

Colour Shifts with pH: Investigating Anthocyanin as a Plaque-Disclosing Agent

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Article

Keywords: Dental plaque, Plaque pH, Plaque disclosing agent, pH indicator, Anthocyanin

Posted Date: October 19th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-11111807/v1>

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Additional Declarations: No competing interests reported.

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Abstract

This study evaluated anthocyanin, a natural pigment, as a pH-sensitive plaque-disclosing agent. Anthocyanin solutions were prepared in deionized water (DIW) and diluted in buffers ranging from pH 1–10. Absorbance at 533 nm was measured to generate a standard curve. Twenty-eight participants aged 8–23 years refrained from toothbrushing for 48 hours and from eating, drinking, or chewing gum for 2 hours before plaque collection. Following a 1-minute rinse with 10% sucrose solution, plaque samples were collected, suspended in DIW, centrifuged, and the supernatant mixed with anthocyanin solution. Absorbance-derived pH values were compared with pH meter readings. Precision was assessed using intraclass correlation coefficients (ICCs, 95% CI). ICCs were 0.766 (0.203–0.948) for pH <4.5, 0.711 (0.229–0.913) for pH 4.5–5.0, and –0.825 (–0.958–0.403) for pH >5.0. Anthocyanin showed good agreement at highly acidic pH, moderate agreement at pH 4.5–5.0, but poor reliability above pH 5.0. These findings demonstrate anthocyanin’s potential as a natural, biocompatible plaque-disclosing agent capable of indicating cariogenic acidity. Further optimization is required to improve sensitivity across the typical plaque pH range before clinical application.

Keywords: Dental plaque; Plaque pH; Plaque disclosing agent; pH indicator; Anthocyanin

Introduction

Dental caries is a significant healthcare problem due to its high prevalence worldwide. The progression of caries leads to impairment of quality of life, such as pain and difficulties in eating and sleeping. Severe caries may result in tooth extraction¹. The ecological plaque hypothesis emphasizes the influence of the oral environment on the development of dental caries. It proposes that dietary sugars are metabolized by oral bacteria, producing acids that lower pH. This acidic shift creates a favourable environment for acidogenic and aciduric bacteria, such as *Streptococcus mutans* and *Lactobacillus* spp., while inhibiting the growth of health-associated species like *Streptococcus sanguinis* and *Streptococcus gordonii*. This microbial imbalance further enhances acid production, perpetuating the cariogenic process². Moreover, an increase in biofilm thickness enhances its capacity to retain acidic substances within its structure, leading to a significant reduction in intra-biofilm pH³. Consequently, tools that facilitate both plaque visualization and an understanding of its acidogenic potential are essential for effective caries prevention and patient education^{4,5}.

Plaque-disclosing agents are widely used in clinical dentistry and oral health education to enhance the visibility of dental plaque and improve oral hygiene performance. Some of these agents can also assess plaque

cariogenicity by measuring its pH⁶. This feature is especially beneficial for assessing a patient's risk of developing caries, as a lower pH indicates a higher risk of dental caries^{7, 8}. There are currently clinical-use plaque disclosing agents that have a property of plaque cariogenicity identification⁶. For instance, a tri-colour plaque disclosing gel can identify new, mature, and acid-producing biofilm by displaying three colours based on the type of dental plaque⁹. However, the products are synthetic chemical products, with high costs, and not generally available over the counter. Enhancing accessibility, particularly for schools or remote areas, could be achieved through the creation of plaque disclosing agents which are simply homemade from natural ingredients. These agents would be biocompatibility, pleasant taste, acceptance, safe, cost-effective, and could be easily purchased. Moreover, repeating assessment of plaque at home may help improve patients' oral hygiene compliance¹⁰. There are various kinds of plants which have been studied to develop plaque disclosing agents, such as butterfly pea¹¹, red dragon fruit¹², and red cabbage. These plants contain pigment known as "anthocyanins", which are naturally occurring, water-soluble flavonoid pigments responsible for the red, purple, and blue coloration of many fruits and vegetables. A distinctive property of anthocyanins is their pronounced colour change in response to pH variation, resulting from structural transformations under different acidic and alkaline conditions¹¹. This pH-dependent chromatic behaviour has been extensively applied in food science and analytical chemistry as a natural pH indicator. Moreover, anthocyanins are generally regarded as safe and possess additional biological properties, including antioxidant and antimicrobial effects, which further support their potential use in oral applications¹². Despite the promising properties of anthocyanins, limited evidence exists regarding their applicability as pH-sensitive indicators in dental plaque. In particular, their accuracy, sensitivity, and reliability across plaque pH ranges relevant to caries development require systematic evaluation. Understanding these characteristics is essential before considering clinical or educational implementation.

Therefore, the aim of this study was to evaluate the potential of anthocyanin as a pH-sensitive plaque-disclosing agent by examining its colorimetric response across a range of pH values and assessing its agreement with pH meter measurements in plaque samples following sucrose exposure. This investigation seeks to provide foundational evidence for the development of a natural, biocompatible plaque-disclosing agent capable of indicating cariogenic acidity.

Materials and Methods

Phase 1: Development of the plaque-disclosing agent

Initially, fresh red cabbage was selected as the pigment source for developing a plaque-disclosing agent, owing to its superior colour-changing efficacy compared to other anthocyanin-containing plants¹³. However, using a crude extract caused precipitation, which interfered with accurate colour measurement by a spectrophotometer. Therefore, a commercially available anthocyanin extract powder was used instead of the crude extract, owing to its superior stability and consistency in colorimetric analysis.

Preparation of anthocyanin solution

A stock solution of anthocyanin was prepared by dissolving 1 g of anthocyanin powder in 10 mL of deionized water and was kept at –20 °C until use. To determine the precise concentration of anthocyanin, the stock solution was diluted 200-fold in deionized water then the solution was titrated with standard 0.1 M NaOH with monitoring the change of absorption spectrum. Titration continued until no further spectral change was observed, indicating complete deprotonation. The total volume of NaOH added corresponded stoichiometrically to the anthocyanin content. The extinction coefficient at 533 nm was calculated from the absorption spectrum at pH 7.0 using the Beer-Lambert Law (Equation 1) and was $2.43 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$. This extinction coefficient is used to determine the concentration of anthocyanin.

$$A = \epsilon c \quad \text{Equation 1}$$

A = the absorbance of anthocyanin solution at 533 nm

ϵ = the extinction coefficient at 533 nm

c = the concentration of anthocyanin

Preparation of pH colour-shade guide

Different pH buffers ranging from pH 1 to 10 were prepared as follows:

- 1 M KCl; pH 1.0, 1.5, 2.0, 2.5
- 1 M Citric acid; pH 3.0, 3.5
- 1 M Ammonium acetate; pH 4.0, 4.5
- 1 M Sodium dihydrogen phosphate; pH 5.0, 5.5, 6, 6.5
- 1 M Potassium dihydrogen phosphate; pH 7.0, 7.5
- 1 M Tris (hydroxymethyl aminomethane); pH 8.0, 8.5, 9.0, 9.5, 10.0

The pH colour-shade guide was prepared by diluting the anthocyanin stock solution 200-fold in these different pH buffers. Photographs of the resulting colour shades were then captured and processed using Procreate

Version 5.3.7 to create a visually refined pH colour-shade reference guide (pH 3-8, Fig. 1). Based on the pH colour-shade guide, anthocyanin exhibited minimal visible colour change within the pH range of 4–6, which corresponds to the clinical plaque pH observed in this study (Fig. 1); therefore, absorbance measurements were used for accurate measurement.

A plot of anthocyanin absorbance changes at 533 nm against pH

The pH shade guide was prepared as previously described. The absorbance of anthocyanin colour change was measured at 533 nm, where its highest sensitivity wavelength, using an ultraviolet-visible (UV-vis) spectrophotometer (SHIMADZU TCC-240A, Japan). To accurately determine pH from anthocyanin colour change, this curve was used as a reference for the pH shade guide, which was plotted from the relationship between maximum absorbance change at 533 nm and a series of known pH buffer solutions. The Marquardt–Levenberg algorithm (Program Kaleida Graph version 3.5) was used to get a curve fit for the experimental data. The resulting parameters were then applied to a Henderson–Hasselbalch equation as shown in the equation below (Equation 2).

$$A = \frac{\varepsilon_A c_T \times 10^{(pK_a - pH)} + \varepsilon_{A^-} c_T}{10^{(pK_a - pH)} + 1} \quad \text{Equation 2}$$

A = absorbance change at 533 nm

ε_A = extinction coefficient of neutral form of anthocyanin

ε_{A^-} = extinction coefficient of the deprotonated form of anthocyanin

c_T = total concentration of anthocyanin ($c_A + c_{A^-}$)

Phase 2: Validating the accuracy of anthocyanin solution as a plaque-disclosing agent

Sample size calculation

The sample size for the intraclass correlation coefficient (ICC) was determined using the Sample Size Calculator (web) taken from the website <http://wnarifin.github.io>¹⁴. The predicted reliability is assumed to have a coefficient of 0.5. The estimated sample size is $n = 28$, with a significance level of $\alpha = 0.05$ and a power of $\beta = 0.2$.

Subject recruitment

A total of 28 individuals, aged 8 to 23 years, were recruited with informed consent obtained through a recruitment infographic shared on social media. Participants included individuals in normal general health with at

least 20 natural teeth. Exclusion criteria comprised individuals with xerostomia, long-term use of medications known to cause oral side effects, or a habitual gum-chewing behaviour.

Plaque collection and pH determination

Participants were instructed to refrain from toothbrushing for 48 hours and to avoid eating, drinking, or using chewing gum for at least 2 hours before the experiment to ensure sufficient plaque accumulation. During the testing appointment, participants were instructed to rinse with 10 ml of 10% sucrose solution for 1 minute. Plaque samples were then collected from all teeth using a spoon excavator. Each sample was transferred to a microcentrifuge tube and mixed with 300 μ L of deionized water using a vortex mixer for 5 minutes, then left to stand for 10 minutes. The mixture was centrifuged at 14000 rpm for 10 minutes (Hettich, Germany) to separate the plaque from the supernatant. The wet weight of the collected plaque was measured using an analytical balance (Precisa, Switzerland) to quantify the amount of plaque obtained from each participant. This measurement was used to evaluate whether the amount of plaque correlated with its acidity. After separation, 250 μ L of the supernatant was transferred to a new microcentrifuge tube. For pH and absorbance measurements, 22 μ L of anthocyanin solution prepared in phase I was mixed with 228 μ L of the plaque supernatant in a 96-well plate. The absorbance was measured using a UV–vis spectrophotometer (SHIMADZU TCC-240A, Japan). and the plaque pH was calculated using the equation described in the “Standard Curve Between Absorbance and Measured pH” section. Additionally, the pH of the solution was measured using a handheld pH meter (LAQUAtwin pH-22) for ICC analysis.

Data analysis

The data was analysed using SPSS Statistical software for Windows (version 28, IBM, United States). The agreement between measured plaque pH and calculated plaque pH was assessed using ICC. The agreement level determined by the ICC was categorized as poor (<0.50), moderate (0.50-0.75), good (0.75-0.90), and excellent (>0.90).

Results

Changes in the absorption spectra of anthocyanin at different pHs

The anthocyanin absorption spectra show a decrease in absorbance with higher pHs, with an absorption peak at 533 nm (Fig. 2a). Therefore, this wavelength was used to determine the effect of pH on anthocyanin absorption. A plot of absorbance at 533 nm versus pH from 1 to 6 showed that the absorbance change at 533 nm

is sensitive to pH changes in the range of 2 to 4.5 (black dots, Fig. 2b). The non-linear curve fit using Equation 2 yields a pK_a of 3.30 ± 0.07 for the anthocyanin (red line, Fig. 2b). This curve was used as a reference for determining pH from absorbance change at 533 nm.

Participants' characteristics

The characteristics of the 28 participants, including age, number of erupted teeth, plaque weight, gender, dental student status, and presence of caries, are summarized in Table 1. The median age is 15 years (range: 8–23). The median erupted teeth are 25.8 teeth (range: 20–30). The median plaque weight is 101.05 grams (range: 68–165.7). The gender distribution consists of 8 males (28.57%) and 20 females (71.41%). Among the participants, 9 (32.14%) are dental students, while 19 (67.86%) are not. Additionally, 7 participants (25%) have caries (cavity and white lesions), whereas 21 (75%) do not.

Determination of plaque pH using anthocyanin solution

The colour of the anthocyanin solution transitions from red to purple to blue across the pH range of 3 to 8 (Fig. 1). However, colour changes between pH 4 and 6 are small, making visual discrimination with the naked eye difficult. Consequently, as demonstrated by the results from participants' plaque samples, it is not feasible to accurately determine plaque pH based solely on visual colour assessment.

Therefore, the absorbance at 533 nm of the anthocyanin solution used to dissolve each participant's plaque sample was measured, and the corresponding pH was calculated using equation as described in the Materials and Methods section.

Reliability assessment of measured and calculated plaque pH

The plaque pH of participants, measured directly using a pH meter (measured plaque pH) and indirectly calculated using Equation 3 (calculated plaque pH), is presented in Table 2. The differences between the two methods could be grouped into three ranges based on the magnitude of pH differences: 1) Minimal differences (–0.28 to 0.08), observed when $pH < 4.5$, 2) Moderate differences (–0.03 to 0.41), for $pH 4.5–5.0$, and 3) Largest differences (0.47 to 1.63), when $pH > 5.0$. Further statistical analyses were conducted to assess the significance of the observed differences.

The precision of the standard equation was evaluated using ICCs with 95% confidence intervals: 0.766 (0.203–0.948) for $pH < 4.5$, 0.711 (0.229–0.913) for $pH 4.5–5.0$, and –0.825 (–0.958–0.403) for $pH > 5.0$ (Table

3). These results indicate good precision for $\text{pH} < 4.5$, moderate precision for $\text{pH} 4.5\text{--}5.0$, and poor precision for $\text{pH} > 5.0$.

Discussion

This study evaluated the potential of anthocyanin as a pH-sensitive indicator for dental plaque and demonstrated that anthocyanin exhibits good agreement with pH meter measurements at highly acidic pH levels, moderate agreement in the transitional range of $\text{pH} 4.5\text{--}5.0$, and poor reliability at pH values above 5.0. These findings indicate that anthocyanin may be particularly effective for identifying strongly acidogenic plaque, which is most relevant to the initiation and progression of dental caries. The observed pH responsiveness is consistent with previous reports on the pH sensitivity range of anthocyanins^{13,15}. However, its precision declined at pH levels above 4.5, a range more commonly associated with early plaque acidification post-sucrose exposure. This limitation underscores the need for further refinement of the anthocyanin formulation to enhance sensitivity within the clinically relevant $\text{pH} 5.0\text{--}6.5$ range, where caries risk remains significant⁸.

The high ICCs observed at pH values below 4.5 indicate that anthocyanin provides reliable pH estimation under strongly acidic conditions. This is clinically significant, as plaque pH in this range is well below the critical threshold for enamel demineralization and reflects highly cariogenic biofilm activity following sugar exposure. The strong performance of anthocyanin in this acidic range is consistent with its known chemical behaviour, whereby the flavylium cation form predominates at low pH, resulting in stable and intense coloration with predictable absorbance characteristics. The colour change of anthocyanin across different pH levels is due to its pH-dependent ionic structure; in acidic conditions, it predominantly exists as the red flavylium cation, whereas in more basic conditions it shifts toward blue anionic quinoidal forms¹¹. In contrast, agreement between anthocyanin-based measurements and pH meter readings decreased in the pH range of $4.5\text{--}5.0$ and was poor above $\text{pH} 5.0$. This reduction in reliability may be explained by structural transformations of anthocyanin molecules as pH increases. At higher pH levels, anthocyanins exist in multiple equilibrium forms, including quinoidal bases and colourless hemiketals, leading to reduced colour intensity and increased instability¹¹. These molecular transitions likely contribute to diminished sensitivity and greater variability in absorbance measurements, resulting in weaker correlation with pH meter values. From a practical perspective, this limitation suggests that anthocyanin is less suitable for precise pH discrimination in near-neutral plaque conditions.

Despite this limitation, the observed pH-dependent performance aligns well with the intended preventive application of a pH-sensitive plaque-disclosing agent. From a caries risk perspective, identifying plaque that

exhibits a pronounced acid response after sucrose exposure is arguably more clinically meaningful than detecting minor pH differences at near-neutral levels. Anthocyanin's strong performance in the highly acidic range supports its potential role as a functional indicator of cariogenic plaque rather than a universal pH measurement tool.

The use of sucrose rinsing prior to plaque collection in this study further strengthens the relevance of the findings, as it simulates a common dietary challenge that induces rapid plaque acidification. The ability of anthocyanin to detect low pH under these conditions suggests potential utility in educational or screening contexts, where visual or semi-quantitative feedback on plaque acidity could enhance patient understanding of sugar-related caries risk. This approach may be particularly valuable in paediatric and adolescent populations, who often benefit from visual learning tools and for whom acceptance of synthetic dyes may be limited.

Previous studies have suggested that the amount or thickness of dental plaque may influence its acidogenicity. Borgström et al. reported that acid production per milligram of plaque decreased as plaque mass increased, possibly due to restricted nutrient and oxygen diffusion within thicker biofilms¹⁶. In contrast, another study found that individuals with lower plaque pH had higher total bacterial counts in their plaque, implying that larger plaque biomass may be associated with increased acidogenic potential⁸.

Several limitations should be acknowledged. First, anthocyanin solutions were evaluated under controlled laboratory conditions using plaque suspensions rather than direct intraoral application. Factors such as saliva, pellicle formation, and inter-individual differences in plaque composition may influence colour response in clinical settings. Second, the negative ICC observed at pH values above 5.0 indicates poor agreement and suggests that anthocyanin, in its current formulation, lacks sufficient sensitivity in this range. Optimization strategies, such as modifying anthocyanin concentration, combining pigments from different botanical sources, or incorporating stabilizing agents, may improve performance across a broader pH spectrum. Finally, the relatively wide confidence intervals observed in some ICC estimates may reflect variability in plaque samples or the limited sample size within specific pH categories.

Future research should focus on refining anthocyanin formulations to enhance colour stability and sensitivity in the pH range of 4–7, which encompasses most plaque conditions encountered clinically. Additionally, studies evaluating direct intraoral application, visual colour interpretation, and patient and clinician acceptability are warranted. Longitudinal studies assessing whether anthocyanin-based disclosure can influence oral hygiene behaviour or caries outcomes would further clarify its clinical value.

Conclusion

This study provides preliminary evidence that anthocyanin functions effectively as a natural pH indicator for dental plaque under highly acidic conditions. While its reliability diminishes at higher pH levels, anthocyanin shows promise as a biocompatible, functionally informative plaque-disclosing agent capable of highlighting cariogenic risk. With further optimization, anthocyanin-based systems may contribute to more targeted and educationally meaningful approaches to caries prevention.

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Acknowledgments

The authors would like to express their sincere gratitude to the staff of the Departments of Microbiology and Biochemistry for their valuable assistance and support throughout this study. We also thank the Clinical Research Center for its support. The authors are grateful to all participants and colleagues who contributed to the completion of this project.

Authors' contributions

P.T., N.S., Ji.S., J.S., and P.D. contributed to the conceptualization of the study. N.S. and Ji.S. contributed to the methodology. P.T., N.S., Ji.S., J.S., and P.D. performed formal analysis and investigation. N.S. and Ji.S. wrote the original draft of the manuscript. P.T., N.S., Ji.S., J.S., and P.D. reviewed and edited the manuscript. P.T., P.D., and J.S. supervised the study. All authors read and approved the final manuscript.

Funding

This study was funded by the Dental Research Fund, Dental Research Project, Faculty of Dentistry, Chulalongkorn University under grant number 320050/#18/2023.

Data Availability

The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

Ethics Approval

All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki Declaration and its later amendments. Approval was obtained from the Ethics Committee of the Faculty of Dentistry, Chulalongkorn University (HRDC-2024-049).

Competing interests

The author(s) declare no competing interests

Consent to Participate

Written informed consent was obtained from all participants or their legal guardians prior to participation in the study.

Consent to Publish

All participants or their legal guardians provided consent for publication of any data and images included in this manuscript.

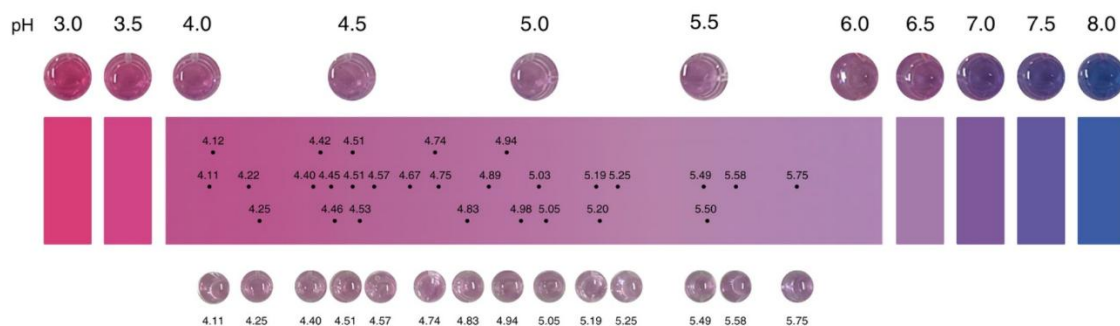


Fig. 1 The upper spots and the colour band represent the anthocyanin pH colour-shade guide. The lower spots show plaque pH values measured from each participant, with the band indicating the overall pH range observed among them.

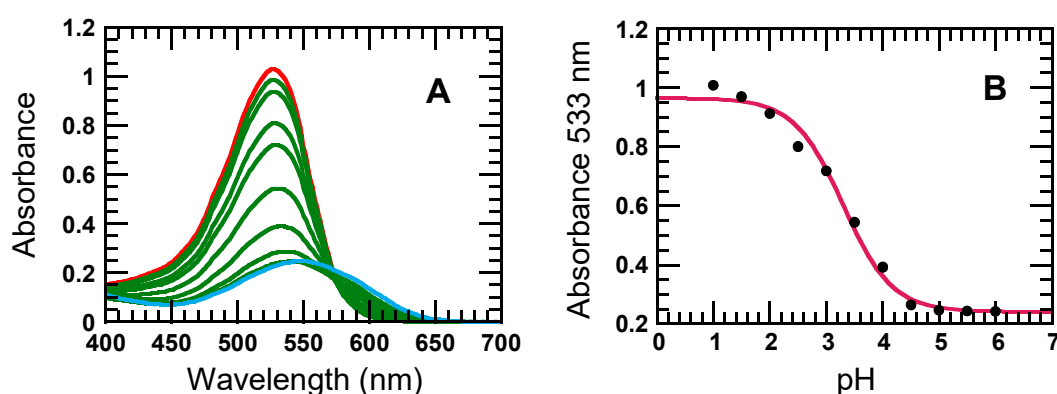


Fig. 2 The effect of pH on the absorption spectra of anthocyanin (a) Anthocyanin was mixed with different pH buffers to achieve a final concentration of 141 μ M. The red line represents the absorption spectrum of anthocyanin in buffer pH 1. The green lines show a decrease in absorbance with increasing pH from 1.5 to 5.5. The blue line represents the absorption spectrum of anthocyanin in buffer pH 6.0 (b) An absorbance at 533 nm was plotted against pHs (black dots). The red line represents a non-linear curve fit using Equation 2.

Table 1 Characteristics of study participants

Characteristics	Total (N=28)
Age, years: Median (Range)	15 (8, 23)
Number of erupted teeth: Median (Range)	25.8 (20, 30)
Plaque weight, gram: Median (Range)	101.05 (68, 165.7)
Gender	
Male	8 (28.57%)
Female	20 (71.41%)
Dental student	
Yes	9 (32.14%)
No	19 (67.86%)
Caries (cavity and white lesion)	
Yes	7 (25%)
No	21 (75%)

Table 2. Comparison of plaque pH values obtained by measurement and calculation

Participants	Measured Plaque pH	Calculated Plaque pH	Difference (Measured - Calculated)
1	4.11	4.16	-0.05
2	4.12	4.17	-0.05
3	4.22	4.30	-0.08
4	4.25	4.21	0.04
5	4.40	4.41	-0.01
6	4.42	4.70	-0.28
7	4.45	4.37	0.08
8	4.46	4.43	0.03
9	4.51	4.24	0.27
10	4.51	4.30	0.21
11	4.53	4.22	0.31
12	4.57	4.57	0.00
13	4.67	4.51	0.16
14	4.74	4.33	0.41
15	4.75	4.53	0.22
16	4.83	4.42	0.41
17	4.89	4.62	0.27
18	4.94	4.53	0.41
19	4.98	5.01	-0.03
20	5.03	4.53	0.50
21	5.05	4.58	0.47
22	5.19	4.44	0.75
23	5.20	4.64	0.56
24	5.25	4.30	0.95
25	5.49	4.41	1.08

26	5.50	4.16	1.34
27	5.58	4.15	1.43
28	5.75	4.12	1.63

Table 3 Intraclass Correlation Coefficient (95% Confidence intervals)

Plaque pH	ICC (95% CI)	p-value
< 4.5	0.766 (0.203-0.948)	0.008
4.5 - 5.0	0.711 (0.229-0.913)	0.005
> 5.0	-0.825 (-0.958--0.403)	0.998