

 Total Quality. Assured.	Laboratory Report
Study Number: 150-LAB-SMH-22-002	Report Authors: G. Thomas, T. Badrock & C. Thompson.
Report Version: Final 2.0	Report Date: 13 th October 2025

Study Title: An *In Vitro* Study to Evaluate the Ability of a Toothpaste Formulation to Repair and Protect Demineralised Enamel Samples

Analytical Objectives

- To use artificially demineralised human enamel samples as the test substrate to compare the ability of a toothpaste formulation, versus a negative control (Deionised water) to repair and protect the demineralised enamel samples during a 3 day treatment and pH cycling protocol.
- To use changes in the surface microhardness of the enamel samples to assess the ability of the treatments to repair and protect the demineralised human enamel samples.

Study Overview

In vitro pH-cycling models are used to compare the ability of test articles to repair and protect tooth enamel, by mimicking test article treatments, saliva immersions and dietary erosive challenges. There are a variety of pH cycling protocols available within the literature, with this treatment protocol being based upon Huang *et al.* (2009)¹.

The study aimed to compare the ability of a toothpaste formulation versus a negative control (deionised water) to repair and protect human enamel samples during 3 days of treatment and pH cycling. The study has been summarised below.

Human enamel blocks were sectioned from acellular human teeth, lapped until their surfaces were flat and then highly polished. The preparation steps produced enamel specimens that were suitable for surface microhardness assessments.

The baseline surface microhardness of the enamel samples was measured using a calibrated Wilson Tukon 1202 microhardness indenter and Knoop diamond indenter tip. Enamel samples that met the baseline surface microhardness criteria progressed to the next stage of the study.

Enamel samples were demineralised in a demineralisation solution, for 1 hour at 37 °C, to produce early stage lesions in the enamel surfaces. The post-demineralisation surface microhardness of the enamel samples was measured using a calibrated Wilson Tukon 1202 microhardness indenter and Knoop diamond indenter tip.

 Total Quality. Assured.	Laboratory Report
Study Number: 150-LAB-SMH-22-002	Report Authors: G. Thomas, T. Badrock & C. Thompson.
Report Version: Final 2.0	Report Date: 13 th October 2025

Twenty enamel samples were stratified between two treatment groups, prior to treatment using the post-demineralisation surface microhardness values of the enamel samples (10 samples per treatment group).

The enamel samples in each treatment group were attached to a modified sample holder and subjected to 3 days of treatment and pH-cycling designed to mimic the oral environment. Following treatment, the surface microhardness of the enamel specimens was measured using a calibrated Wilson Tukon 1202 microhardness indenter and Knoop diamond indenter tip.

The percentage surface microhardness recovery (% SMHR) values achieved by the toothpaste formulation and the negative control were calculated and statistically compared. Higher %SMHR values were indicative of greater repair and protection of the demineralised enamel samples.

Test Products

Table 1: Treatments

Treatment Group	Raw Chemical	Solution Prepared
1	Carifree® Pro Gel 5000 - 1.1% Neutral Sodium Fluoride Anti-Cavity Toothpaste Crimp Code: 682213 Exp: 04/24	1:1.6 Paste to DI water ratio (w/w) Paste: 39.22g DI Water: 62.75g
2	Negative Control - Deionised Water Lot N°: 846747 Exp: 10/06/23	Used as supplied by SLS.

Block Preparation

20 enamel blocks, measuring 4 x 4 mm, were cut from acellular human teeth, and lapped planar-parallel using a Logitech PM5 lapping machine. The enamel surfaces of the samples were machine-polished using a MetPrep Saphir 550 to a final finish of 0.3 microns. One corner of each block was removed to ensure correct orientation of the samples on the SMH machine.

The enamel samples were sonicated, and surface checks were performed with an objective lens of the Wilson Tukon 1202 microhardness indenter. Suitable enamel samples were stored refrigerated, inside individual 7 ml Sterilins, on tissue dampened with 0.1 % thymol solution.

Baseline SMH Measurements

The baseline surface microhardness of the enamel samples was measured using a calibrated Wilson Tukon 1202 microhardness indenter and Knoop diamond indenter tip.

 Total Quality. Assured.	Laboratory Report
Study Number: 150-LAB-SMH-22-002	Report Authors: G. Thomas, T. Badrock & C. Thompson.
Report Version: Final 2.0	Report Date: 13 th October 2025

The enamel samples were orientated with the abraded corner in the top right. Baseline surface microhardness indents were placed in the centre of the enamel samples. Five Knoop indents were placed in a vertical line (under a load of 50 g, for 10 seconds). Surface imperfections were avoided.

Each enamel block was required to have an average baseline Knoop SMH > 250.0 HK (Hardness Knoop) and a standard deviation of < 20.0 to progress to the demineralisation phase of the study.

Initial Enamel Block Demineralisation

A demineralisation solution was prepared according to the following specifications:

- 50 mM Acetic acid
- 2.2 mM Calcium nitrate
- 2.2 mM Potassium phosphate monobasic
- 0.1 ppm Sodium fluoride
- Final pH of the solution was adjusted to 4.5 with sodium hydroxide.

Enamel samples were mounted onto acetate squares using double-sided tape. Enamel samples were immersed in 8 ml demineralisation solution per sample and placed into an oven for 1 hour (37 °C). The enamel samples were removed from the demineralisation solution, rinsed with deionised water for 2 minutes, and returned to their original containers.

Post-demineralisation SMH Measurements

The post-demineralisation surface microhardness of the enamel samples was measured using a calibrated Wilson Tukon 1202 microhardness indenter and Knoop diamond indenter tip.

The enamel samples were orientated with the abraded corner in the top right. Post-demineralisation Knoop surface microhardness indents were placed approximately 100 microns from the baseline indents. Five Knoop indents were placed in a vertical line at each timepoint (under a load of 50 g, for 10 seconds). Surface imperfections were avoided.

Removal of Outliers and Stratification

Specimens with either 'outlier' baseline/demineralised SMH values or standard deviations > 4.0 were removed from the study. Twenty blocks were stratified between 2 treatment groups ($n = 10$) to create groups with similar mean post-demineralisation surface microhardness values.

 Total Quality. Assured.	Laboratory Report
Study Number: 150-LAB-SMH-22-002	Report Authors: G. Thomas, T. Badrock & C. Thompson.
Report Version: Final 2.0	Report Date: 13 th October 2025

Treatment and pH Cycling

The enamel samples in each treatment group were subjected to 3 days of the following treatment and pH cycling regime:

<u>Duration</u>	<u>Treatment & pH Cycling Regime</u>
5 min	Specified treatment
55 min	Remineralisation solution
5 min	Specified treatment
55 min	Remineralisation solution
60 min	Demineralisation solution
2 hr	Remineralisation solution
5 min	Specified treatment
55 min	Remineralisation solution
5 min	Specified treatment
Overnight	Remineralisation solution

All pH cycling took place inside a hotbox heated to 37°C with the samples agitated during all treatment and immersions.

Post-treatment (SMH) Measurements

The post-treatment surface microhardness of the enamel samples was measured with a calibrated Wilson Tukon 1202 microhardness indenter and Knoop diamond indenter tip.

The enamel samples were orientated with the abraded corner in the top left. Post-treatment Knoop surface microhardness indents were placed approximately 100 microns from the baseline indents. Five Knoop indents were placed in a vertical line at each timepoint (under a load of 50 g, for 10 seconds). Surface imperfections were avoided.

Data Management

The day to day running of the study was documented in laboratory notebooks, which contained the study number 150-LAB-SMH-22-002. All laboratory notebook pages were signed by the study analysts.

The formula used to calculate the %SMHR values can be seen below:

$$\%SMHR = (\text{Post treatment SMH} - \text{Post Demin SMH}) / (\text{Baseline SMH} - \text{Post Demin SMH}) * 100$$

All Excel datasets and formulas used in this study were entered, merged, and checked in accordance with data management SOPs. All data-check records were scanned into an electronic study folder.

A copy of the Excel dataset can be seen in Appendix 1.

 Total Quality. Assured.	Laboratory Report
Study Number: 150-LAB-SMH-22-002	Report Authors: G. Thomas, T. Badrock & C. Thompson.
Report Version: Final 2.0	Report Date: 13 th October 2025

Statistical Analysis

Minitab 18 was used to calculate descriptive statistics (mean, standard deviation, median, minimum, maximum, and sample size) for the %SMHR data achieved by each treatment.

A 2-Sample t-Test was selected to make pairwise statistical comparisons between the %SMHR values achieved by the two treatments. A copy of the statistical analysis report can be found in Appendix 2.

Results

The mean percentage %SMHR values achieved by each treatment after 3 days of treatment and pH cycling is shown in Table 2.

Table 2: Post Treatment Mean %SMHR

Treatment	Mean %SMHR	StDev
Carifree® Pro Gel 5000	62.99	6.94
DI Water	37.64	12.90

After 3 days of treatment and pH cycling, the mean %SMHR value achieved by the Carifree Pro Gel toothpaste formulation was 63%, versus 38% for the negative control. The mean %SMHR value achieved by the Carifree Pro Gel toothpaste formulation was approximately 67% higher than the mean %SMHR value achieved by the negative control.

Statistical analysis of the %SMHR data showed that the Carifree Pro Gel toothpaste formulation achieved a statistically significantly higher %SMHR value than the negative control.

Conclusions

The %SMHR data showed the Carifree Pro Gel toothpaste formulation was able to achieve statistically significantly greater repair and protection of the enamel samples than the negative control during this treatment and pH cycling study.

Reference

1. Huang, S. B., Gao, S. S., & Yu, H. Y. (2009). Effect of nano-hydroxyapatite concentration on remineralization of initial enamel lesion *in vitro*. *Biomedical Materials*, 4(3), 1-6. doi:10.1088/1748-6041/4/3/034104

 Total Quality. Assured.	Laboratory Report
Study Number: 150-LAB-SMH-22-002	Report Authors: G. Thomas, T. Badrock & C. Thompson.
Report Version: Final 2.0	Report Date: 13 th October 2025

Appendix 1: Surface Microhardness Raw Data

Sample Number	Treatment Group	Baseline							Post Demin							Post Treatment										
		HK1	HK2	HK3	HK4	HK5	Average	StnDev	HK1	HK2	HK3	HK4	HK5	Average	StnDev	Change in SMH	HK1	HK2	HK3	HK4	HK5	Average	StnDev	Change in SMH	% SMHR	
002	Carifree® Pro Gel 5000	299	326	299	332	323	315.80	15.67	68	63	71	69	67	67.60	2.97	-248.20	239	207	227	225	224	224.40	11.44	156.80	63.17	
010	Carifree® Pro Gel 5000	297	318	310	287	294	301.20	12.56	76	88	83	85	81	82.60	4.51	-218.60	231	249	200	227	267	234.80	25.12	152.20	69.62	
004	Carifree® Pro Gel 5000	329	332	323	307	335	325.20	11.10	110	107	98	106	123	108.80	9.09	-216.40	245	241	227	265	249	245.40	13.74	136.60	63.12	
007	Carifree® Pro Gel 5000	320	320	341	341	310	326.40	13.94	110	131	119	146	110	123.20	15.39	-203.20	203	251	201	241	265	232.20	28.87	109.00	53.64	
017	Carifree® Pro Gel 5000	335	335	335	347	360	342.40	11.13	161	147	142	149	132	146.20	10.57	-196.20	252	245	234	260	273	252.80	14.79	106.60	54.33	
020	Carifree® Pro Gel 5000	312	332	335	307	299	317.00	15.80	126	110	129	152	131	129.60	15.01	-187.40	245	216	217	249	225	230.40	15.61	100.80	53.79	
023	Carifree® Pro Gel 5000	312	364	354	332	312	334.80	23.82	181	150	152	140	182	161.00	19.26	-173.80	275	310	287	326	239	287.40	33.53	126.40	72.73	
011	Carifree® Pro Gel 5000	318	318	307	315	294	310.40	10.21	139	158	149	140	132	143.60	10.06	-166.80	269	243	247	258	249	253.20	10.40	109.60	65.71	
003	Carifree® Pro Gel 5000	320	312	323	320	302	315.40	8.53	157	163	156	151	162	157.80	4.87	-157.60	251	271	267	278	267	266.80	9.91	109.00	69.16	
015	Carifree® Pro Gel 5000	271	267	263	265	273	267.80	4.15	132	129	145	129	112	129.40	11.76	-138.40	231	208	236	227	192	218.80	18.35	89.40	64.60	
														Average	-190.66								Average	119.64		62.99
														StnDev	32.71								StnDev	22.46		6.94
016	DI Water	304	285	310	299	323	304.20	13.99	54	69	61	62	75	64.20	8.04	-240.00	197	179	183	150	182	178.20	17.22	114.00	47.50	
005	DI Water	312	344	329	323	304	322.40	15.47	98	109	110	98	101	103.20	5.89	-219.20	211	204	205	192	194	201.20	7.98	98.00	44.71	
013	DI Water	292	287	307	312	318	303.20	13.22	85	94	96	96	83	90.80	6.30	-212.40	194	194	219	198	185	198.00	12.67	107.20	50.47	
021	DI Water	271	299	302	271	280	284.60	15.01	74	77	64	80	75	74.00	6.04	-210.60	157	168	179	184	200	177.60	16.29	103.60	49.19	
001	DI Water	294	302	312	332	312	310.40	14.24	112	127	131	94	108	114.40	14.98	-196.00	181	203	239	204	191	203.60	21.93	89.20	45.51	
024	DI Water	302	302	315	307	310	307.20	5.54	101	116	125	105	128	115.00	11.90	-192.20	188	182	196	179	182	185.40	6.77	70.40	36.63	
012	DI Water	299	323	297	320	302	308.20	12.32	133	145	150	137	126	138.20	9.52	-170.00	217	203	216	178	210	204.80	15.99	66.60	39.18	
006	DI Water	323	312	292	320	304	310.20	12.58	154	124	137	141	156	142.40	13.13	-167.80	192	182	141	145	162	164.40	22.37	22.00	13.11	
008	DI Water	357	354	354	344	347	351.20	5.45	234	219	236	185	182	211.20	26.15	-140.00	229	217	239	273	232	238.00	21.12	26.80	19.14	
014	DI Water	287	315	294	320	323	307.80	16.24	166	168	177	150	178	167.80	11.28	-140.00	216	198	221	205	216	211.20	9.42	43.40	31.00	
														Average	-188.82								Average	74.12		37.64
														StnDev	33.66								StnDev	33.86		12.90

 Total Quality. Assured.	Laboratory Report
Study Number: 150-LAB-SMH-22-002	Report Authors: G. Thomas, T. Badrock & C. Thompson.
Report Version: Final 2.0	Report Date: 13 th October 2025

Appendix 2: Statistical Analysis

Descriptive Statistics: % SMHR
Statistics

Variable	Treatment Group	N	Mean	StDev	Minimum	Median	Maximum
% SMHR	Carifree® Pro Gel 5000	10	62.99	6.94	53.64	63.89	72.73
	DI Water	10	37.64	12.90	13.11	41.94	50.47

Two-Sample T-Test and CI: % SMHR_Carifree® Pro Gel ... R_DI Water
Method

μ_1 : mean of % SMHR_Carifree® Pro Gel 5000

μ_2 : mean of % SMHR_DI Water

Difference: $\mu_1 - \mu_2$

Equal variances are not assumed for this analysis.

Descriptive Statistics

Sample	N	Mean	StDev	SE Mean
% SMHR_Carifree® Pro Gel 5000	10	62.99	6.94	2.2
% SMHR_DI Water	10	37.6	12.9	4.1

Estimation for Difference

Difference	95% CI for Difference
25.34	(15.33, 35.35)

Test

Null hypothesis $H_0: \mu_1 - \mu_2 = 0$

Alternative hypothesis $H_1: \mu_1 - \mu_2 \neq 0$

T-Value	DF	P-Value
5.47	13	0.000

 Total Quality. Assured.	Laboratory Report
Study Number: 150-LAB-SMH-22-002	Report Authors: G. Thomas, T. Badrock & C. Thompson.
Report Version: Final 2.0	Report Date: 13 th October 2025

Report signature:

I declare that this report constitutes a true and faithful account of the procedures adopted and the results obtained in the performance of this study.

Gavin Thomas



(Laboratory Manager, Intertek CRS)

Date..... 13th Oct 2025

Intertek CRS
Elm House
Unit A4, Oaklands Office Park
Hooton Road
Hooton
Cheshire
CH66 7NZ
UK

+44 151 347 4288

Last page of document