APPENDIX D: BONE BIOAPATITE PRETREATMENT TRIALS

D.1 **Background**

Despite the drawbacks connected with bone bioapatite (see below and Chapter Three), extraction was undertaken for the purpose of comparing early forming enamel bioapatite signature to that of the potentially later forming bone bioapatite. It would also offer a similar age related signature to that of bone collagen, and therefore, more directly associated isotope signatures and collagen-bioapatite spacing than that of enamel bioapatite.

Unlike enamel bioapatite, bone bioapatite is variable in its preservation of the fossil biogenic signature. Koch *et al.* (1997) noted significant differences in $\delta^{13}C$ values from fossil bone and dentin compared to that of enamel bioapatite from the same individuals. They concluded that the differences were not related the dietary changes over life, but due to recrystallisation or contamination by non-biogenic bioapatite carbonates. Lee-Thorp and van der Merwe (1987) and Saliège *et al*. (1995), in contrast, found fossil bone bioapatite to have undergone almost no alteration. The preservation of the signature may be related to favourable depositional environment and the continued presence of bone collagen as a protective layer. As the depositional environment of the SLRB has proven to be conducive to bone and bone collagen preservation (Voigt 1983; Lee-Thorp *et al.* 1993) it was decided to further pursue the extraction of bone bioapatite.

Previous methods for bone bioapatite extraction (Lee-Thorp 1989) were modified to take into account the smaller sample size now required for mass spectrometry, and to minimize future damage to archaeological specimens. Based on enamel micro-sampling, it was necessary to establish the minimum amount of time the reduced bone sample required in NaOCl and CH_3COOH solutions. The steps of the trial are detailed in the proceeding section. The resulting protocol and mass spectrometry adheres to that outlined for enamel bioapatite (see Chapter 3), with the following exceptions. Prior to chemical pretreatment, bone was reduced to powder via Spex Mill cooled in liquid nitrogen. Bone powder samples weighing ~5mg were placed into micro-centrifuge tubes and soaked in 2ml of NaOCl solution for 3 to 4 hours, at which point reaction had ceased. The samples were then soaked in 0.5ml of CH_3COOH solution for 10 minutes.

The protocol developed from the trials, generated reliable $\delta^{13}C$ and $\delta^{18}O$ values. These values were reproduced within <1.5‰, except for $\delta^{18}O$ values for the *Bos taurus* from Schroda, which were<1.8‰. Furthermore, the bone bioapatite values were in line with enamel bioapatite values from the same individual, and had acceptable collagen-bioapatite spacing of ~5.5 to~8‰ (Krueger and Sullivan 1984; Lee-Thorp *et al*. 1989).

D.2 **Outline of Pretreatment Trials**

A series of timed trials were carried out on modern faunal bone, as well as 2 archaeological specimens, *Bos taurus* and *Ovis/Capra*, from Schroda. Bone pieces were powdered in a Spex Mill cooled by liquid nitrogen. Approximately 1000mg of bone powder from each specimen was required for the initial stage of the trial, but the sample:solution remained proportional to that for enamel bioapatite, e.g. 5mg of powder in 2ml of NaOCl . Each sample was divided into 4 subgroups of 250mg, and soaked in 40ml of NaOCl for 0.5, 1, 2 and 4 hours. Samples were frequently shook. In the modern samples between 0.5 and 4 hours reaction was still visible, but from 4 to 5 hours the reaction was weak or was completed. Specimen UCT 7119 had a further 250mg set for 24 hours. In the archaeological samples reaction was less vigorous after 2 hours and had ceased by 4 hours. In each case the NaOCl solution was replace with new solution to make sure that reaction had actually ceased, and was not the result of being spent. Due to the bone having a higher organic content than enamel, the comparatively longer NaOCl soak was expected.

After their respective soak times samples were rinsed, centrifuged and freeze-dried as per Method 2. Each subgroup was then further subdivided into three subgroups. Allots of ~5mg were weighed into micro-centrifuge tubes and treated with 0.5ml of CH_3COOH for 10, 15 and 30 minutes, respectively. One sample from each specimen was divided into four subgroups, with the additional sample being left in CH_3COOH solution for 60 minutes. As before, samples were shook frequently until finished and then rinsed, centrifuged and freeze-dried. The $\delta^{13}C$ and $\delta^{18}O$ values for each time sequence is present in Table D.1.

Fourier Transform Infra-Red (FTIR) spectroscopy analysis was conducted on selected

Table D.1: FTIR Indices

Indices	**Description**	**Bands Measured cm^{-1}**
API	The amount of “A” type carbonate to phosphate	1540/605
BPI The amount of organic to phosphate	The amount of “B” type carbonate to phosphate	1415/605
BAI	Relative amount of “B” to “A” carbonate	1415/1540
OPI(1)	The amount of organic to phosphate	1640/605
OPI(2)	The amount of organic to phosphate	1650/605
PCI	Crystallinity	(565+605)/590

extraction stages from one modern and one archaeological specimen to determine at which point both organic and inorganic contaminates were removed. To ready samples for FTIR analysis, ~1.8mg of powder was ground together with 300mg of potassium bromide (KBr) and place under 9 tons of pressure for 5 minutes to create a transparent pellet. Through irradiating the prepared sample, FTIR records the intensities of specific absorption bands, which are a reflection the organic and inorganic constituents. Sponheimer and Lee-Thorp (1999b) have set forth following indices that are useful for detecting changes in these bands (Table D.2). The other band of concern is 711cm^{-1}. A peak at this band indicates the presence of exogenous calcite.

The indices indicate for both modern and archaeological samples that after 3 hours NaOCl and 5 to 10 minutes CH_3COOH the API and OPI are approximately half the value of the untreated samples, as was the archaeological BPI (Table D.3). There was little change in theses indices between 3 and 24 hours NaOCl. Use of OPI values below 0.7 as an indicator of organic removal are consistent with those found elsewhere for bone bioapatite (M. Sponheimer 2000, pers. comm.). In the FTIR graphs a small peak still persists at 5 minutes CH_3COOH for the 711cm^{-1}, but had nearly flattened out after 3 hours/10 minutes. Based on this, it is suggested that by 15 minutes the peak should be completely removed, but there is the risk of altering the sample's composition with further time in acid.

Further sets of soaks at 3 and 4 hours for NaOH, and 10, 15, 30 minutes for CH3COOH were completed to determine if the additional soak times would alter the isotope values. This time ~5mg of bone powder was used as the starting weight. This allowed for the assessment of repeatability and whether the larger sample size used in the NaOCl step of the initial trial had a different affect on isotope values. Reactions rates were similar to that of the first trial. Results indicate a <1‰ difference in $\delta^{13}C$ and $\delta^{18}O$ values between the two time series, and <1.5‰ between the first and second trial (Table D.4). Based on the the stable isotope results in combination with the FTIR analysis and isotope data from other skeletal tissues, it is suggested that a pretreatment soak of 3 to 4 hours in NaOCl and 10 to 15 minutes in CH_3COOH would provide consistent results in both modern and archaeological samples from the research area.

Table D.2: Bone bioapatite pretreatment trial 1 $\delta^{13}C$ and $\delta^{18}O$ (‰) values

Location and Sample ID	**2% NaOCl (hours)**	**0.1M CH_3COOH (mins)**	**$\delta^{13}C$**	**$\delta^{18}0$**
Venetia - Modern Fauna				
***Aepyceros melampus*, UCT 7200**				
	0.5	10	na	na
	0.5	15	na	na
	0.5	30	-9.2	3.1
	1	10	-8.3	4.8
	1	15	na	na
	1	30	-10.4	-15.6
	2	10	-10.3	-8.6
	2	15	-10.2	-1.7
	2	30	-9.8	1.3
	4	10	-8.3	4.8
	4	15	-9.5	3.3
	4	30	-8.8	2.9
	4	60	-9.0	2.9
***Equus quaaga*, UCT 7119**				
	0.5	10	-15.8	-8.5
	0.5	15	-14.2	0.5
	0.5	15	-14.7	-2.6
	0.5	30	-14.8	-3.5
	1	10	-13.4	-4.4
	1	10	-15.1	-2.5
	1	15	-11.5	na
	1	15	-15.8	-5.7
	1	30	-12.5	-5.8
	1	30	-14.9	-5.2
	2	10	-14.9	-2.2
	2	10	-14.5	-2.3
	2	15	-12.9	-0.4
	2	15	-15.2	-3.0
	2	30	-14.3	-1.3
	4	10	-14.9	-3.0
	4	15	-14.8	-2.2
	4	30	-15.0	-2.6
	4	60	-14.4	-2.7
	24	10	-15.0	-3.0
	24	15	-15.3	-2.9
	24	30	-15.4	-3.6
	24	60	-15.3	-2.9

Table D.2 con't.

Schroda - Archaeological Fauna				
***Ovis/Capra*, TSR 4242**				
	0.5	10	-3.5	-0.1
	0.5	10	-3.8	-0.8
	0.5	15	-3.9	1.1
	0.5	30	-3.8	0.3
	1	10	-3.6	-0.7
	1	15	-3.5	0.7
	1	30	-3.7	-0.2
	2	10	-4.4	-2.1
	2	10	-4.0	-0.8
	2	15	-3.5	-0.5
	2	30	-3.6	-0.6
	4	10	-3.7	0.5
	4	15	-4.1	0.4
	4	30	-3.6	0.4
	4	60	-3.6	1.1
***Bos taurus*, TSR 4465**				
	0.5	10		
	0.5	15	-2.1	-1.0
	0.5	30	-2.4	-1.9
	1	10	-2.4	-1.7
	1	15	na	na
	1	30	na	na
	2	10	na	na
	2	15	-2.6	-1.8
	2	30	-2.2	-1.3
	4	10	-2.3	-2.4
	4	15	na	na
	4	30	-2.7	-2.3
	4	60	-2.5	-1.9

Table D.3: Bone bioapatite trial 1 FTIR indices and $\delta^{13}C$ and $\delta^{18}O$ (‰) values

Location and Sample ID	2% NaOCl (hours)	0.1M CH_3COOH (mins)	API 1540/605	BPI 1415/605	BAI 1415/1540	PCI (565+605)/590	OPI 1640/605	OPI 1650/605	$\delta^{13}C$	$\delta^{18}O$
Venetia - Modern										
***Equus quaaga*, UCT 7119**										
	0	0	0.86	0.89	1.03	2.20	0.89	0.95	-12.3	1.8
	0	5	0.86	0.79	0.92	2.34	1.01	1.05	-13.9	1.7
	0	0	0.94	0.93	1.00	2.15	0.97	1.02	-12.2	1.9
	3	0	0.45	0.78	1.74	2.81	0.37	0.37	-12.4	-0.7
	4	0	0.46	0.79	1.73	2.74	0.37	0.38	-12.4	-1.7
	24	0	0.61	0.90	1.49	2.54	0.54	0.55	-12.5	-1.8
	0	5	0.85	0.84	0.99	2.33	0.95	1.03	-13.6	1.8
	0	10	0.89	0.87	0.98	2.25	0.98	1.05	-13.4	1.6
	0	60	0.80	0.79	0.98	2.51	1.02	1.06	-12.3	2.5
	3	5	0.48	0.71	1.48	2.78	0.51	0.52	-13.8	0.2
	3	10	0.46	0.70	1.52	2.81	0.47	0.49	-14.0	-0.9
	3	60	0.46	0.70	1.52	2.81	0.47	0.48	-14.1	-0.6
Schroda - Archaeological										
Ovis/Capra TSR 4242										
	0	0	0.66	1.44	2.18	2.50	0.64	0.67	-2.2	-0.6
	3	0	0.43	1.48	3.45	2.75	0.37	0.36	-3.0	-1.0
	4	0	0.39	1.35	3.41	2.73	0.33	0.32	-2.9	-0.7
	3	5	0.57	0.87	1.53	2.57	0.53	0.53	-3.7	0.9
	3	10	0.36	0.77	2.14	2.92	0.32	0.30	-3.7	0.7
	3	60	0.37	0.73	1.96	2.92	0.34	0.33	-4.0	2.3

Table D.4: Bone bioapatite trial 2 $\delta^{13}C$ and $\delta^{18}O$ (‰) values

Location and Sample ID	**2% NaOCl (hours)**	**0.1M CH_3COOH (mins)**	**$\delta^{13}C$**	**$\delta^{18}0$**
Mara - Modern Fauna				
***Ovis/Capra*, UCT 7423**				
	0	0	-1.3	-1.5
	2.5	0	-2.2	0.1
	3	0	-2.2	-0.2
	4	0	-2.4	-0.9
	3	10	-0.9	2.0
	3	15	-0.9	1.4
	3	30	-1.0	1.6
	4	10	-1.4	0.6
	4	15	-1.2	1.1
	4	30	-1.3	0.7
Mara - Modern Fauna				
***Bos taurus*, UCT 7434**				
	0	0	-1.5	0.7
	2.5	0	-3.4	-1.9
	2.5	0	-3.3	-2.6
	3	0	-3.1	-2.2
	4	0	-3.2	-2.7
	5	0	-3.6	-2.1
	3	10	-2.0	-0.8
	3	15	-1.7	0.1
	3	30	-2.0	-0.6
	4	10	-2.1	-1.0
	4	15	-1.9	-1.0
	4	30	-1.8	-0.8
Venetia –Modern Fauna				
***Aepyceros melampus*, UCT 7200**				
	0	0	-7.9	6.2
	2.5	0	-8.6	3.2
	3	0	-8.5	2.0
	4	0	-9.9	-1.6
	5	0	-8.3	2.5
	3	10	-8.1	4.5
	3	15	-8.0	3.3
	3	30	-8.4	3.3
	4	10	-8.0	4.7
	4	15	-8.0	4.7
	4	30	-8.2	3.9

Table D.4 con't.

Venetia - Modern Fauna				
***Connochaetes taurinus*, UCT 7260**				
	0	0	-2.0	4.5
	2.5	0	-3.7	0.4
	3	0	-3.4	0.7
	3	0	-3.5	0.4
	4	0	-3.6	0.2
	5	0	-3.8	0.8
	3	10	-2.8	2.4
	3	15	-2.6	2.3
	3	30	-2.3	2.8
	4	10	-2.7	2.2
	4	15	-2.6	1.8
	4	30	-2.6	2.4
Balerno - Modern Fauna				
***Bos taurus*, UCT 7289**				
	0	0	-3.9	2.1
	2.5	0	-5.0	-0.5
	3	0	-4.8	-0.6
	4	0	-5.0	-0.9
	5	0	-5.1	-1.0
	3	10	-4.3	1.1
	3	15	-4.4	0.6
	3	30	-4.3	0.6
	4	10	-4.4	0.2
	4	15	-4.4	0.7
	4	30	-4.1	0.7
SLRB - Modern Fauna				
***Equus quaaga*, UCT 7119**				
	0	0	-13.2	-0.2
	3	0	-13.0	-2.3
	4	0	-12.8	-2.0
	5	0	-12.4	-2.1
	3	10	-13.8	-0.4
	3	15	-13.2	0.0
	3	30	-13.9	-0.1
	4	10	-14.1	-0.5
	4	15	-13.7	-1.1
	4	30	-14.0	-0.5

Table D.4 con't.

Schroda - Archaeological Fauna				
***Ovis/Capra*, TSR 4242**				
	0	0	-2.3	0.3
	2.5	0	-3.0	-1.6
	2.5	0	-3.3	-1.7
	3	0	-3.0	0.0
	4	0	-3.1	-1.8
	4	0	-3.4	-2.0
	5	0	-3.4	-2.2
	3	10	-3.7	1.8
	3	15	-3.6	1.3
	3	30	-3.9	1.7
	4	10	-3.5	1.1
	4	15	-3.7	1.1
	4	30	-3.9	1.6
Schroda - Archaeological Fauna				
***Bos taurus*, TSR 4465**				
	0	0	-2.8	-2.1
	2.5	0	-3.2	-2.3
	3	0	-3.2	-3.0
	3	0	-3.2	-3.2
	4	0	-3.2	-2.8
	4	0	-3.5	-3.5
	5	0	-3.5	-2.0
	3	10	-2.1	-1.1
	3	15	-1.7	-0.4
	3	30	-1.6	0.0
	4	10	-1.9	-0.7
	4	15	-1.8	0.0
	4	30	-1.7	0.1