

**An enhanced *in vitro* model to study the
effectiveness of dentinal fluid on remineralization**

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Appendix 1: Protocols for Chapter 3

Protocol to make acetate demineralizing solution

An acetate buffered demineralizing solution (ten Cate, 1982), partially saturated with respect to calcium and phosphate ion contains

50 mmol/l acetate, 2.2 mmol/l Ca, 2.2 mmol/l PO₄ at pH 4.3

For 2.2 mmol/l Ca and 2.2 mmol/l phosphate we need 2.2 mmol/l CaHPO₄

For 1 l of solution:

$$2.2 \text{ mmol CaHPO}_4 = 2.2 \times 10^{-3} \text{ mol} = 2.2 \times 10^{-3} \times 136.06 \text{ g} = \underline{0.2993 \text{ g CaHPO}_4}$$

$$50 \text{ mmol acetate} = 50 \text{ mmol pure acetic acid} = 0.05 \text{ mol} = 0.05 \times 60.05 \text{ g} = 3.0025 \text{ g}$$

The volume of pure acetic acid would be 3.0025 g/1.053 g/l = 2.851 ml pure acetic acid

Heat (just enough to warm the solution) and stir in 250 ml conical flask till the CaHPO₄ is dissolved.

Put into 1 l flask or beaker and make up to 900 ml with Distilled De-ionized Water.

Adjust pH to 4.3 using 10% Na OH drop by drop. Use a magnetic stirrer while adjusting the pH.

Make up to 1 l using Distilled De-ionized Water.

Add a few grains of Thymol to solution to prevent growth of mould (0.01%).

Store in bottles sealed tightly at room temperature.

Sample preparation and dehydration protocol

Sectioning samples: Samples from experiments were sectioned mesio-distally to 1.5mm thickness sections using an IsoMet® Slow Speed Saw (Buehler USA) with a Diamond Watering Blade (Van Moppes, England).

Dehydrating samples:

Fixing samples

- Fixative solution was prepared by the staff of Adelaide Microscopy. The contents of fixative solution were 1.25% glutaraldehyde, 4% paraformaldehyde, 4% sucrose, two

drops of 1M sodium hydroxide and the solution was made up to 100ml with phosphate buffered saline.

- Place the sample in a labeled plastic 5ml vial
- Using a pipette, slowly add fixative solution (made less than 1 month prior to use) to the containers of samples so that the fixative solution only just immerses the slices. Leave to fix in fridge overnight.
- Store the remainder fixative solution in fridge in a labeled bottle with the date it was made on it.

Washing samples

- The following day, pipette off the fixative solution from each sample **in the fume hood**, and **discard the fixative solution** in the fix waste bottle at Adelaide Microscopy.
- Replace the fixative solution with washing buffer (use 4 X volume of sample) stored in the Adelaide Microscopy fridge.
- Agitate the bottle by hand to ensure contact with all slides of the tooth slice.
- Keep samples in the fridge between washes.
- Continue with washing after 30 minutes (or leave in fridge until next working day) by replacing the buffer solution.
- Another 30 minutes later replace the buffer for a second time.
- Discard waste into fixative waste bottle.

Formula of fixative solution made by a staff in Adelaide, microscopy

Formula of washing buffer solution made by a staff in Adelaide Microscopy

Dehydrating samples

- Add correct amounts of dry ethanol and distilled water to make up the required solutions. To avoid contamination, never pipette straight from the Winchester bottle.
- In the fume hood, replace the washing buffer with 25% ethanol.
- Discard the washing buffer into the fixative waste container.
- Place samples in the fridge between solution changes.
- Ethanol waste is discarded in the alcohol waste bottle.
- Dehydrate with the following ethanol concentrations:
 - 25% for 20 minutes

- 50% for 20 mins
- 75% for 20 mins (if required, the samples may be left overnight at this point).
- 95% for **30** mins, and
- 100% for **1 hour**.
- After being dehydrated using ethanol, each sample was placed in a separate vial allowed ethanol to evaporate and then samples were ready to be embed into epoxy resin.

Protocol to polish samples using Abraham polishing machine

Put six samples into a holder.

Polish the base of samples using sandpaper # 180, program 1, for 3 minutes

Polish the samples

- Using sandpaper # 500, program 1 with 10N load, for 1 minute. Wash the samples with tap water. Check the samples under microscope until the whole sample are exposed.
- Change to program 3 with 20 N load, using diamond disc # 15 microns with diamond paste # 15 microns and lubricant to polish the samples for 5 minutes. Add lubricant drop by drop. Wash the samples carefully with tap water after polishing. Dry the sample before the next polishing.
- Change to program 4 with 20N load, using diamond disc # 3 microns with diamond paste # 3 microns and lubricant to polish the samples for 3 minutes. Add lubricant drop by drop. Wash the samples carefully with tap water after polishing. Dry the sample before the next polishing.
- Change to program 5 with 20N load, using diamond disc # 1 micron with diamond paste # 1 micron and lubricant to polish the samples for 3 minutes. Add lubricant drop by drop. Wash the samples carefully with tap water after polishing and check the samples under the microscope to see if the smooth surface was achieved.

Appendix 2: Additional Figures for Chapter 3

Figure 40 shows Alizarin red penetrated into the artificial demineralized dentine. The area close to the pulp horn shows more Alizarin red.



Figure 41: Penetration of Alizarin red into artificial demineralized dentine

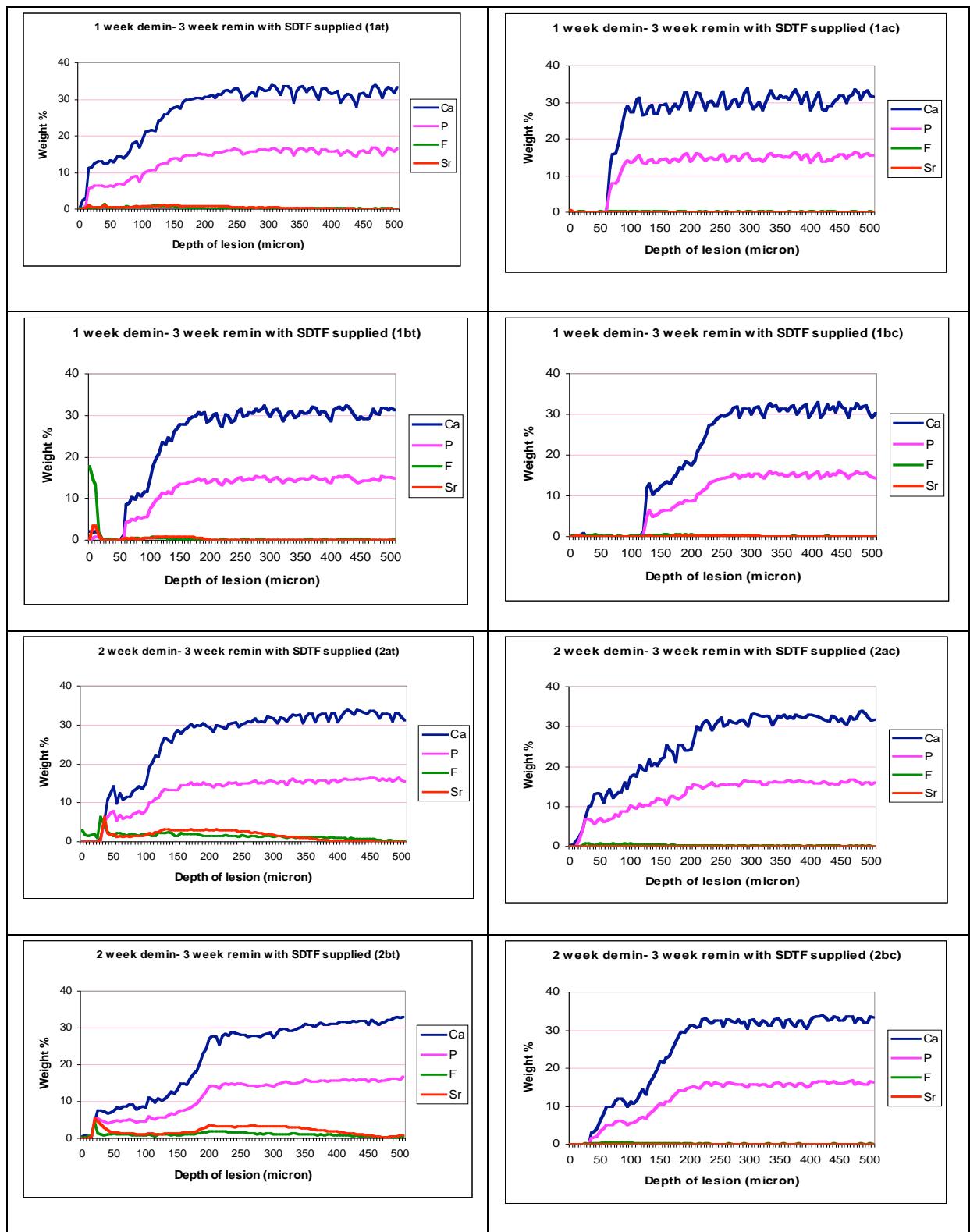
Figure 41 shows that silver nitrate did not penetrate well into dentine as Alizarin red did. The left corner of the pulp chamber shows no penetration of silver nitrate. It could be because an air bubble was present when silver nitrated was perfused into the pulp chamber.

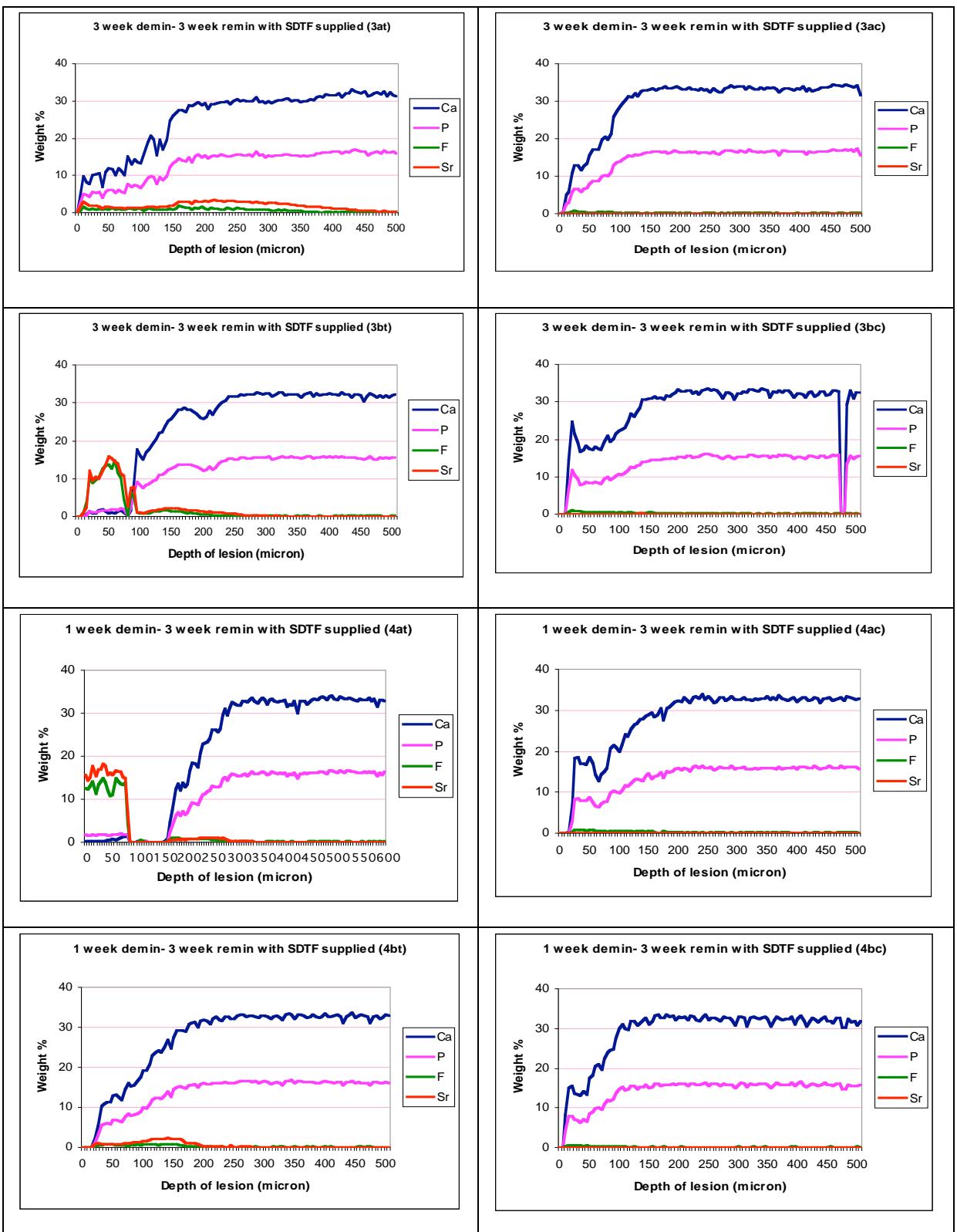


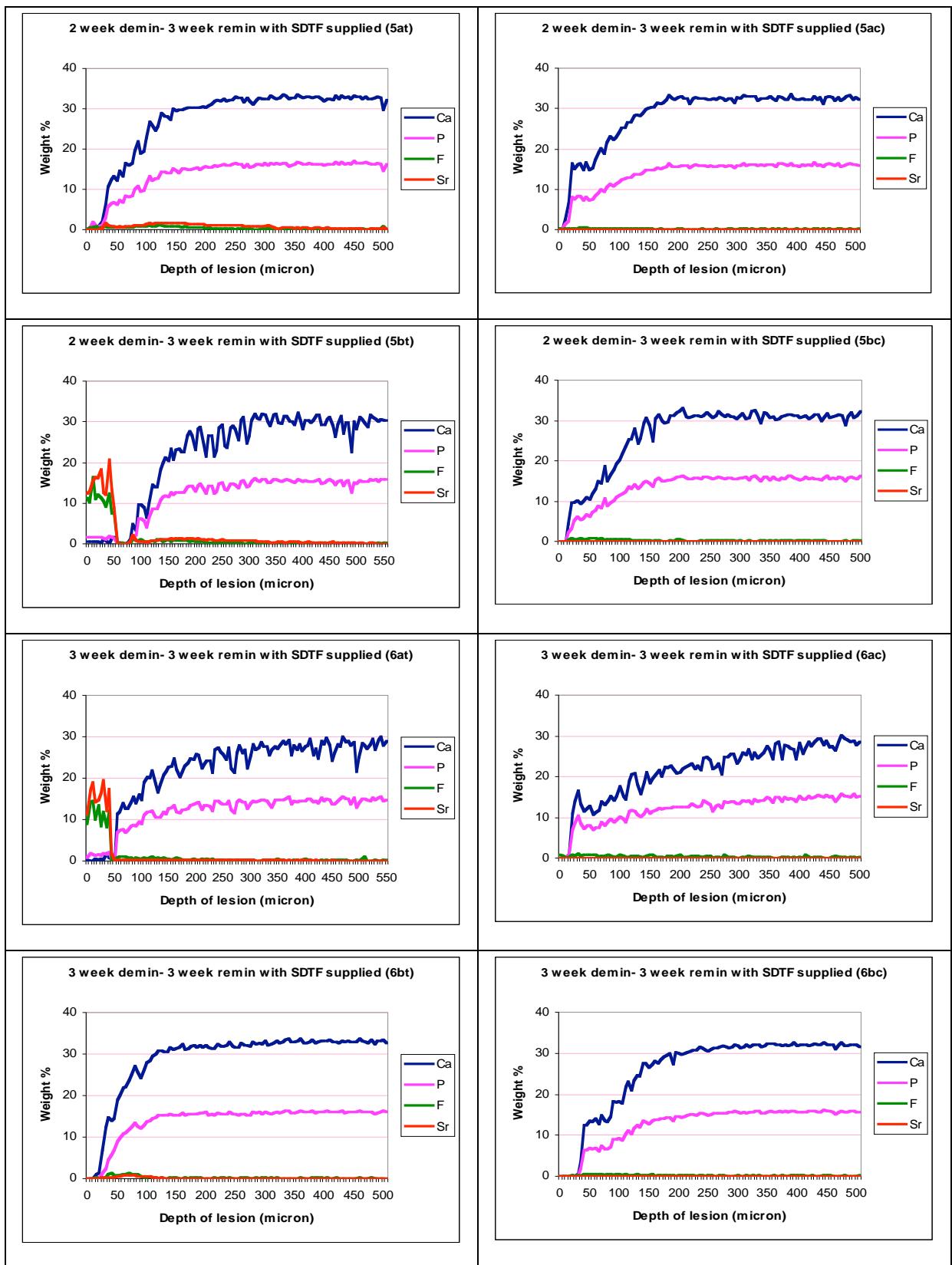
Figure 42: Penetration of silver nitrate into dentine

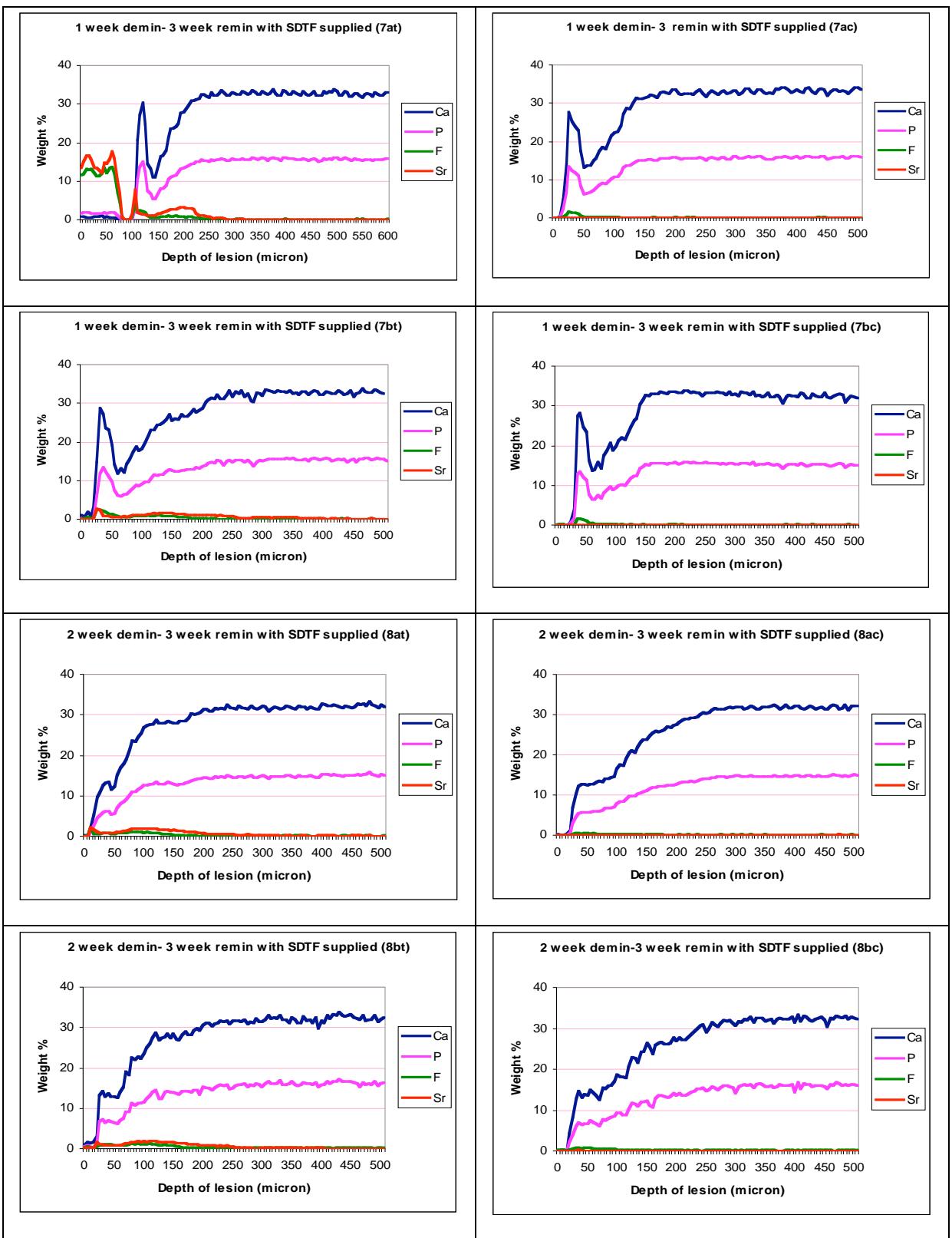
Appendix 3: Additional results for Chapter 5

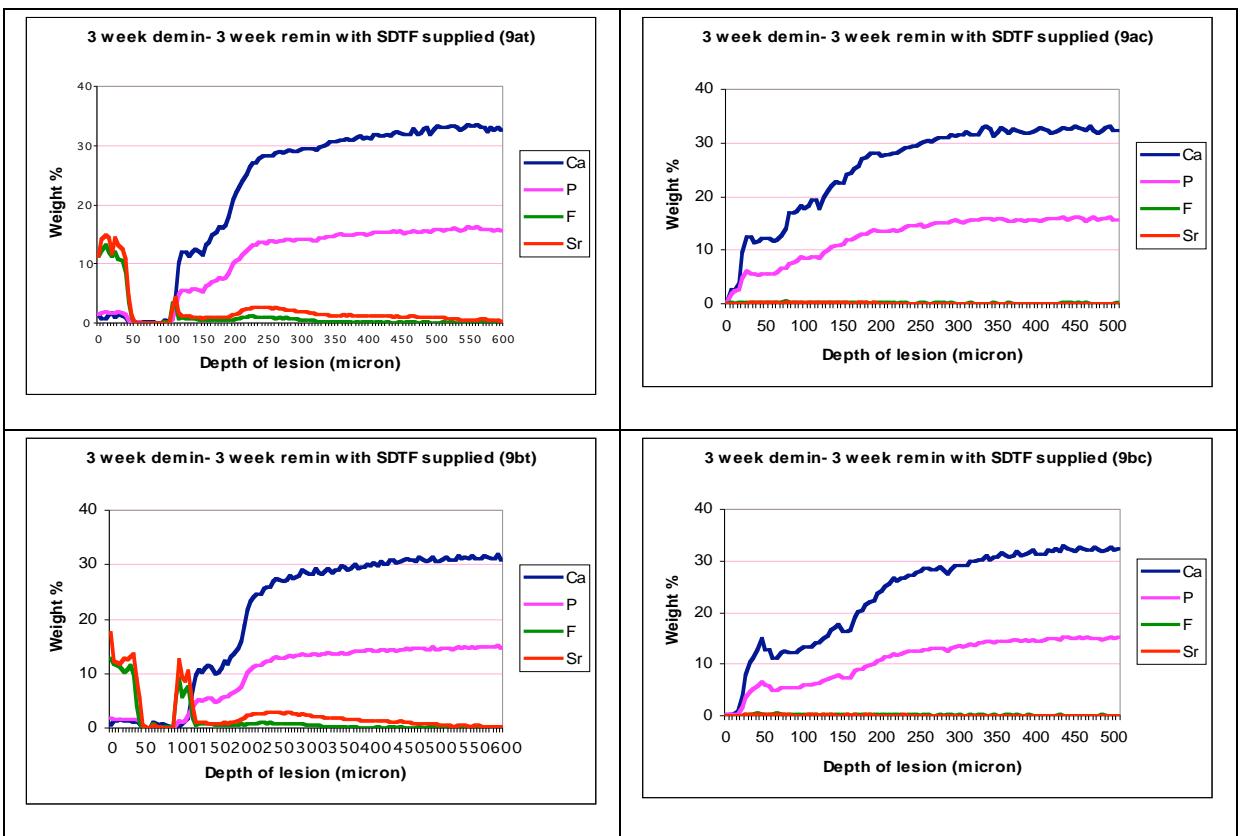
Mineral profiles of samples restored in Fuji IX and supplied with SDTF





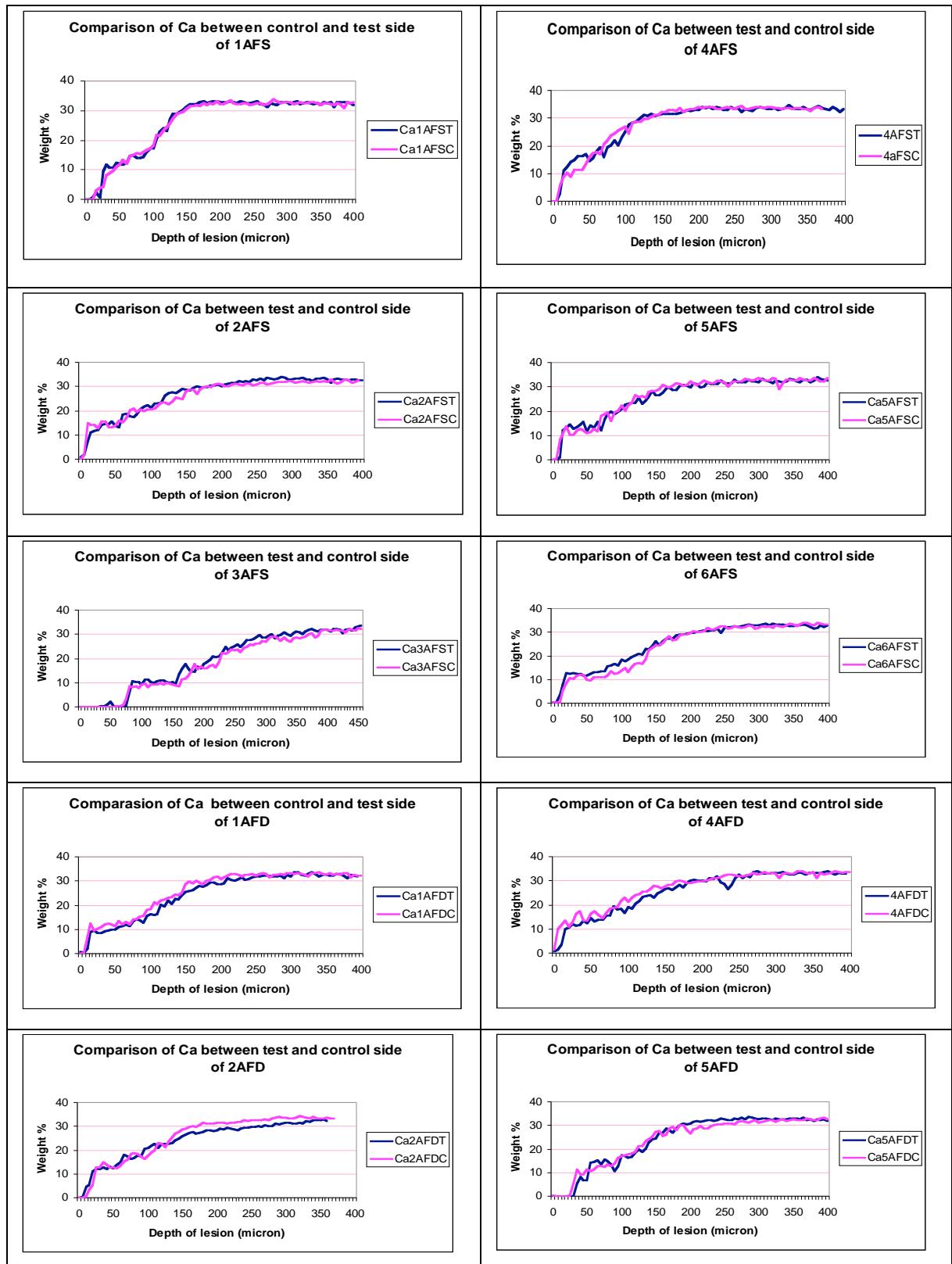


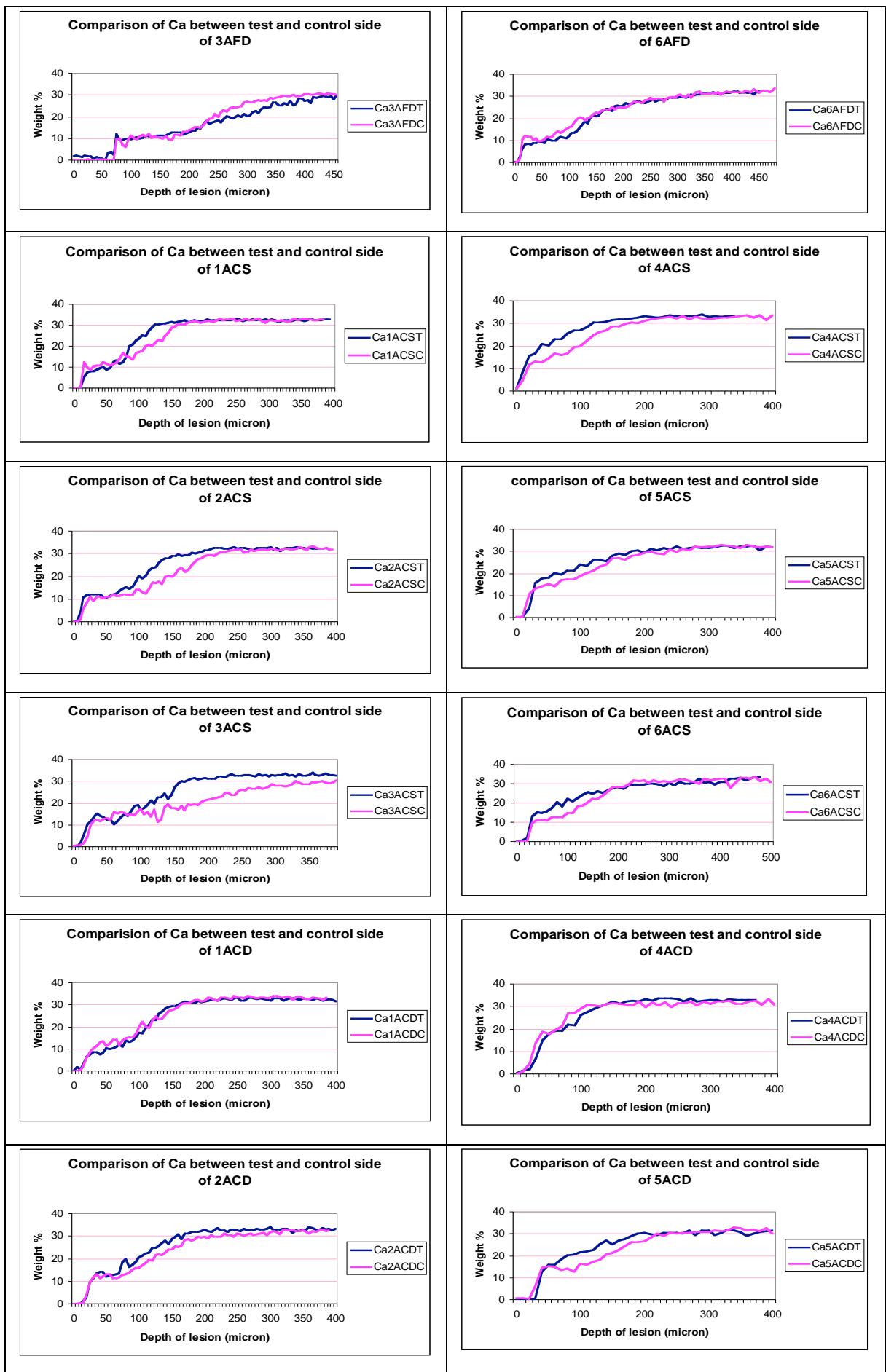


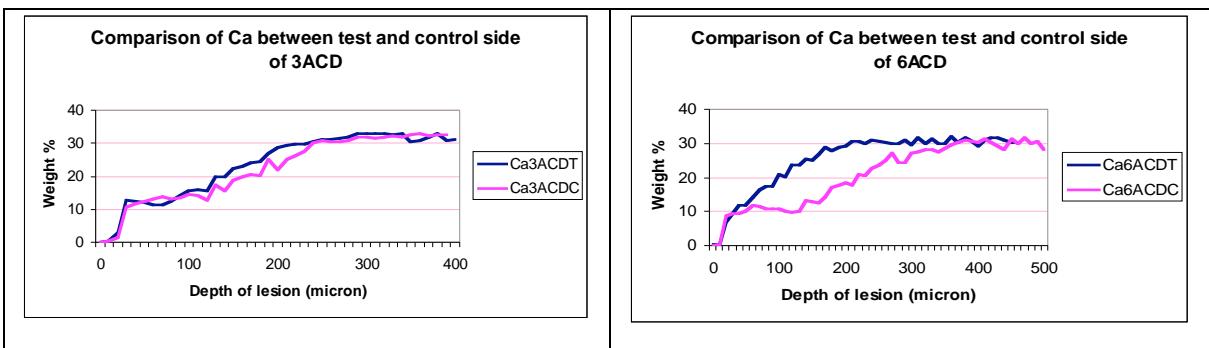


Appendix 4: Additional results for Chapter 6

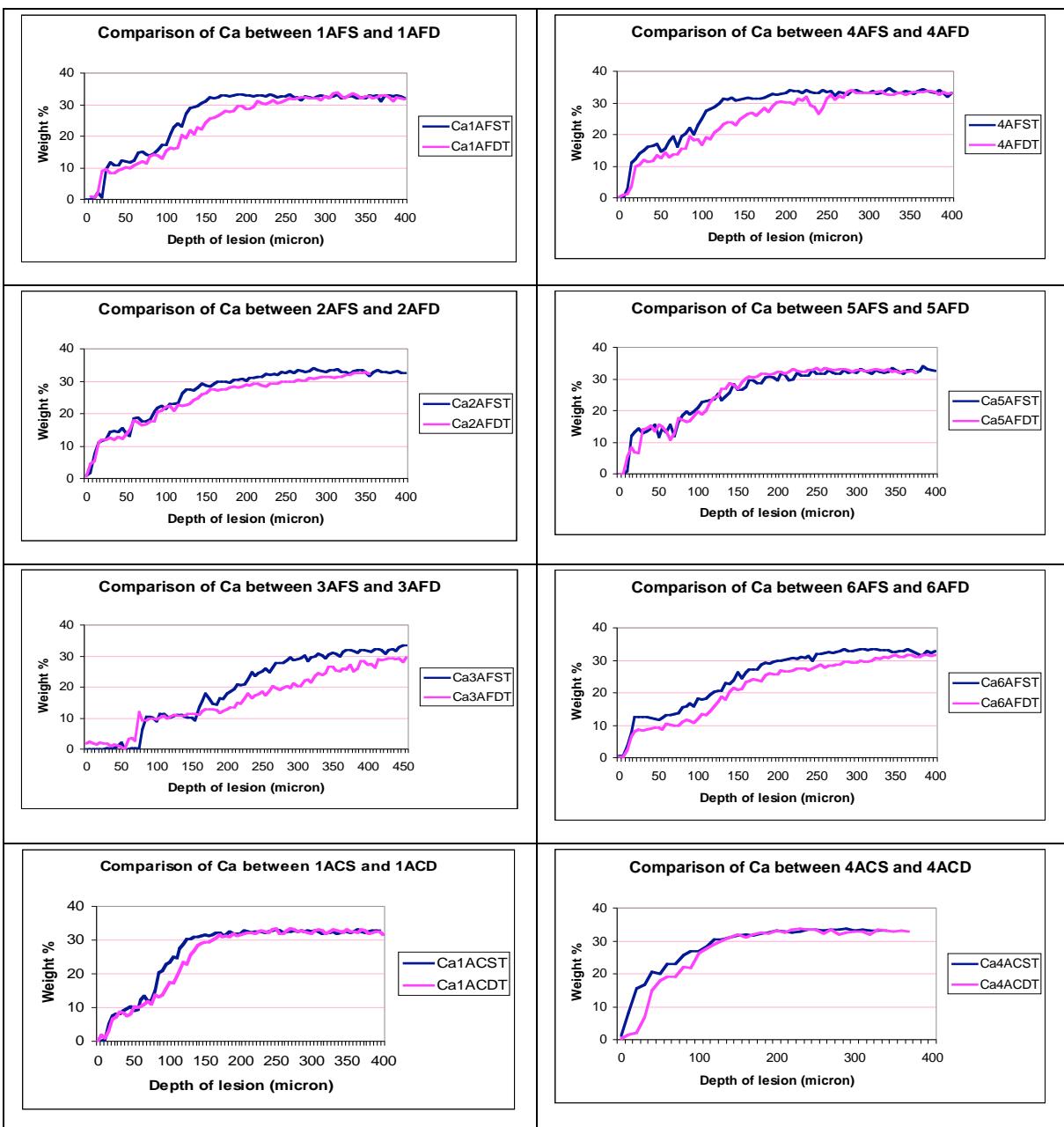
Comparison of calcium profiles between the test and control sides of all samples

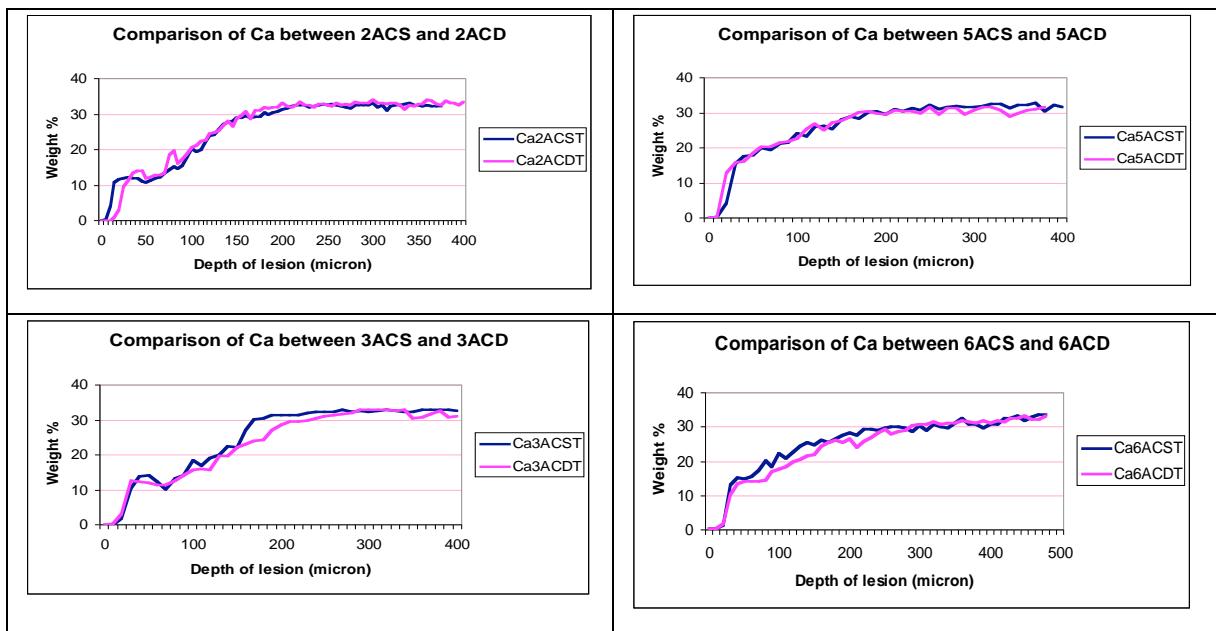






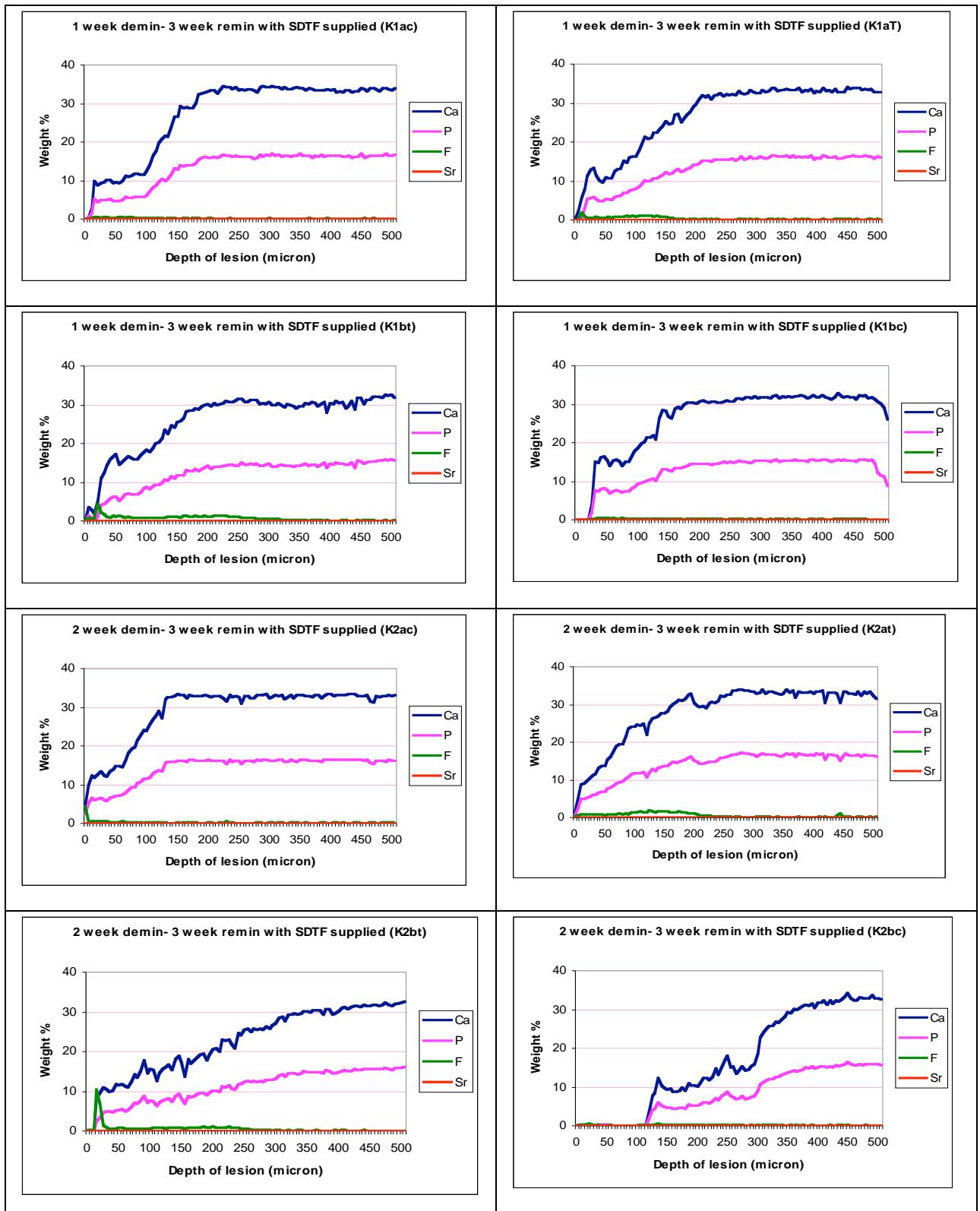
Comparison of calcium profiles between the samples supplied with SDTF/DDW

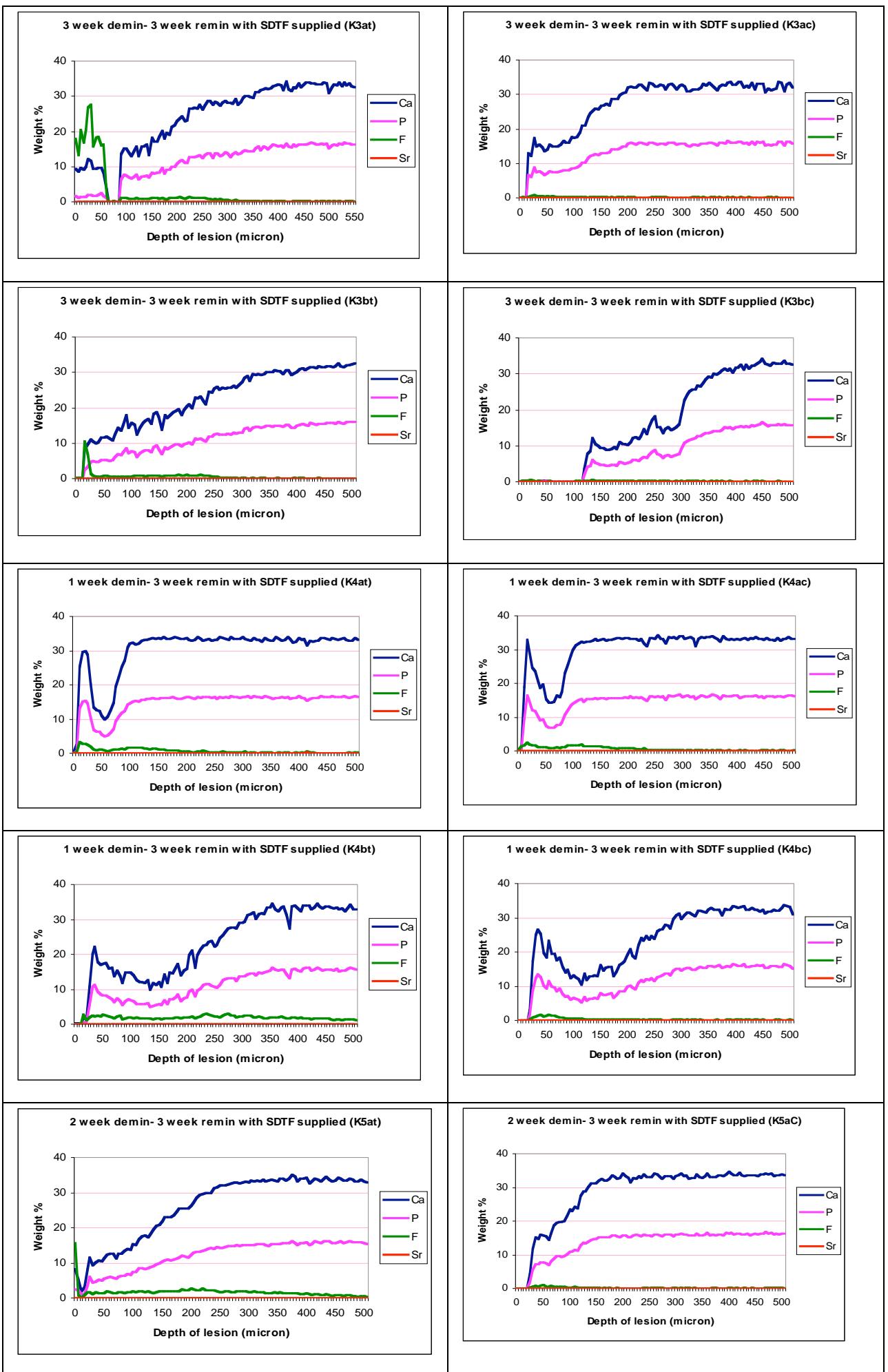


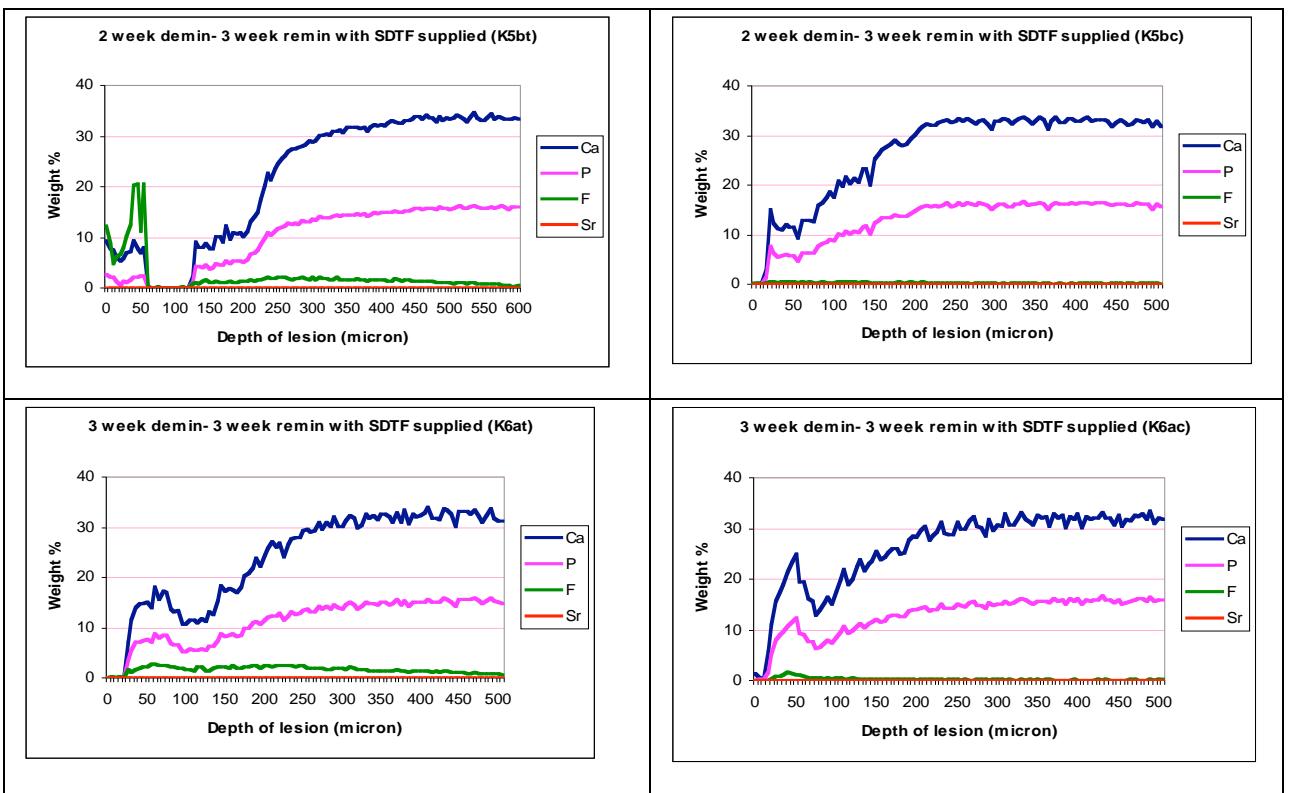


Appendix 5: Additional results for Chapter 7

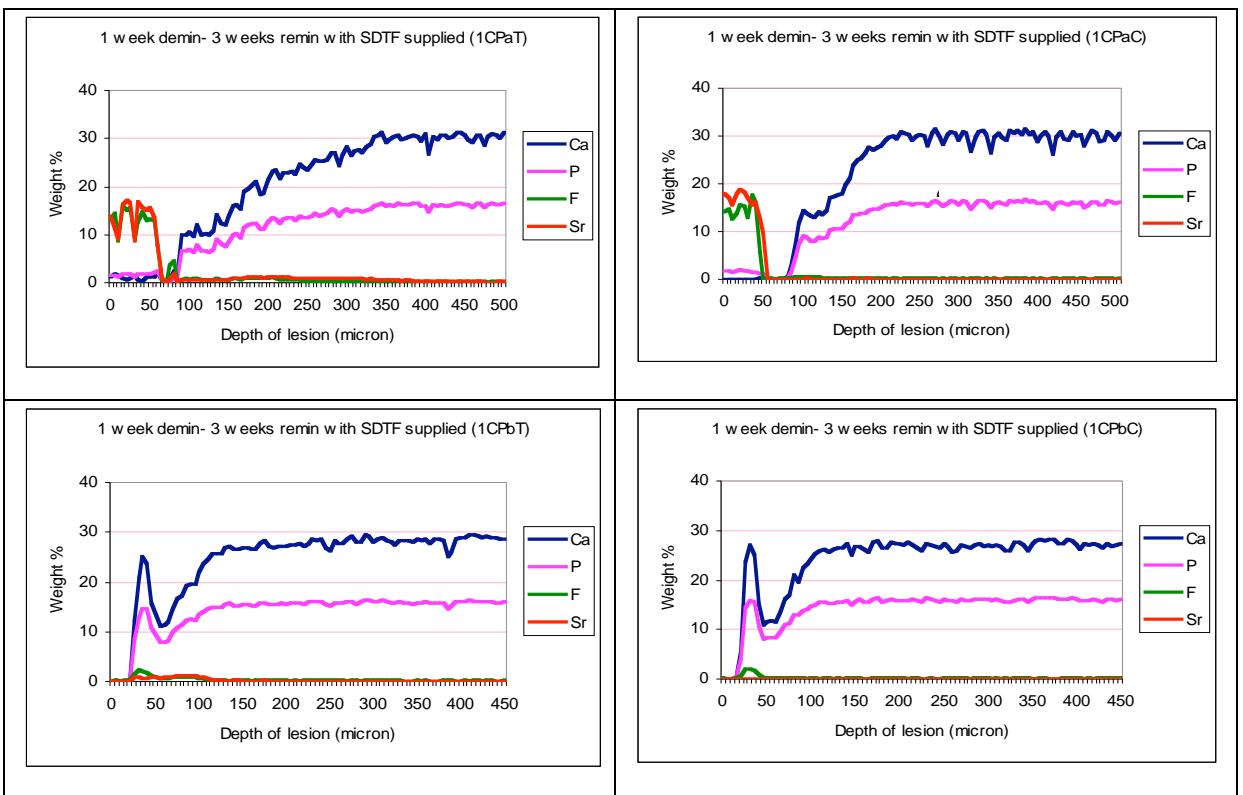
Mineral profiles of samples restored in Ketac Molar and supplied with SDTF

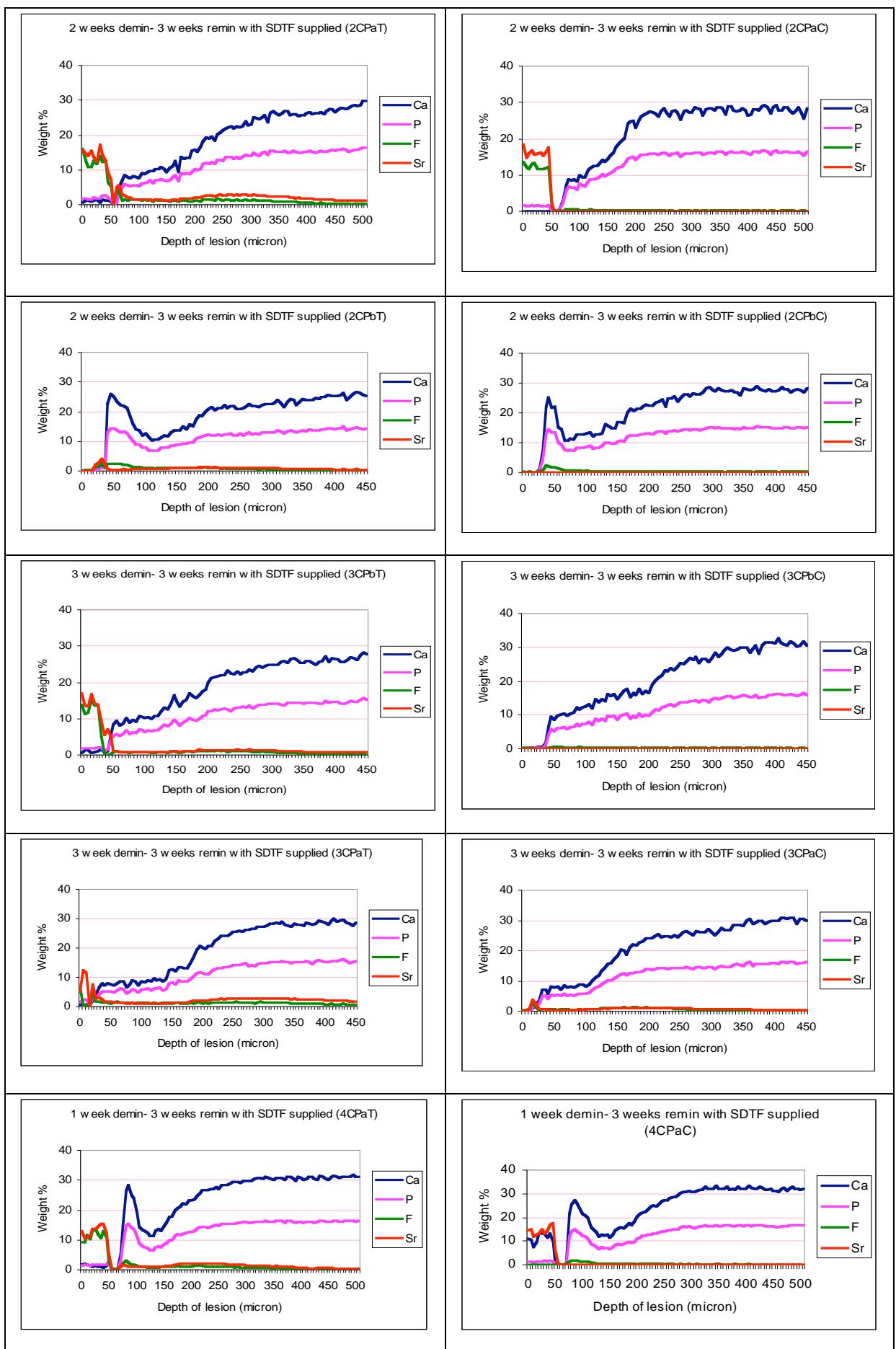


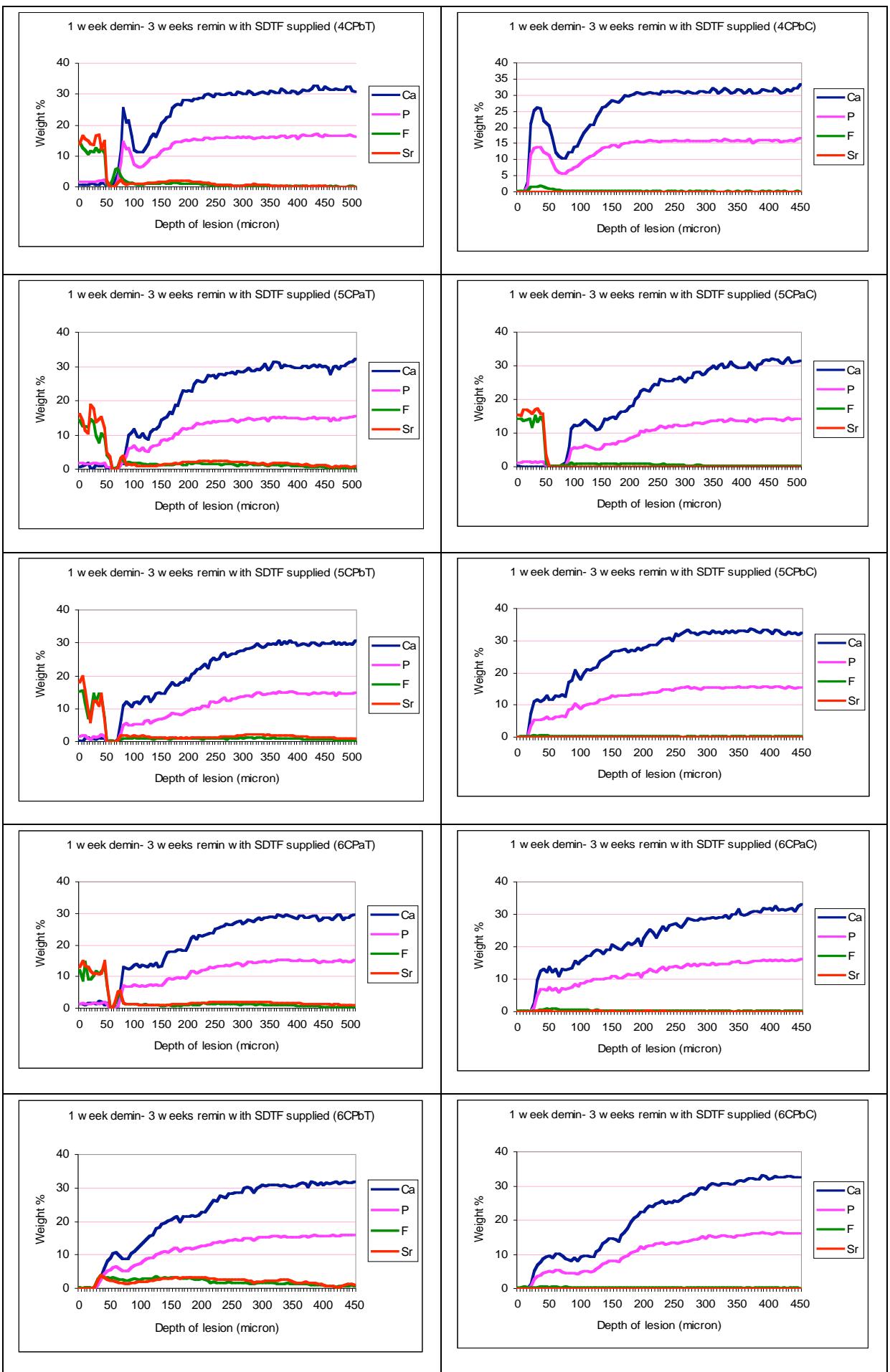




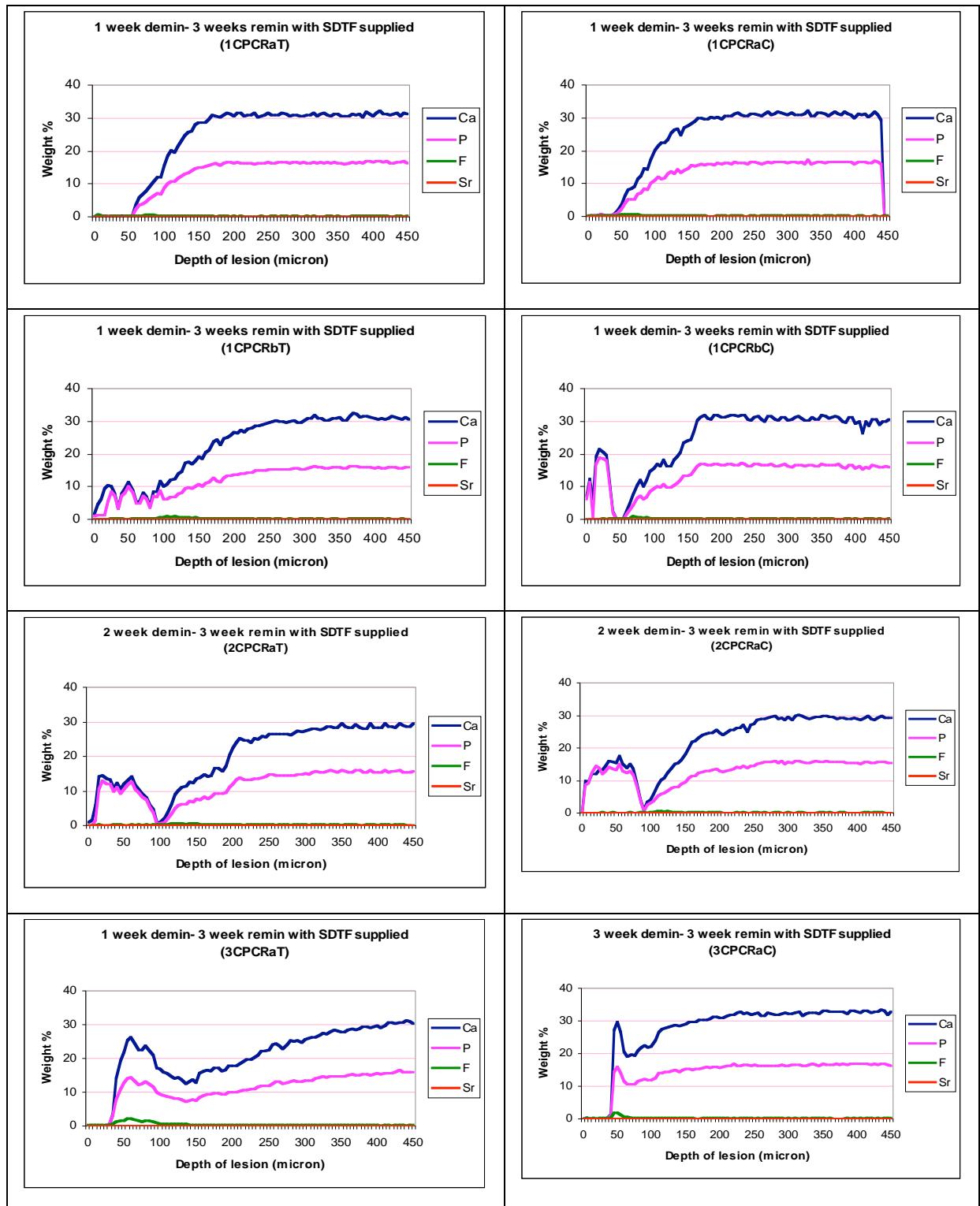
Mineral profiles of samples restored in Fuji IX with CPP-ACP application and supplied with SDTF

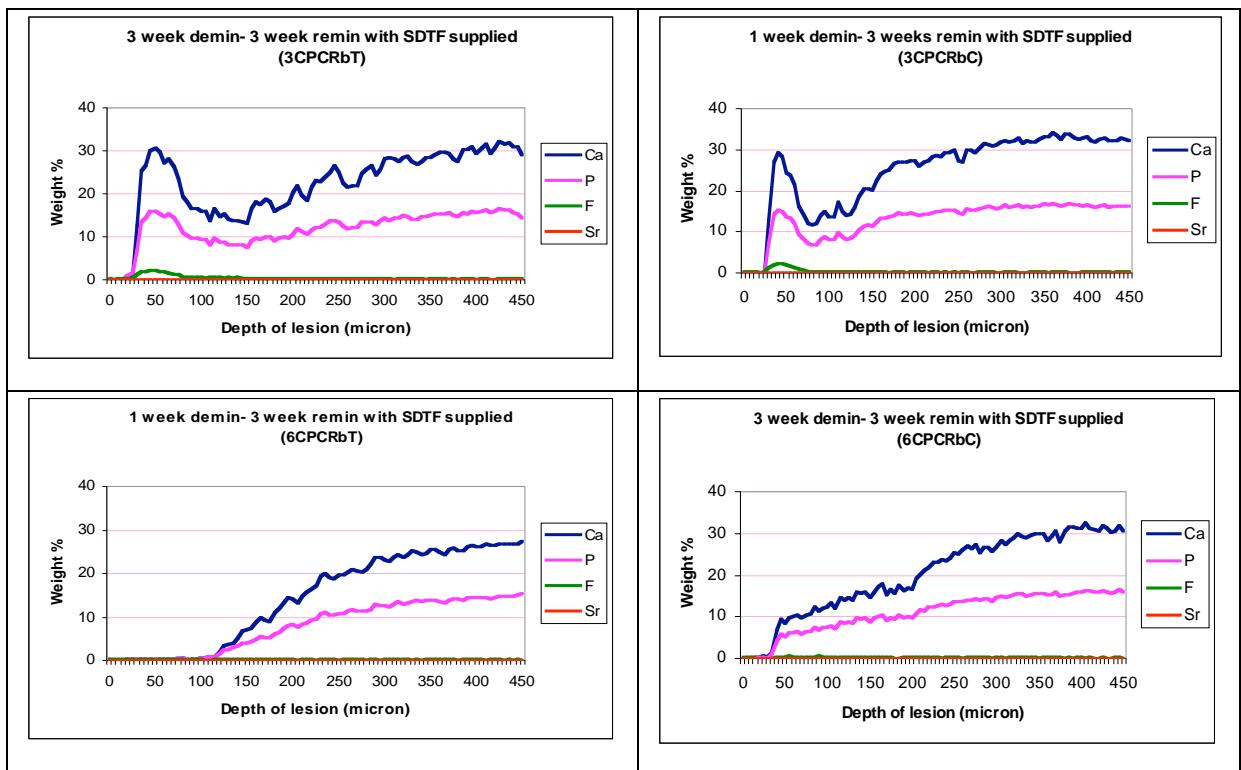




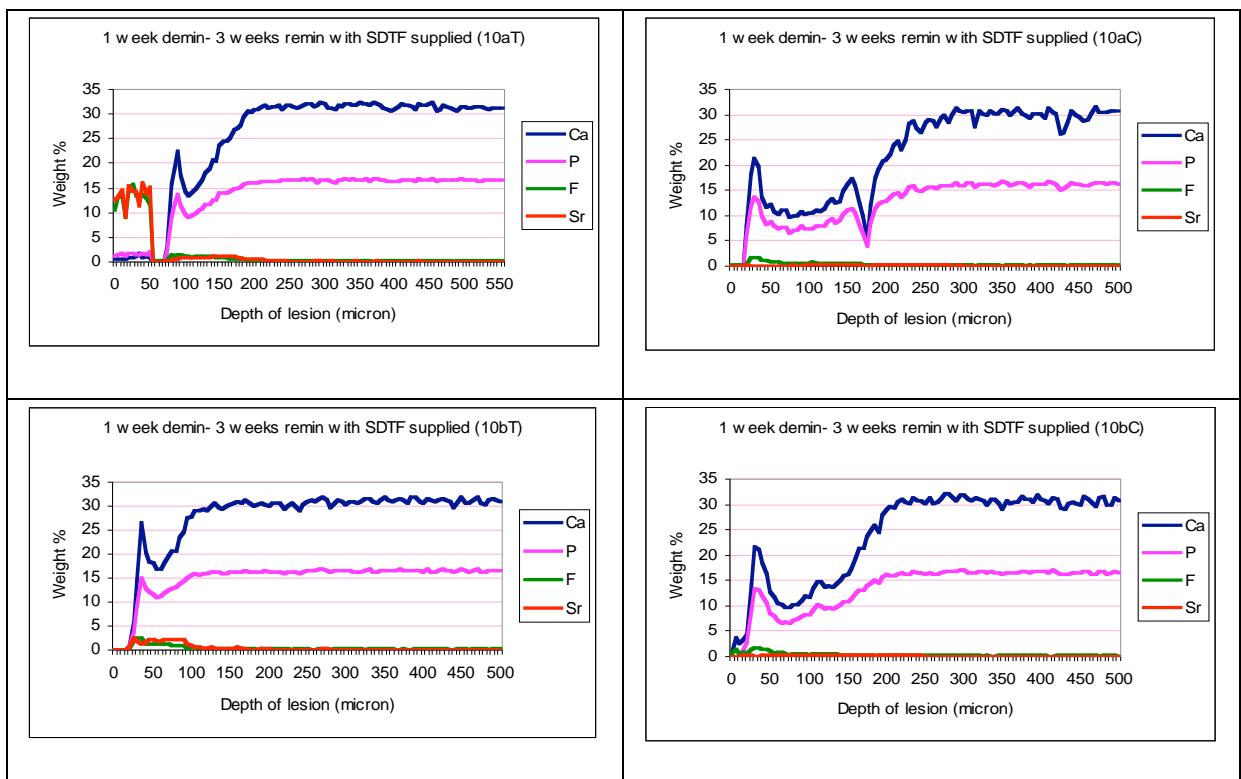


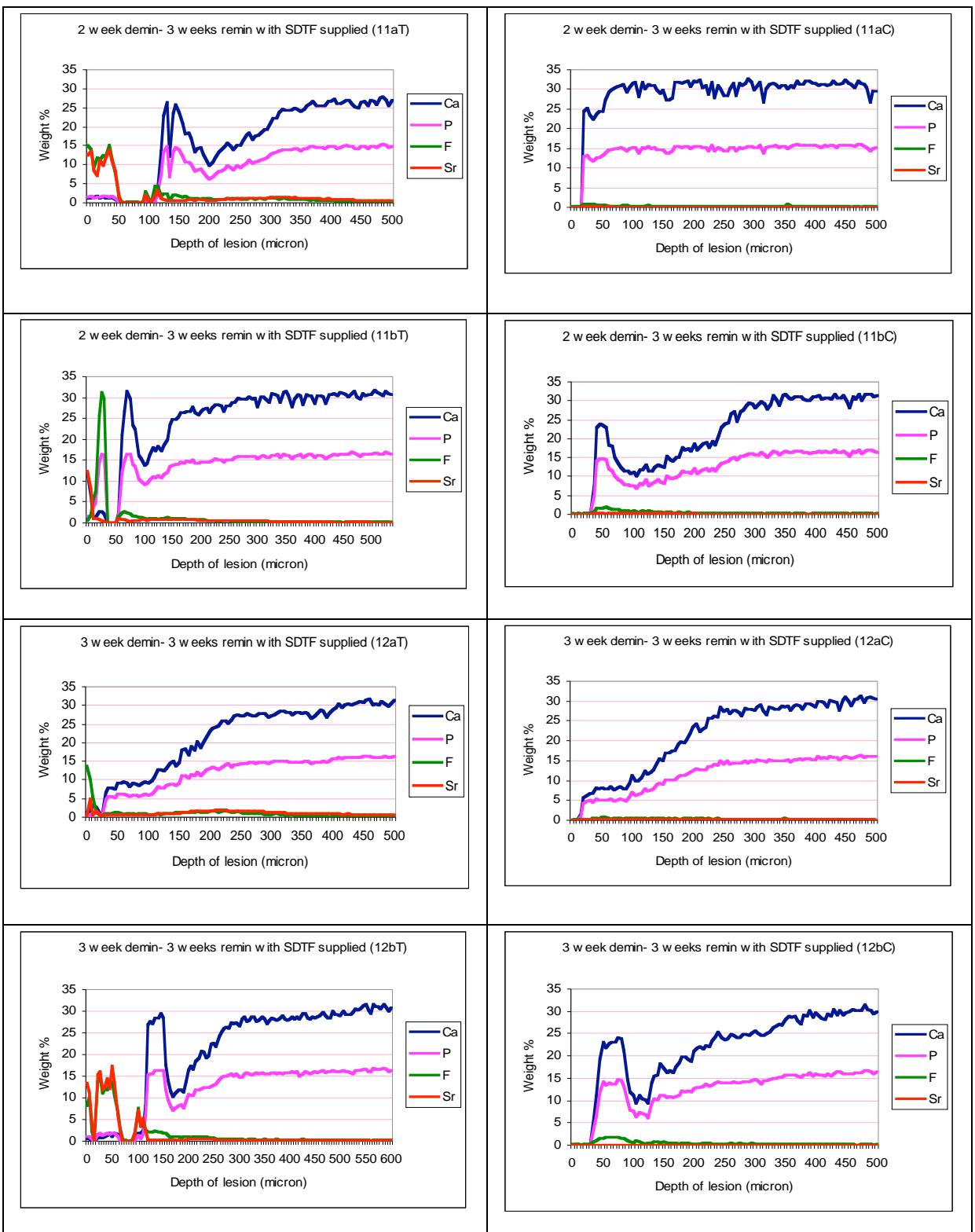
Mineral profiles of samples restored in composite resin with CPP-ACP application and supplied with SDTF



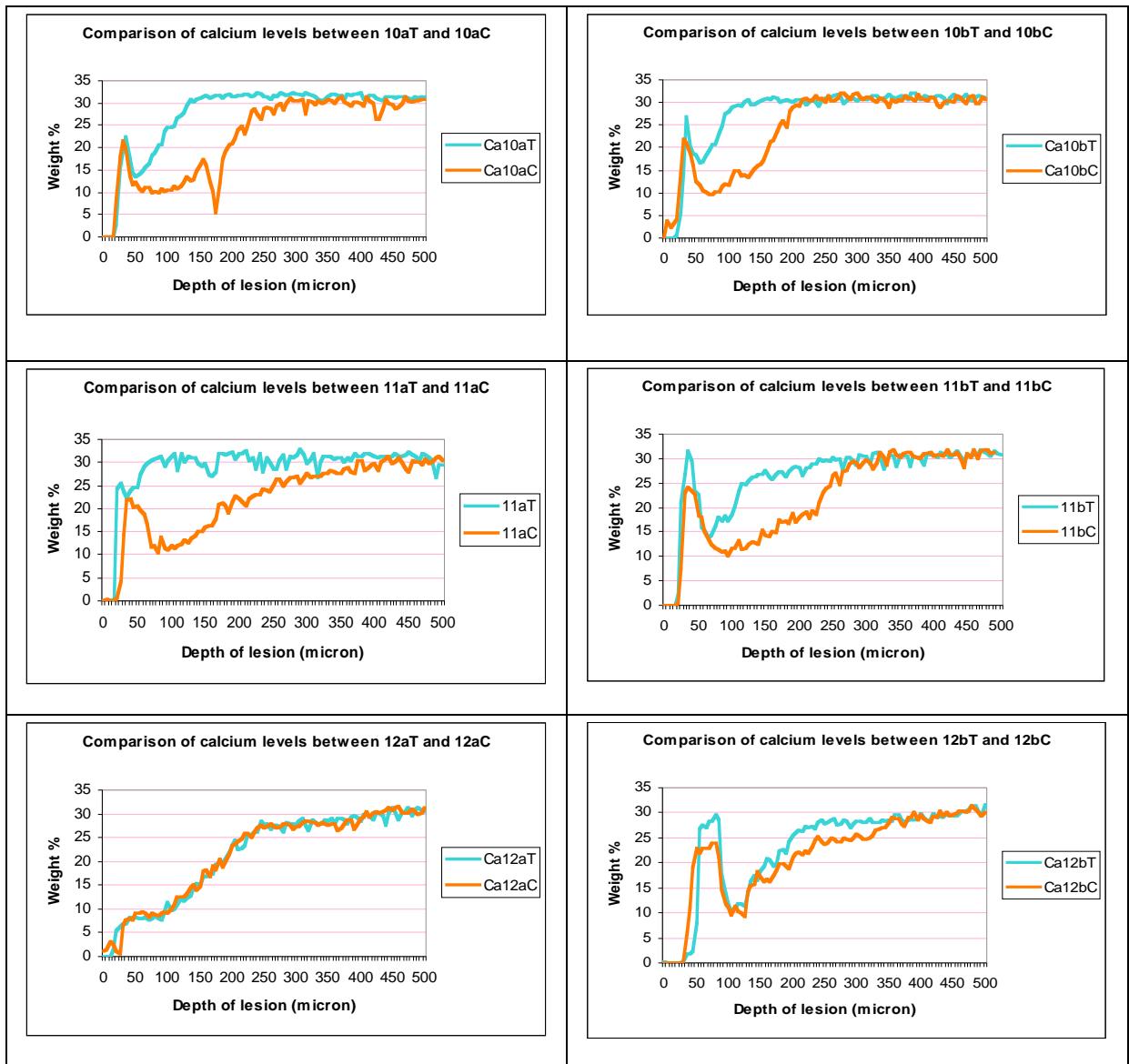


Mineral profiles of samples restored in Fuji IX and supplied with SDTF for six weeks





Comparison of calcium levels between the test and control sides



Additional statistical results for Chapter 7: AUC values of P, F and Sr (CPP-ACP and Ketac Molar groups)

Table 40: Comparison of P, F, Sr levels (CPP-ACP group)

AUC	P Test	P Control	F Test	F Control	Sr Test	Sr Control
1CPa	4332.9	4848.6	200.4	84.2	260.7	21.8
1CPb	5037.1	5085.2	137.6	94.7	93.5	4.1
4CPa	4609.2	4567.6	321.8	150.8	490.2	7.1
4CPb	4720.4	4628.3	307.7	125.9	341.6	10.6
2CPa	3820.2	4641.1	434.3	85.2	767.1	6.3
2CPb	3856.9	4165.5	350.2	159.2	338.5	14.7
5CPb	3736.7	4188.9	334.1	101.3	544.4	15.5
3CPa	3490.7	3846.5	474.8	248.6	759.9	292.7
3CPb	3775.3	3802.3	266.0	91.5	431.2	8.5
6CPa	4074.8	3450.3	788.6	100.8	861.0	25.2
6CPb	4009.6	3931.9	424.5	126.9	589.0	46.8
Mean(SD)	4133.1 (481)	4286.9 (505)	367.3 (171)	124.5 (48)	497.9 (236)	41.2 (84)
P values	0.2		0.0008		<0.0001	

Table 41: Comparison of P, F, Sr levels (Ketac Molar group)

AUC	P Test	P Control	F Test	F Control	Sr Test	Sr Control
K1a	4055.7	4257.5	155.1	69.5	1.3	1.2
K1b	3830.1	4330.7	338.4	88.0	1.2	1.3
K2a	4535.3	4741.8	268.5	87.5	0.9	1.3
K2b	3331.9	3392.0	283.6	64.2	1.5	1.2
K3a	4160.1	4380.1	229.8	75.6	1.4	1.1
K3b	3331.9	3392.0	283.6	64.2	1.5	1.2
K4a	4993.9	4912.5	299.3	297.6	1.0	0.9
K4b	3209.9	3482.6	701.8	141.8	0.6	1.0
K5a	3845.2	4674.0	617.5	88.3	0.4	1.0
K5b	3865.8	4281.1	537.6	110.7	0.5	1.8
K6a	3628.7	4221.8	681.5	119.5	0.3	0.7
Mean(SD)	3889.9(538.1)	4187.8(538.9)	399.7(195.9)	109.7(66.9)	0.9(0.4)	1.2 (0.2)
P	0.003		0.0006		0.2	

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