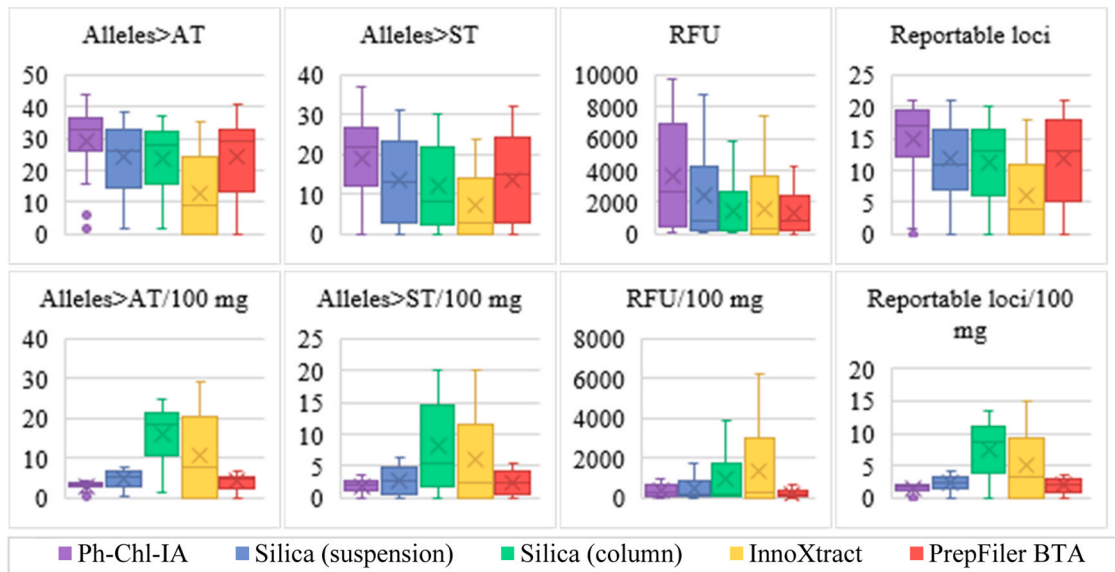
Fig. 2. DNA profile parameters plots, both raw data (above) and normalized by skeletal remain quantity input in each protocol (below).

column, p-value < 0.001, between PrepFiler™ BTA and silica in column, p-value < 0.001, and between silica in column and silica in suspension, p-value < 0.001), by RFU/mg (between InnoXtract™ and phenol/chloroform, PrepFiler™ BTA, and silica in suspension, p-value < 0.01), and by reportable loci (between InnoXtract™ and phenol/chloroform and PrepFiler™ BTA, p-value < 0.01, between phenol/chloroform and silica in column, p-value < 0.001, and between silica in column and phenol/chloroform, PrepFiler™ BTA, and silica in suspension, p-value < 0.001). No statistically significant differences were observed by heterozygotic balance.

In addition, statistically significant differences were observed among samples by skeletal remain type, obtaining higher quantities of DNA and more informative profiles with petrous bone (ANOVA Welch's Post Hoc p-value < 0.05).

Fig. 3 shows the average RFU obtained in each locus by every DNA extraction protocol. In all extraction protocols, a 'ski-slope' profile is obtained: the smaller loci are more amplified than the largest. Secondly, RFU results are consistent: there are loci with high fluorescence in proportion to each extraction protocol' average RFU results, and there are markers where little amplification has been obtained (as expected since the main factor behind this behavior is the commercial kit's chemistry itself). Thirdly, the established rank of each protocol' success by RFU is repeated in every locus. No

statistically significant differences were observed among loci RFU by extraction protocol.

Revisiting the literature, better mitochondrial DNA results were obtained with silica in suspension, while the worst results were achieved by Ph-Chl-IA protocol [10]. Comparing PrepFiler™ BTA and Ph-Chl-IA and silica in suspension, this magnetic particles commercial kit outperforms both DNA extraction protocols, producing more informative profiles in terms of threshold requirements and reportable loci than organic extraction [27], and around three times more detected alleles than silica in suspension [28]. Silica in suspension and PrepFiler™ BTA outperformed InnoXtract™ in massively parallel sequencing, with InnoXtract™ being more variable, with the lowest number of reads and mapped targets [29]. So according to the literature, a possible ranking of DNA extraction methods may be PrepFiler™ BTA, silica in suspension, Ph-Chl-IA, and InnoXtract™. However, in our study Ph-Chl-IA was found to be the method with the highest number of detected alleles, reportable alleles, RFU and reportable markers, however being silica in column and InnoXtract™ were the methods with the highest efficiency. Furthermore, negative profile was obtained in samples 1–3 by organic extraction and PrepFiler™ BTA, while approximately 10 more alleles were obtained with InnoXtract™ and silica in suspension.
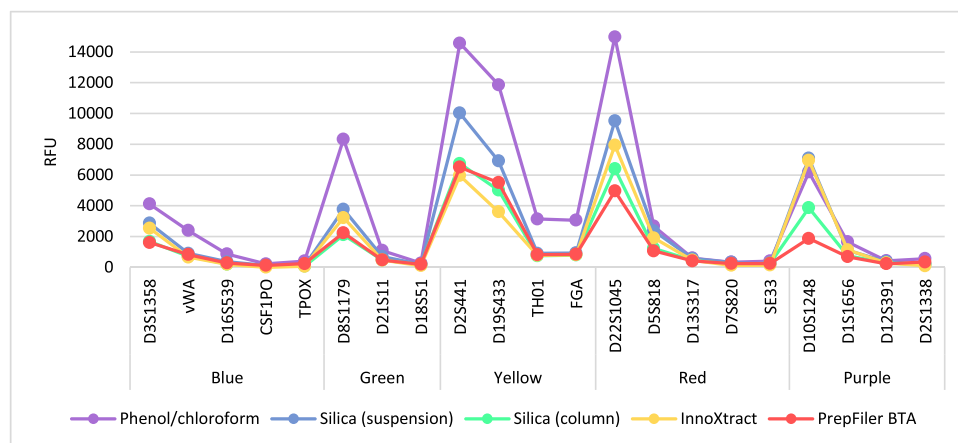

Fig. 3. Average RFU by locus and by extraction protocol.

Table 5
Advantages and disadvantages of the tested DNA Extraction protocols.

DNA Extraction protocol	Advantages	Disadvantages
Ph-Chl-IA	– High yield. – Cellular components elimination. – Standard protocol.	– Higher amounts of sample are required. – Extra purification step is needed. – Toxic compounds. – Laborious.
Silica (suspension)	– Moderated yield. – No toxic compounds are used. – Commonly used in ancient DNA.	– Extra purification step may be needed. – pH measurement may be required – Longer lysis time. – Requires reagents preparation. – Laborious.
Silica (column)	– Best efficiency (less bone powder required). – Less laborious than silica in suspension. – Less reagents preparation. – Inhibitors removed efficiently.	– Longer lysis time. – Susceptible to bone powder interferences.
InnoXtract™	– Automatable – No reagents preparation is needed. – Inhibitors removed efficiently.	– Less yield. – Sample handling.
PrepFiler™ BTA	– Automatable – No reagents preparation is needed. – Inhibitors removed efficiently. – Fast protocol.	– Less yield. – High-cost.

Some methodological concerns may also be discussed. Firstly, the amount of bone powder input: protocol or manufacturer's recommended input quantities were chosen over the using the same amount of bone powder in each method since each one's chemistry is optimized for a certain amount of substrate. Secondly, InnoXtract™ Bone extraction method (three aliquots concentrated in a vacuum centrifuge) was developed with the manufacturer during internal validation. Thirdly, PrepFiler™ BTA and AutoMate™ Express protocol was developed after internal validation, in which several approaches were tested [30]: 50 mg bone powder input, 200 mg bone powder input, three 200 mg aliquots and five 500 mg aliquots, being the method presented in this research the best cost/benefit balance. Finally, regarding the number of detected alleles, homozygous alleles are not duplicated (only peaks are counted) since many peaks are situated below the stochastic threshold and allelic drop-out could not be discarded.

Overall, assuming each analyzed parameter as equally important and considering not only the average results but also the statistical significances, InnoXtract™ and Ph-Chl-IA were the best methods in terms of amount of DNA recovered, while Ph-Chl-IA, silica in suspension and silica in column were the best protocols in terms of both qPCR and profiles results. In general, our results suggest Ph-Chl-IA as the best performing DNA extraction protocol, whereas silica in column and InnoXtract™ were the most efficient methods in proportion to the quantity of DNA input. Additionally, considering quantification results, InnoXtract™ seems really promising as an efficient DNA extraction method with small DNA fragments applications such as SNPs analysis or next generation sequencing. Until now, this method has not been systematically tested with human remains in the literature.

PrepFiler™ BTA in AutoMate™ Express had a reasonably good performance, obtaining 20% fewer reportable markers than Ph-Chl-IA. This is a protocol that should be specially considered since it is automated, reducing hands-on time with samples and cross-contamination risk, and no hazardous chemicals are used. In this sense this method may be suitable as a routine DNA extraction protocol with this kind of samples if implemented with another method with higher efficiency (e.g., Ph-Chl-IA or silica in suspension) to be used with extra-challenging bones or where no results were obtained with this automated option. The same conclusion was claimed by an earlier work [31].

Nevertheless, Ph-Chl-IA has always been considered as the gold standard in DNA extraction because its versatility, DNA yield and effective cleanup of PCR inhibitors, despite being toxic (Ph-Chl-IA residues should be treated properly in laboratories), labor-intensive (it involves many transfers among tubes/filters) and having an increased risk of contamination. Paramagnetic particles methods are usually rapid, they remove most of the PCR inhibitors and are adaptable to automation, reducing contamination risk and saving time. However, DNA yields are more limited [32]. Ultimately, each laboratory must validate its own DNA extraction protocol with its routine samples [7]. All things considered, a summary of advantages and disadvantages of the different methods tested is shown in Table 5.

4. Conclusion

Degraded skeletal remains are a challenge for any DNA typing laboratory due to endogenous DNA fragmentation into small fragments. DNA extraction is a key step, and there is no consensus in the literature about which DNA extraction methods has a better performance, or which protocols works with which sample.

There are not many comparisons performed with critical human remains. Previous studies put silica in suspension and PrepFiler™ BTA as the most efficient methods compared to the traditional Ph-Chl-IA.

Our study compares five DNA extraction protocols in critical human remains: phenol/chloroform/isoamyl alcohol, silica in suspension, silica in column, InnoXtract™ and PrepFiler™ BTA automated with AutoMate™ Express. Phenol/chloroform/isoamyl alcohol was the most efficient method in both quantification and DNA profile obtention, reaching on average the highest number of reportable loci. Nevertheless, data normalization by bone powder input quantity reveals silica in column as the most efficient, reaching considerably good results considering the little quantity of bone powder used. Furthermore, our in-house PrepFiler™ BTA extraction protocol with AutoMate™ Express reveals as a good quality procedure for routine high-throughput DNA extraction from degraded human remains.

Heeding this information, our laboratory extracts DNA from skeletal remains using two parallel protocols: an automated one with PrepFiler™ BTA and AutoMate™ Express and an organic

extraction by phenol/chloroform/isoamyl alcohol, obtaining two profiles by different extractions in order to elaborate a consensus profile.

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CRediT authorship contribution statement

CH: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – Original Draft. **XG:** Validation, Investigation. **DCVE:** Software, Validation, Data curation, Formal analysis., Writing – review and editing. **MIML:** Validation, Investigation. **MS:** Software, Validation, Data curation, Formal analysis, Writing – review & editing. **JAL:** Conceptualization, Methodology, Writing – review & editing, Supervision, Project Administration. **JCA:** Conceptualization, Methodology, Writing – review & editing, Supervision, Project Administration.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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