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HOW CLEAN IS ULTRAFILTRATION CLEANING OF BONE COLLAGEN?

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ABSTRACT. As part of our bone dating development, we have tested the ultrafiltration of bone gelatin using 2 different filters—Vivaspin 20™ (VS20), a polyethersulfone, and Vivaspin 15R™ (VS15R), a cellulose, both with a 30,000 molecular weight cutoff—and bone collagen from dated samples ranging in age from 1.5 to >50 kyr BP. A direct accelerator mass spectrometry (AMS) measurement yielded radiocarbon concentrations of ~0.5 pMC (~42 kyr) for the polyethersulfone, ~14.4–17.5 pMC (~15.6–14 kyr) for the cellulose, and ~107.4 pMC for the glycerin. The filters were cleaned before use similar to the Oxford protocol (Bronk Ramsey et al. 2004), and a series of freeze-dried archaeological bone gelatin samples and a modern pig-skin gelatin were passed through VS20 and VS15R filters (Vivascience™). We recovered both the eluent (<30-kD fraction) and the liquid that stayed above the filter (>30 kD) in order to obtain a carbon mass and isotope balance. While the >30-kD collagen fraction that is usually selected for AMS analysis does not appear to be significantly contaminated, measurements show significant age differences between the eluent <30 kD and the unfiltered bone collagen, indicating that, despite cleaning, both glycerin and filter still give off contaminants in the eluent. Ultrafiltration with young collagen from pig skin generally confirms these results for the <30-kD fraction but also shows the possibility of small contaminations in the >30-kD fraction. Until a contamination with filter carbon of the >30-kD collagen fraction can be excluded, we would recommend caution in the use of ultrafiltration for cleaning bone collagen with VS20 or VS15R ultrafilters.

INTRODUCTION

Collagen is generally used for the radiocarbon dating of bones. The routine bone pretreatment in the Leibniz laboratory (Grootes et al. 2004; see Figure 1) includes the removal of the mineral phase of the bone with 1% HCl followed by a gentle base-acid extraction to remove humic acids, which could contain significant amounts of foreign carbon, from the organic bone residue. Finally, the collagen, with a molecular weight (M) around 300,000 is dissolved as gelatin (M 100,000), filtered through a pre-cleaned 0.45-μm pore-size silver filter to remove non-soluble contaminants (Longin 1970), and freeze-dried. Dissolving the bone collagen is not a totally selective process (van Klinken and Mook 1990 and citations therein). The gelatin solution may contain not only endogenous collagen material but also various amounts of exogeneous proteins and hot-water-soluble organic compounds (e.g. humics, altered collagen parts). A final cleaning step using the high molecular weight of gelatin to remove contaminants with M <30,000 by ultrafiltration is therefore used by some laboratories.

Ultrafiltration to remove low molecular weight contaminants from the gelatin solution was initially tested with positive results by Brown et al. (1988). Recent experiences at Oxford indicate, however, the filters may not only remove contaminants but also release organic material into the solution and thus act as a source of contamination (Bronk Ramsey et al. 2004). Filter membranes and filters are made of organic material that could contribute particles and/or dissolved carbon to both the filtrate and the supernatant. Filters come coated with glycerin to keep them flexible and will contribute this glycerin to the sample solution, especially to the filtrate.

As a preparation for adding ultrafiltration to our routine bone sample preparation, we tested the effect of 2 different types of filters and different filter cleaning procedures with a series of freeze-dried bone gelatin fractions obtained from bones dated from 1645 to 54,000 ^{14}C yr BP, and with modern gelatin produced from pig skin (MBP Medical Biomaterial Products GmbH, Schwerin, Germany).

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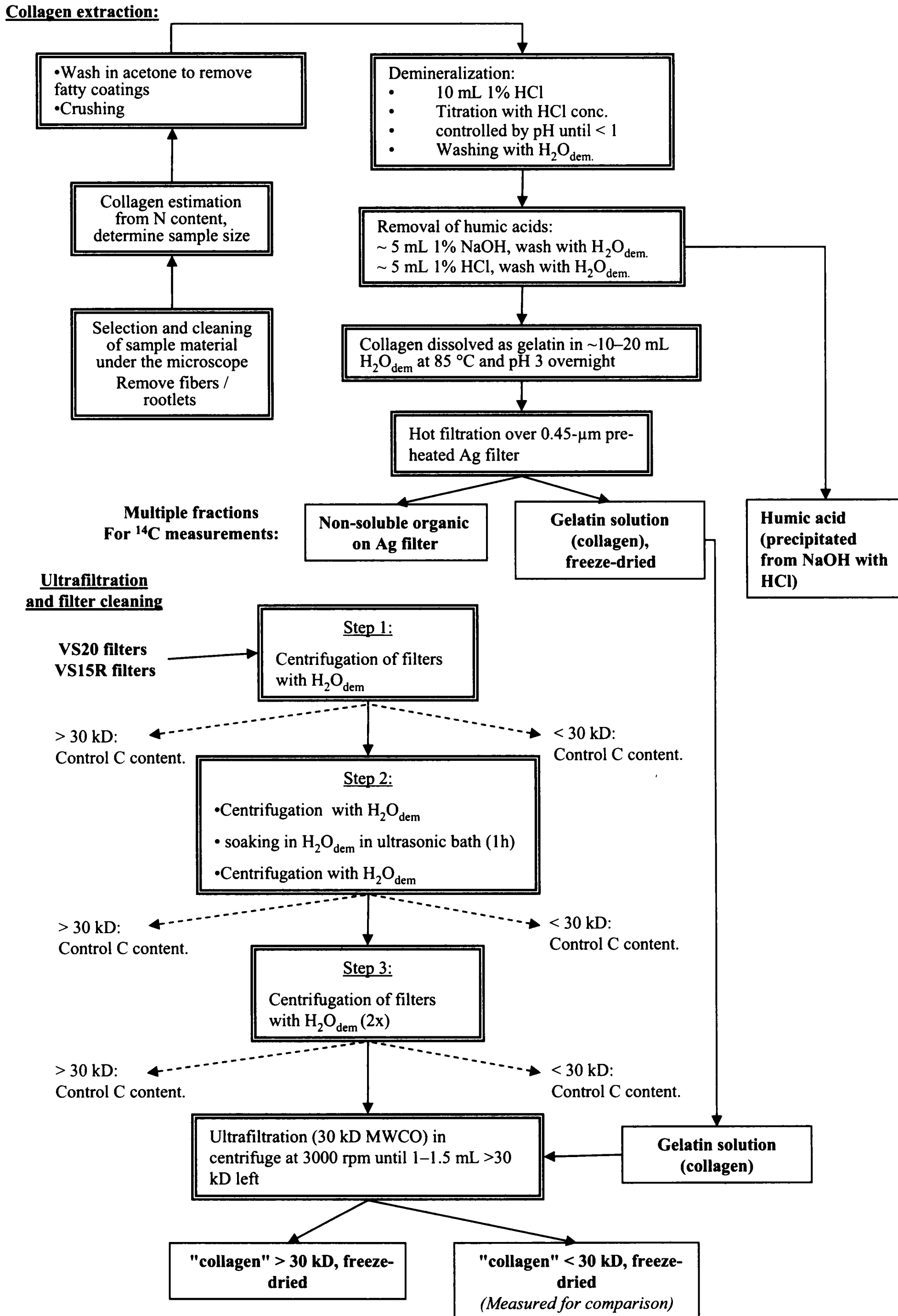

Figure 1 Schematic overview of collagen extraction, ultrafilter cleaning, and samples taken to assess the removal of glycerin.

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METHODS AND MATERIAL

Collagen Extraction

At the Leibniz laboratory, extraction of bone collagen starts with a demineralization of the bone in HCl, which is controlled by pH measurements (see Figure 1). A gentle (at room temperature for 1 hr) base extraction with 1% NaOH, followed by HCl, will remove humic acids. The organic residue is then heated at pH 3 in ultrapure water to 85 °C, gelatinizing and dissolving the collagen proteins. The dissolved gelatin is filtered over a 0.45-μm pore-size Ag filter to remove non-dissolved organic material, which could contain significant amounts of non-contemporaneous organic material. The dissolved gelatin is then freeze-dried and ready for combustion (Longin 1970; Grootes et al. 2004).

Ultrafiltration: Filter Cleaning

Two filter types with a 30,000 molecular weight cutoff (MWCO) were tested:

1. Vivaspin 20™ 30,000 MWCO (Vivascience™) (polyethersulfone, VS20).
2. Vivaspin 15R™ 30,000 MWCO (Vivascience™) (regenerated cellulose, VS15R).

Both filter types come coated with glycerin to keep the filters flexible. Since early 2005, the glycerin contains young carbon, whereas before 2005 the glycerin contained a fossil carbon signal (Brock et al. 2007), documenting changes in the production. Because glycerin is soluble in water, the ultrafilters to be used were cleaned by centrifugation and ultrasonication in a large volume of ultrapure water, closely similar to the Oxford protocol (Bronk Ramsey et al. 2004) (Figure 1). The cleaning steps are the following:

- Step 1: Centrifugation of filters with ultrapure water at 3000 rpm until all water has gone through the filters.
- Step 2: Centrifugation of filters with ultrapure water at 3000 rpm, then soaking of filters in a large volume of ultrapure water in an ultrasonic bath for 1 hr, followed by a third centrifugation with ultrapure water.
- Step 3: Centrifugation with ultrapure water at 3000 rpm until all water has gone through the filters (2×).

Samples of the filtrate (<30 kD) and the supernatant (>30 kD) were taken after the successive cleaning steps 1–3, freeze-dried, and analyzed for their carbon content. For 3 samples, we also determined the ^{14}C concentration.

For our ultrafiltration experiments, the dissolved gelatin solution was pipetted into an ultrafilter (either VS20 or VS15R) and centrifuged at ~3000 rpm until 1–1.5 mL of the molecular weight >30,000 (>30 kilo Dalton [kD]) gelatin fraction remains. The ultrafiltered gelatin solutions (>30 kD and <30 kD) are freeze-dried and ready for combustion to CO_2. For our tests, we have measured the ^{14}C concentration of both >30-kD and <30-kD ultrafiltered fractions (UF-coll.)

RESULTS

The removal of glycerin by the cleaning steps 1–3 above is documented by measurements of the carbon and ^{14}C content of the ultrapure water after cleaning (Table 1). For both filters, the carbon content in the wash water (both <30 kD and >30 kD) decreased below 5 μg already after cleaning step 2. A young carbon source for the contamination is confirmed by accelerator mass spectrometry (AMS) measurements on aliquots of water from step 1 cleaning (~106–108 pMC, see Table 1) and by direct

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measurements on glycerin used for the coating, a sample that was kindly provided by Vivascience (107.32 ± 0.35 pMC). The complete 3-step cleaning should thus be sufficient for the ultrafiltration of samples with more than 5 mg collagen, giving <0.1% contamination, well within the statistical uncertainty of the AMS measurements (usually >0.2%).

Table 1 Carbon and ^{14}C in cleaning water.[a]

	VS20				VS15 R	
	>30 kD		<30 kD		>30 kD	<30kD
Cleaning step	C (mg)	pMC	C (mg)	pMC	C (mg)	C (mg)
1	0.08				0.01	0.35
	0.03					
	0.96	108.30 ± 0.38	14.1	108.08 ± 0.33		
	0.34		11.01	106.94 ± 0.32		
2	<0.005		<0.005		<0.005	0.01
	<0.005		0.01		0.01	0.01
	0.01					
3	<0.005					
	<0.05					

[a]Glycerin ^{14}C is 107 ± 0.35 pMC.

Another source of contamination may be the filter membrane itself. Cleaned filter membranes of VS20 show ^{14}C concentrations of 0.3–0.5 pMC (Table 2), close to the pure and untreated membrane material ("glycerin-free"), suggesting an almost complete removal of the glycerin. Interestingly, filter membrane material cleaned with methanol and water gave an even lower ^{14}C concentration. Whether this cleaning step affects the filter properties needs to be assessed. The ^{14}C measurements for VS15R filter material show higher concentrations around 11–18 pMC (Table 2) and large variability without an apparent connection to cleaning or origin (pure vs. removed from filter). This large variability may result from changes in the production of the filters with varying ^{14}C concentration of the regenerated cellulose used for the filter, as has been seen for the glycerin.

Table 2 ^{14}C content of filter membranes before and after cleaning.

Description	VS20 Corrected pMC[a]	VS15R Corrected pMC[a]
Pure membranes	0.54 ± 0.09	14.36 ± 0.11
	—	17.48 ± 0.15
Pure membranes cleaned with methanol and H_2O	0.33 ± 0.07	18.49 ± 0.12
	0.26 ± 0.09	18.80 ± 0.1
Membranes removed from filter containers cleaned with methanol (3×) and water in US	0.13 ± 0.04	15.5 ± 0.1
	0.13 ± 0.04	15.90 ± 0.15
After cleaning step 3	0.49 ± 0.06	5.72 ± 0.09
	0.50 ± 0.05	11.10 ± 0.11
	0.31 ± 0.04	17.69 ± 0.14
	0.34 ± 0.04	—

[a]pMC corrected for $\delta^{13}C$ and background.

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To test the effect of ultrafiltration on the ^{14}C concentration and age measured for a collagen sample, we re-dissolved and filtered the freeze-dried gelatin of a set of 7 collagen samples with ages measured in our laboratory from 1645 to 54,000 ^{14}C yr BP and determined the apparent age of the fractions >30 kD and <30 kD (Table 3). To distinguish between a real cleaning of the gelatin and filter contamination, we repeated the filtration on several of the >30-kD fractions (Table 3, Figure 2).

Table 3 Results of the ultrafiltration of bone collagen. Given are the ^{14}C concentrations in pMC of unfiltered collagen (modified Longin method, L-coll.), ^{14}C concentration of ultrafiltered collagen fractions (UF-coll.), and modeled ^{14}C concentration.[a]

				1st ultrafiltration			2nd ultrafiltration	
Sample ID	Filter	L-coll ^{14}C (pMC)	L-coll age (BP)	UF-coll. >30 kD ^{14}C (pMC)	UF-coll. <30 kD ^{14}C (pMC)	Mod. UFC ^{14}C (pMC)	II UF, UF-coll. >30 kD ^{14}C (pMC)	II UF, UF-coll. <30 kD ^{14}C (pMC)
A	VS20	0.12 ± 0.05	54,010 + 3940/ −2630	0.34 ± 0.05	3.07 ± 0.07	1.37	0.27 ± 0.08	2.61 ± 0.26
				0.28 ± 0.05	1.34 ± 0.06	0.64	0.16 ± 0.05	0.82 ± 0.1
B	VS20	0.23 ± 0.06	48,650 + 2380/ −1840	0.28 ± 0.05	1.83 ± 0.07	0.93	—	—
				0.44 ± 0.05	2.01 ± 0.07	1.07	—	—
C	VS15R	0.41 ± 0.07	44,160 + 1400/ −1190	0.32 ± 0.06	0.64 ± 0.05	0.59	—	—
				0.38 ± 0.06	0.81 ± 0.06	0.71	—	—
D	VS20	18.21 ± 0.14	13,680 ± 60	17.66 ± 0.13	18.54 ± 0.29	17.84	—	—
E	VS20	33.67 ± 0.16	8745 ± 35	33.03 ± 0.17	32.35 ± 0.17	32.89	33.19 ± 0.16	33.10 ± 0.18
F	VS20	52.14 ± 0.16	5230 ± 25	51.79 ± 0.24	52.14 ± 0.21	51.82	—	—
	VS15R			52.25 ± 0.19	52.14 ± 0.19	52.21	—	—
G	VS20	81.46 ± 0.26	1645 ± 25	81.14 ± 0.26	76.53 ± 0.23	80.35	81.30 ± 0.23	77.15 ± 0.28
				80.50 ± 0.29	78.18 ± 0.23	79.72	81.41 ± 0.24	77.04 ± 0.38

[a]Mod. UFC ^{14}C (pMC) = $wt\%_{>30kD} \times pMC_{>30kD} + wt\%_{<30kD} \times pMC_{<30kD}$.

In general, the ^{14}C concentration of the filtered >30-kD fraction—the preferred collagen fraction—is close to that of the unfiltered collagen. The filtrate <30 kD contains significantly more ^{14}C for the 3 old samples and significantly less for the younger test samples E at 33.6 pMC and G at 81 pMC. We filtered the >30-kD fractions A, E, and G a second time in order to discriminate between a real cleaning of a contaminated collagen fraction and contamination introduced by the filtration as the cause of the observed concentration differences between the >30-kD and <30-kD fractions. The results for A and G indicate a contamination of the <30-kD fraction by filter material of intermediate ^{14}C concentration as the A filtrate again contains significantly more ^{14}C and the G filtrate less ^{14}C than the >30-kD fraction before and after filtering, while for E there are no significant differences. The ^{14}C content of the >30-kD fraction after 2 filtrations shows no statistically significant difference D ($D/(\sqrt{\sigma_1^2 + \sigma_2^2})$ <2) with that after 1 filtration or, for one A and one G duplicate, gets closer to that of the original material. This suggests the >30-kD fraction is not significantly contaminated by the ultrafiltration.

For the samples B, E, F, and G, we also measured the ^{14}C concentration of the non-soluble residue left on the 0.45-µm silver filter after the gelatin filtration. The residues B, F, and G showed no statistically significant differences in ^{14}C content with the gelatin fraction. This indicates these samples were not contaminated and supports the conclusion that the differences after ultrafiltration must be attributed to contamination by the filter. The residue E contained significantly more ^{14}C, indicating a young contamination of the sample. The lower ^{14}C concentration of the >30-kD collagen fraction may thus show a real cleaning of the sample by ultrafiltration. The filter contamination of the <30-kD fraction precludes the use of this fraction to verify the purity of the collagen fraction >30 kD.

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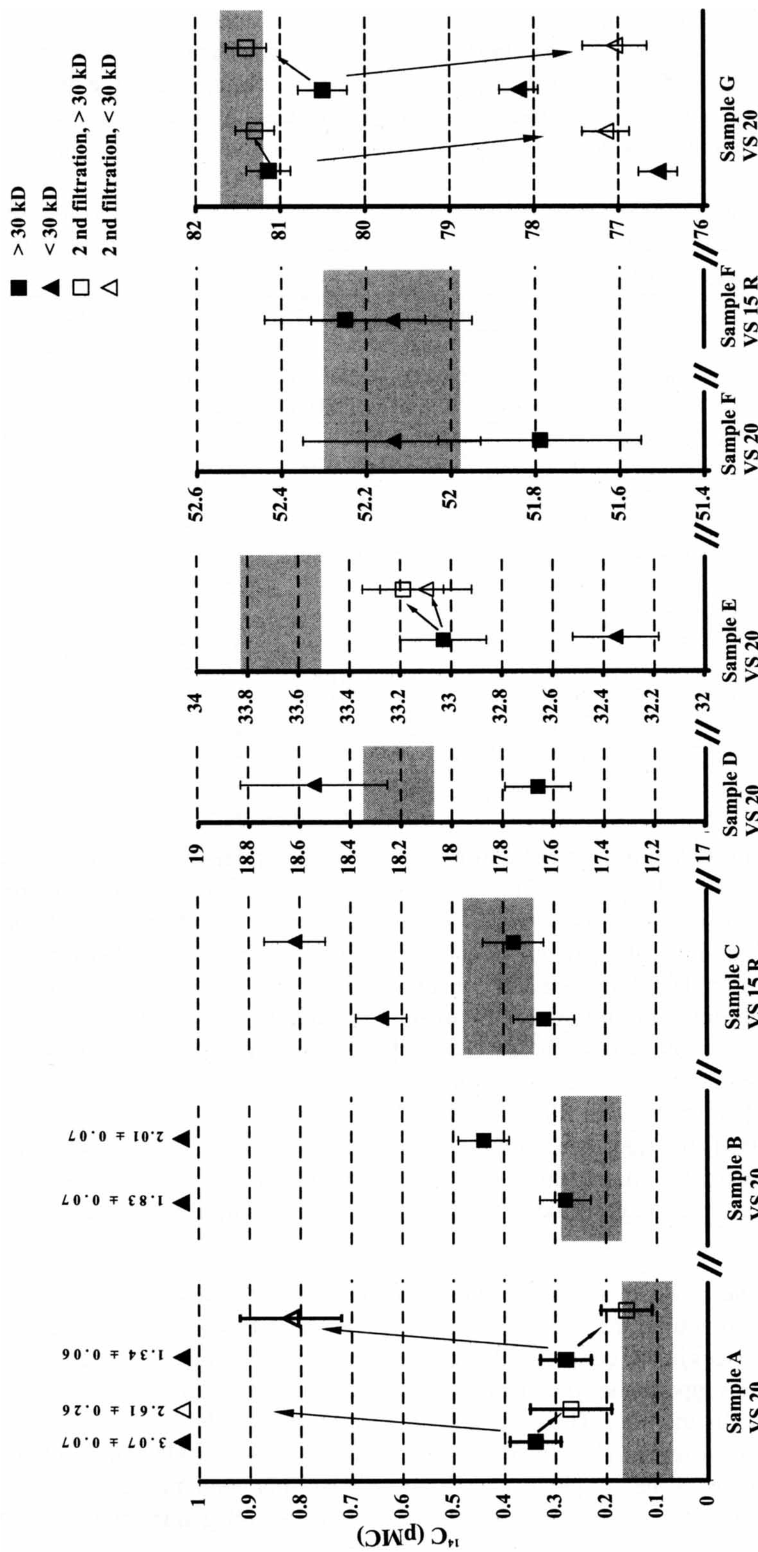

Figure 2 Results of the ^{14}C measurements of bone collagen after ultrafiltration and after repeated filtration of the >30-kD fraction. The ^{14}C concentration of the unfiltered bone collagen is referenced for each sample with a gray area (measurement ±1 σ).

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As a further check on contamination by the filtering process, we reconstructed the ^{14}C composition of the starting material from the measured mass and ^{14}C content of the 2 filter fractions by a mass balance calculation (Table 3, column 7, "Mod. UFC"). In ideal circumstances, this mass balanced-calculated ^{14}C concentration of the collagen should be similar to the ^{14}C concentration of the unfiltered collagen. One of the C samples, the D sample, and the F samples do not show statistically significant ^{14}C differences. Otherwise, older samples are shifted towards higher ^{14}C concentrations (= younger), while younger samples are shifted towards smaller ^{14}C concentrations (= older), which indicates a contribution of intermediate ^{14}C content to the measured material.

The disadvantage of old collagen for contamination tests is its unknown age and, in most cases, its limited availability. Since the filter ^{14}C concentrations are ~0.5 pMC for VS20 and ~14–15 pMC for VS15R, we used modern collagen produced in large quantities for pharmaceutical products from pig skin (MBP Medical Biomaterial Products) for additional ultrafiltration tests to investigate a possible addition of old filter carbon. First results of the ultrafiltration of 10 mg, 30 mg, and 80 mg collagen with the VS20 filter are shown in Figure 3. These results clearly indicate a contamination in the <30-kD fraction, which had a larger effect on the smaller-sized filtration fractions. Assuming a similar ^{14}C concentration for the collagen <30 kD of all ultrafiltration fractions, the calculated amount of carbon from the filters is about 0.05 mg.

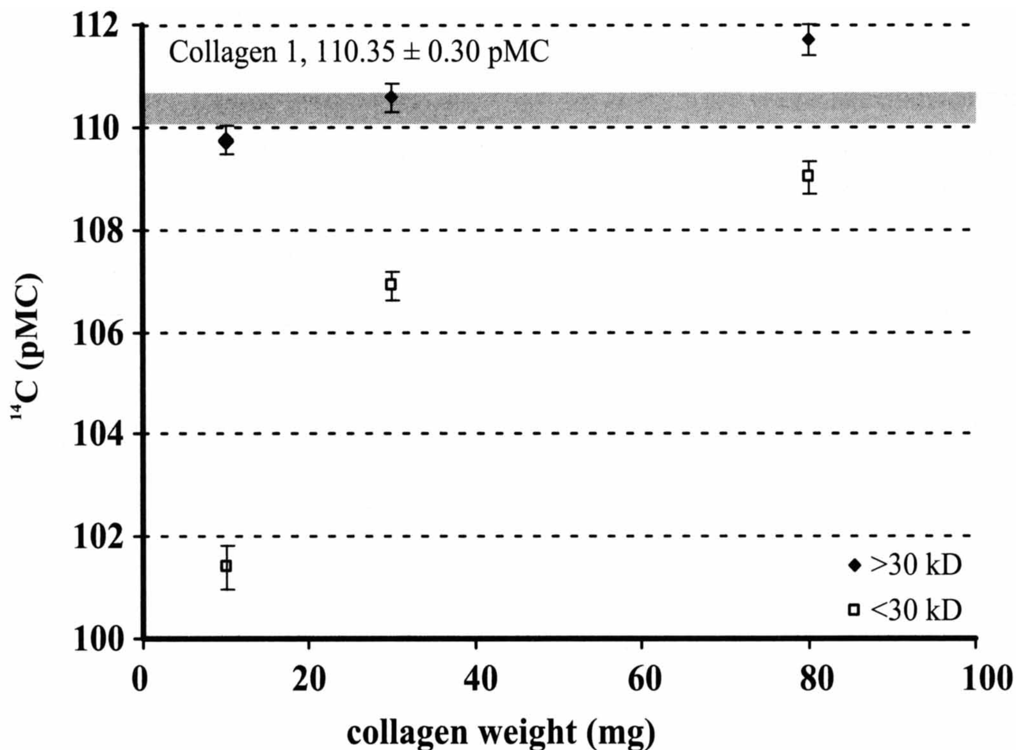

Figure 3 ^{14}C concentrations of 10 mg, 30 mg, and 80 mg of modern collagen after ultrafiltration with VS20 filters. The ^{14}C concentration of the unfiltered collagen is shown as the gray area (measurement ±1 σ).

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CONCLUSION

The 3-step filter cleaning protocol may give an almost complete removal of glycerin as indicated by carbon measurements on aliquots of the used ultrapure water after the final cleaning steps.

Beside the glycerin with young carbon, both filter types VS20 and VS15R contain fossil carbon (^{14}C concentration: VS20 ~0.5 pMC; VS15R ~11–18 pMC), a potential source for contamination during ultrafiltration. The high variability in ^{14}C of VS15R filter membranes may indicate changes in production as noted for the glycerin, which emphasize the need of control measurements before using a new batch of filters. The higher and variable ^{14}C content of the VS15R membrane makes it less desirable for ultrafiltration of old bone collagen.

The ultrafiltration of 7 freeze-dried collagen samples of varying ages revealed a statistically significant contamination with filter carbon of intermediate ^{14}C content in the <30-kD fraction. No significant contamination was seen in the seven >30-kD fractions.

Ultrafiltration of modern collagen also indicates a contamination of the <30-kD fraction by 0.05 mg carbon of intermediate ^{14}C content from the VS20 filters. The >30-kD fraction may contain small amounts of young carbon, possibly from glycerin left on the filters. Recent results (to be published in a future paper) indicate that this contribution of foreign carbon may be significant. In the meantime, we recommend caution in the use of ultrafiltration of bone collagen and prior testing of any filter type used.

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