Acta Odontologica Scandinavica. 2015; 73: 595–601

informa
healthcare

ORIGINAL ARTICLE

Changes in composition and enamel demineralization inhibition activities of gallic acid at different pH values

JINGYANG ZHANG[1*], XUELIAN HUANG[1,2*], SHENGBIN HUANG[1], MENG DENG[1,3], XINCHENG XIE[1], MINGDONG LIU[4], HONGLING LIU[1], XUEDONG ZHOU[1], JIYAO LI[1] & JACOB MARTIEN TEN CATE[2]

[1]*State Key Laboratory of Oral Diseases, West China Hospital of Stomatology, Sichuan University, Chengdu, PR China,* [2]*Department of Preventive Dentistry, Academic Centre for Dentistry Amsterdam (ACTA), University of Amsterdam and Free University Amsterdam, Amsterdam, The Netherlands,* [3]*Oral Biology Program, School of Dentistry, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA, and, and* [4]*Department of Analytical Toxicology, West China School of Preclinical and Forensic Medicine, Sichuan University, Chengdu, PR China*

Abstract

Background. Gallic acid (GA) has been shown to inhibit demineralization and enhance remineralization of enamel; however, GA solution is highly acidic. This study was to investigate the stability of GA solutions at various pH and to examine the resultant effects on enamel demineralization. ***Methods.*** The stability of GA in H_2O or in phosphate buffer at pH 5.5, pH 7.0 and pH 10.0 was evaluated qualitatively by ultraviolet absorption spectra and quantified by high performance liquid chromatography with diode array detection (HPLC-DAD). Then, bovine enamel blocks were subjected to a pH-cycling regime of 12 cycles. Each cycle included 5 min applications with one of the following treatments: 1 g/L NaF (positive control), 4 g/L GA in H_2O or buffered at pH 5.5, pH 7.0 and pH 10.0 and buffers without GA at the same pH (negative control), followed by a 60 min application with pH 5.0 acidic buffers and a 5 min application with neutral buffers. The acidic buffers were analysed for dissolved calcium. ***Results.*** GA was stable in pure water and acidic condition, but was unstable in neutral and alkaline conditions, in which ultraviolet spectra changed and HPLC-DAD analysis revealed that most of the GA was degraded. All the GA groups significantly inhibited demineralization ($p < 0.05$) and there was no significant difference of the inhibition efficacy among different GA groups ($p > 0.05$). ***Conclusions.*** GA could inhibit enamel demineralization and the inhibition effect is not influenced by pH. GA could be a useful source as an anti-cariogenic agent for broad practical application.

Key Words: *enamel, demineralization, gallic acid, stability, polyphenol*

Introduction

Gallic acid (3,4,5-trihydroxybenzoic acid, GA), a common naturally occurring polyphenol, is widely available in various plants and foods and it possesses a variety of biological properties, such as antioxidant, anti-mutagenic and anti-carcinogenic effects [1,2]. GA has the potential to enhance enamel remineralization [3] and was revealed to be the main component effective inhibitor of demineralization in *Galla chinensis* extract (GCE) [4], which was a promising finding in the development of anti-caries agents [3,5–8]. While the general picture of tooth protection by GA is now clear, some questions remain.

As a polyphenol, aqueous solutions of GA are highly acidic; for example, a 4 g/L GA solution has a pH of ~3.1. A decrease in dental plaque pH is thought to be responsible for the demineralization of teeth and solutions low in pH may cause dental erosion [9]. Given the limitations of using acidic solutions, GA solutions should be considered and tested at higher pH levels if this compound is to be developed into a clinical application.

However, storage of GA at higher pH may result in instability because many polyphenols, such as green tea catechins [10], caffeic acid and chlorogenic acid [11], are not stable at high pH, which may result in the destruction of polyphenolic molecules and the

Correspondence: Jiyao Li, Department of Operative Dentistry and Endodontics, West China College of Stomatology, NO.14, Unit 3, Renmin Nan Road, Chengdu city, Sichuan province, 610041, PR China. Tel: +86 28 85501439. Fax: +86 28 85582167. E-mail: jiyao_li@126.com
*Jingyang Zhang and Xuelian Huang contribute equally to this article.

(Received 14 May 2014; accepted 7 January 2015)

ISSN 0001-6357 print/ISSN 1502-3850 online
DOI: 10.3109/00016357.2015.1007478

formation of pigment [10,11]. It is not clear whether GA is stable at high pH and how potential chemical changes of GA will impact on its anti-caries effect.

On the other hand, as GA is expected to reduce the risk of disease and provide health benefits through daily intake [2], GA may find practical applications for the improvement of dental health when added to foods and beverages. Additionally, if GA could protect teeth under acidic conditions, in which it is more stable, it may be used as an ingredient in acidic foods or soft drinks.

Therefore, the purpose of this study was to investigate the stability of GA solutions at various pH levels and to examine the resultant effects on enamel demineralization.

Materials and methods

Gallic acid

Gallic acid (GA; reference substance) was purchased from The National Institute for the Control of Pharmaceutical and Biological Products (Chengdu, China). GA solutions in H_2O (pH 3.1) and in phosphate buffers with pH 5.5, pH 7.0 and pH 10.0 were employed in the present study.

Spectrophotometric analysis

Changes in the chemical composition of GA at different pH levels were qualitatively studied with ultraviolet (UV) absorption spectroscopy. The spectra were measured in 1-nm increments at wavelengths ranging from 200–400 nm and they were recorded on a Perkin-Elmer Lambda 35 UV−VIS spectrophotometer (Perkin Elmer, Norwalk, CT). Sodium dihydrogen phosphate and NaOH were mixed in various ratios to prepare buffers at concentrations of 0.01 M at pH 5.5, pH 7.0 and pH 10.0.

After the solutions were prepared, the UV spectra were obtained immediately and after 1 h, 8 h, 24 h, 48 h and 72 h. In addition, after 24 h, an aliquot of GA at pH 10.0 was reduced to pH 7.0 with concentrated hydrochloric acid. Additional measurements were taken immediately and again after 24 h. All samples were analysed in triplicate.

HPLC-DAD analysis

After different incubation time in H_2O or phosphate buffers, the remaining GA was further detected quantitatively by high performance liquid chromatography with diode array detection (HPLC-DAD). The Agilent 1200 series (Agilent Technologies, Santa Clara, CA) high performance liquid chromatography (HPLC) instrument consisted of a model G1315D diode array detector (DAD), a G1312B binary Pump, a G1379B vacuum degasser, a G1367C autosampler and a G1316B column heater. Gradient elution HPLC was applied at a flow rate of 0.4 mL/min with detection at 270 nm. Two solvents were used for the mobile phase: (A) 0.1% formic acid and 10 mM ammonium formate (pH3.0) and (B) acetonitrile. The compounds were separated using the following gradient: 0–5 min, 5% B; 5–8 min, 20% B; 20–30 min, 30% B; 30–40 min, 20% B; 40.0–40.1 min, 5% B. The injection volume was 10 μL. Data processing was performed using CHEMSTATION B.04.02 software (Agilent Technologies, Santa Clara, CA, USA).

Specimen preparation

Bovine enamel specimens were prepared according to a previous study [12]. Briefly, permanent bovine incisors were obtained from a local slaughterhouse. Cubic enamel blocks were prepared with a diamond-coated band saw under continuous water cooling (Struers Minitom; Struers, Copenhagen, Denmark) and then embedded in Orthodontic Resin (L.D. Caulk, Milford, CT). The labial surface of enamel was serially smoothed with waterproof SiC abrasive papers up to 5000 grit, followed by polishing on a felt cloth impregnated with diamond paste (1–5 μm; Struers). Subsequently the enamel surface was covered with a double layer of nail varnish, except for a 2×2 mm^2 window area. Then the surface microhardness (SMH) of the enamel was evaluated with a Knoop diamond indenter (Duramin-1/-2; Struers) at 50 g load for 15 s dwell time. Ninety specimens with SMH between 388.7–436.1 Knoop hardness number (KHN) were selected and stored in Hank's balanced salts solution until use.

pH cycling

The specimens were randomly divided into the following nine groups (each $n = 10$): 1 g/L NaF (positive control), 4 g/L GA in H_2O (pH 3.1) and in 60 mM phosphate buffers at pH 5.5, pH 7.0 and pH 10.0 and buffers without GA at the same pH (pH 3.1, pH 5.5, pH 7.0 and pH 10.0), respectively (negative control). The treatment solutions were stored at room temperature for 24 h before use for pH-cycling to get the chemical changes. Then the specimens were pH-cycled following the procedure described in previous studies [7,8,13]. Briefly, after immersion of the specimens in one of the treatment solutions for 5 min, the blocks were placed in an acidic buffer (50 mmol/L acetic acid, 1.5 mmol/L potassium dihydrogen orthophosphate, pH 5.0) for 1 h (2 mL per block) and then in a neutral buffer (20 mmol/L HEPES, 1.5 mmol/L potassium dihydrogen orthophosphate, pH 7.0) for 5 min (2 mL per block). This procedure was repeated for 12 cycles. After pH cycling, the acidic buffers were retained for analysis. The calcium concentration of the acidic solutions was determined using atomic absorption spectrophotometry (AAS) (Thermo

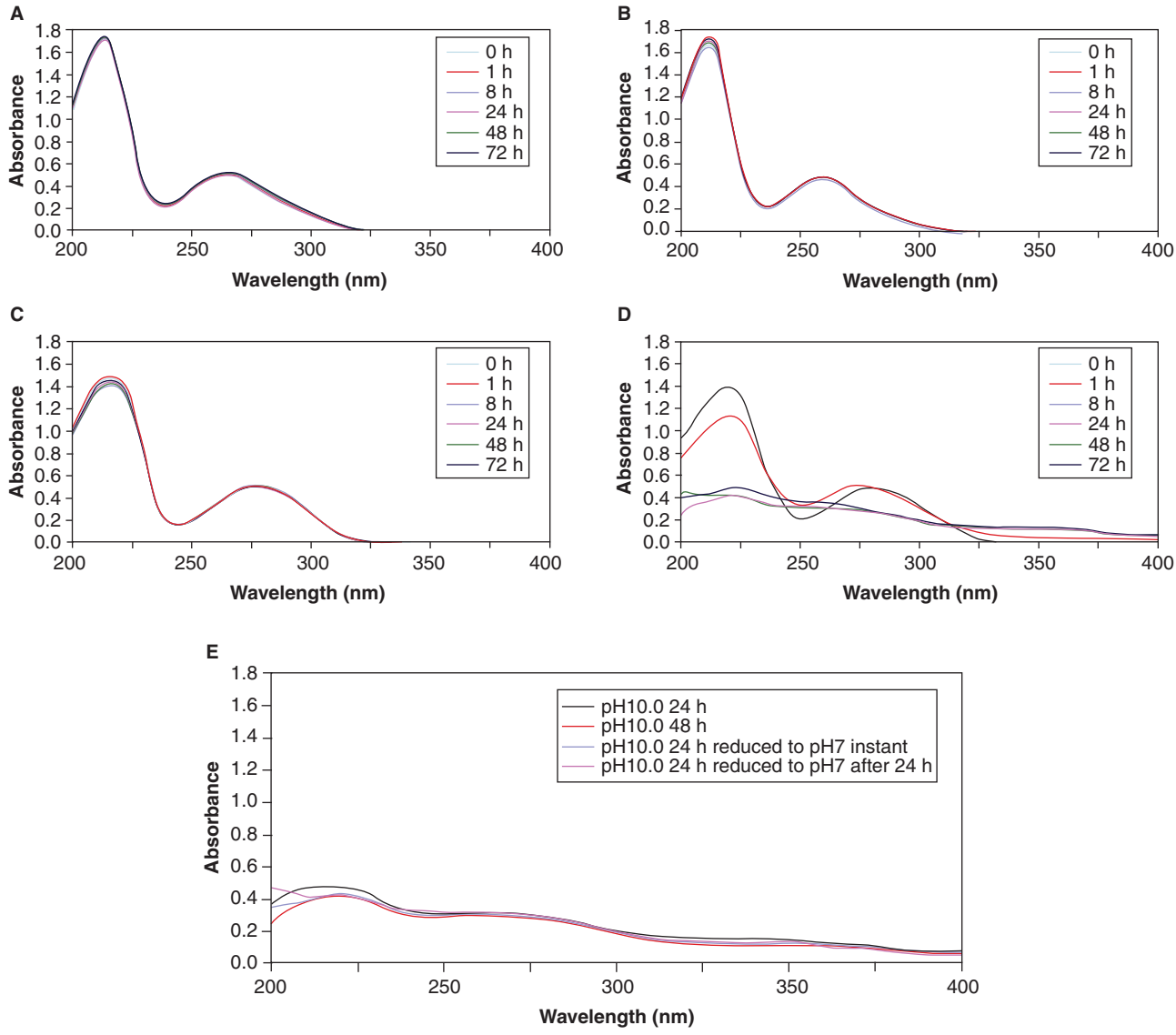

Figure 1. The effect of pH and time (0-time, 1 h, 8 h, 24 h, 48 h and 72 h) on the UV spectra of GA (10 μg/mL): (A) GA in H_2O; (B) pH 5.5; (C) pH 7.0; (D) pH 10.0; (E) pH 10.0 and then lowered to pH 7.0.

Element MKII-M6, Thermo Electron, Waltham, MA). Then, the calcium depletion rate (CDR) for each sample was calculated. This is a measure of the calcium extracted per unit area per unit time per mL acid and is calculated as:

$$\mathrm{CDR} = \frac{\text{mass of calcium extracted}}{(\text{time in acid})(\text{area exposed})} (\mu g / (h\,mm^2))$$

Statistical analysis

Statistical evaluations were performed using SPSS 16.0 software (SPSS Incorporated, Chicago, IL). The normal distribution of the data was tested with Kolmogorov–Smirnov tests. A *T*-test was used to compare the GA group with the negative control at the same pH. One-way ANOVA followed by a *post hoc* least significant difference (LSD) test was used to compare the GA groups at different pH levels. The level of significance was set at 5%.

Results

Spectrophotometric analysis

Both the pH and incubation time affected the chemical stability of GA solution, as demonstrated in the UV spectra (Figure 1). The UV spectra of GA in H_2O (Figure 1A), at its intrinsic pH, had absorption maxima at 213 nm and 263 nm, and these peaks did not change over time. When the pH was raised to pH 5.5 (Figure 1B), these absorption maxima shifted to 212 nm and 259 nm, respectively and, after 72 h, there was a small further change, suggesting that GA was nearly stable at pH 5.5. Compared to GA in H_2O, GA in a pH 7.0 buffer and in a pH 10.0 buffer

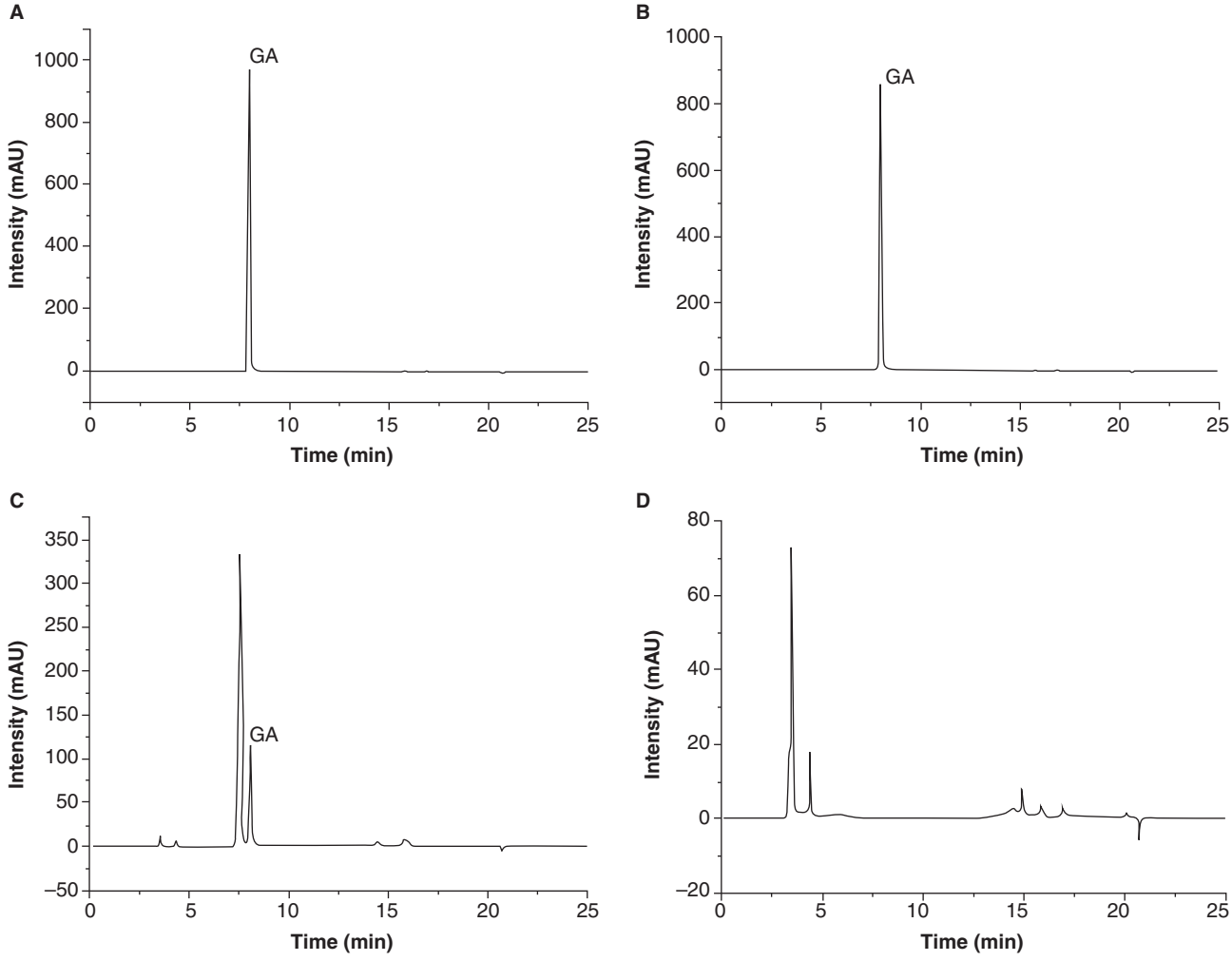

Figure 2. HPLC-DAD of GA solutions incubated for 24 h. (A) GA in H_2O; (B) pH 5.5; (C) pH 7.0; (D) pH 10.0.

displayed a shift to a longer wavelength (red shift). At pH 7.0 (Figure 1C), the absorption maxima shifted to 216 nm and 276 nm; the absorbance continuously decreased at the two wavelengths of maximum absorption, while they increased in 300–400 nm as time went by. At pH 10.0 (Figure 1D), the absorption maxima shifted to 220 nm and 280 nm, respectively, and, after 24 h, the two characteristic bands of phenols in the UV absorption spectra were no longer detected; the spectra were almost the same during the next 48 h. Therefore, we decided to store all the solutions at varying pH levels for 24 h to allow for this change in composition. When the pH 10.0 solution was neutralized to pH 7.0 (Figure 1E), no further spectral changes could be measured, indicating that the composition change was irreversible.

HPLC-DAD analysis

A series of HPLC chromatograms with fresh GA aqueous solutions was prepared. Based on these chromatograms, the regression equations for GA were obtained by plotting the peak area of GA (*y*-axis) vs the GA concentration (1.015–203.04 μg/mL, *x*-axis): $Y = 73.6326x + 0.04986$, $R^2 = 0.9993$, and the retention time of GA in the present HPLC condition was determined (8.174~8.468 min). Typical HPLC chromatograms of GA at different pH levels incubated for 24 h are shown in Figure 2. According to the retention time and regression equations, the GA peak area was identified and the amount of remaining GA data was calculated (Figure 3), respectively. It was found that GA in pH 10.0 was extremely unstable and degraded almost completely after 3 h, whereas in H_2O it remained stable for at least 72 h. The effect of pH on the stability of GA was best illustrated at pH values between 5.5–7.0. GA incubated at pH 5.5 was slightly degraded, with 93.6% GA left after 24 h, while only 13.1% was left at pH 7.0 after 24 h. It was obvious that the stability of GA was pH-dependent under the present experimental conditions.

pH cycling

The average CDR data are presented in Table I, which shows that the CDR in the GA groups were

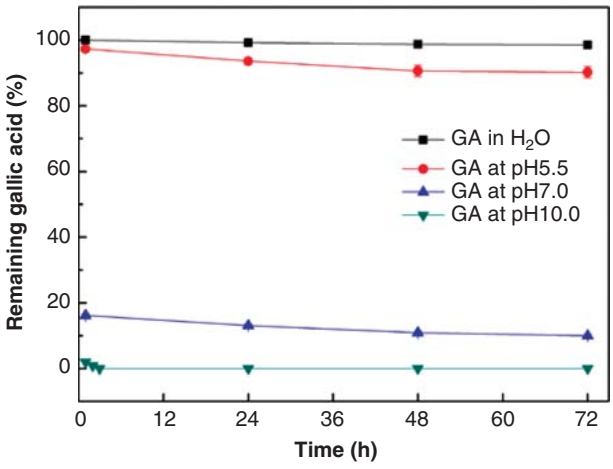

Figure 3. Remaining gallic acid (GA) in 60 mM sodium phosphate buffers with varying pH levels. Data are expressed as the mean ± SD of $n = 3$ samples.

significantly lower than in the corresponding negative control ($p < 0.05$, *t*-test), but no significant differences were observed among the GA treatments ($p > 0.05$, ANOVA). All the GA groups revealed similar values to the NaF group ($p > 0.05$, ANOVA). The statistical results showed that GA could inhibit enamel demineralization at different pH levels.

Discussion

Combining the data from this study, we conclude that GA solutions were efficacious in inhibiting enamel demineralization in acidic conditions, as the GA molecules were relatively stable at pH 3.1 and showed marginal instability at pH 5.5. In contrast, when stored at pH 7.0 and pH 10.0, the GA molecules were degraded, but such GA solutions retained a significant level of efficacy. We did not observe an impact of chemical changes of GA on the inhibition of enamel demineralization.

The experiments in the present study were conducted at pH values of 3.1, 5.5, 7.0 and 10.0, as this wide pH range is found in the oral cavity due to bacterial fermentation of different types of food or to oral care products. An oral plaque pH higher than 6.0 is considered to be safe; a pH between 6.0–5.5 is potentially cariogenic, particularly with respect to dentine, whereas a pH between 5.5–4.0 is within the cariogenic (danger) zone for caries [14]. We found that a 4 g/L aqueous solution of GA has a pH of ~3.1 and the GA level in solution is almost 100%, even after 72 h of incubation. A pH of ~10.0 is the highest acceptable pH value for toothpaste and other oral care products, in accordance with International Standard [15], and, at pH 10.0, no GA polyphenolic molecules remained after 24 h of storage.

We attempted to detect the degeneration products of GA under neutral or alkaline conditions in detail (in preliminary experiments) using silica gel column chromatography and liquid chromatography–time of flight-mass spectrometry (LC-TOF-MS). However, the products were not stable and were difficult to identify. Therefore, spectrophotometric analysis [11] and HPLC-DAD analysis [10] were used to qualitatively measure the composition changes and to quantitatively measure the remaining GA levels.

In UV absorption spectra, phenols usually show two bands between 200–360 nm. Friedman and Jurgens [11] observed similar GA changes in the UV spectra and concluded that these may result from the formation of unstable quinone intermediates. Other documents [16] confirmed that polyphenols are easily oxidized by oxygen and the product is quinone, with pH as one of the most important factors. In parallel, we speculate that quinones were formed in the GA solution at neutral and high pH levels, which caused the compositional change. In HPLC-DAD analysis, gradient elution refers to a continuous change in the mobile phase during separation, such that the retention of later peaks is continually reduced [17]. By regression calculation of the area of GA, the remaining GA amount could be assessed. HPLC analysis clearly demonstrated that GA was rapidly degraded at high pH.

In this study, we followed the pH-cycling regime used in previous studies [7,8,13] and we prepared a negative control at each pH value in order to subtract the influence of pH itself. Despite differing amounts of remaining GA at different pH levels, during pH

Table I. Calcium depletion rates (CDR) in buffers at various pH levels.

	CDR/(μg/h/mm^2)			
Group	pH 3.1	pH 5.5	pH 7.0	pH 10.0
Gallic acid (GA)	0.31 ± 0.19*	0.30 ± 0.07*	0.39 ± 0.09*	0.28 ± 0.13*
Negative control (buffer)	0.55 ± 0.16	0.53 ± 0.14	0.50 ± 0.12	0.47 ± 0.19
Positive control (NaF)	0.29 ± 0.14			

Data expressed as the mean ± standard deviation of the mean ($n = 10$).
No significant differences were found among GA treatments ($p > 0.05$).
All GA groups showed CDR values similar to the NaF group ($p > 0.05$).
*Significant differences were found between GA treatments and buffer treatments at the same pH ($p < 0.05$).

cycling, no impact of pH on ability of GA to inhibit enamel demineralization was detected. GA had an inhibiting effect over a wide pH range, showing that it could have broad practical applications. A proper delivery way of drug is also important to achieve the optimum effect. In previous studies, particularly under acidic conditions, NaF and nano-HA were found to significantly accelerate the rate, depth of penetration and extent of remineralization of artificial incipient lesions [14,18]. Casein-derived phosphopeptides were also demonstrated to protect hydroxyapatite when simultaneously exposed to CPP and acid, even when the mix buffer was adjusted to pH 2.5 [19]. In this study, our results show that the inhibition effect of GA on the enamel demineralization is not pH sensitive, indicating GA could be used at a wide pH range. For instance, it might be incorporated into acidic food or drink, neutral mouth rinse wash water and alkaline toothpaste or used as an edible natural pigment without losing its inhibition function.

It has been suggested that the diffusion pathway of demineralization in enamel is controlled by the organic matrix network [20], which was revealed to be the mechanism of GCE's anti-demineralization effects [21]. A present study further revealed that GCE could maintain the structural integrity of dentin organic matrix and reduce biodegradation against enzymatic digestion [22]. As GA is the main effective component of GCE in inhibiting demineralization [4] and the organic matrix of mature enamel mainly consists of proteins such as enamelin, tuftelin and sheathlin [23], we hypothesize that GA reacts with proteins at different pH levels, thereby slowing the demineralization. In fact, GA can bind with milk protein [24] and can form cross-links with calcium ions and thereby form precipitates [25]. Quinones, which are highly electrophilic compounds, could react with the nucleophilic groups (-NH_2 and -SH) in proteins [16]. Such interactions form covalent bonds and the cross-linked combination is irreversible [26]. This mechanism still needs to be explored in detail.

Despite of the broad application of fluoride therapies, dental caries continues to be the most prevalent and costly oral infectious disease worldwide [8]. Various types of polyphenols have been reported to have potential in caries prevention. As illustrated in this study for GA, the instability resulted in chemical change into pigmentation substrate, which may lead to other side-effects, such as teeth staining. More concerns may be raised when it comes to the applications of many polyphenols.

In conclusion, the present study demonstrated that, although different amounts of GA remained when solutions are prepared at various pH levels, no impact of pH on the ability of GA to inhibit of enamel demineralization was detected. GA could, therefore, be a useful source for the development of promising anti-cariogenic agents with broad practical applications.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (General Program, 81170958) and Science & Technology Pillar Program of Sichuan Province (2011SZ0130).

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

[1] Shahrzad S, Aoyagi K, Winter A, Koyama A, Bitsch I. Pharmacokinetics of gallic acid and its relative bioavailability from tea in healthy humans. J Nutr 2001;131:1207–10.
[2] Hsu C, Yen G. Effect of gallic acid on high fat diet-induced dyslipidaemia, hepatosteatosis and oxidative stress in rats. Br J Nutr 2007;98:727–35.
[3] Cheng L, Li J, Hao Y, Zhou X. Effect of compounds of Galla chinensis and their combined effects with fluoride on remineralization of initial enamel lesion in vitro. J Dent 2008;36:369–73.
[4] Huang X, Liu M, Li J, Zhou X, Ten Cate JM. Chemical composition of Galla chinensis extract and the effect of its main component (s) on the prevention of enamel demineralization in vitro. Int J Oral Sci 2012;4:146–51.
[5] Cheng L, Exterkate R, Zhou X, Li J, Ten Cate JM. Effect of Galla chinensis on growth and metabolism of microcosm biofilms. Caries Res 2011;45:87–92.
[6] Huang S, Gao S, Cheng L, Yu H. Combined effects of nano-hydroxyapatite and Galla chinensis on remineralisation of initial enamel lesion in vitro. J Dent 2010;38:811–19.
[7] Zou L, Zhang L, Li J, Hao Y, Cheng L, Li W, et al. Effect of Galla chinensis extract and chemical fractions on demineralization of bovine enamel in vitro. J Dent 2008;36:999–1004.
[8] Huang X, Cheng L, Exterkate R, Liu M, Zhou X, Li J, et al. Effect of pH on Galla chinensis extract's stability and anticaries properties in vitro. Arch Oral Biol 2012;57:1093–9.
[9] Cate JMT. Current concepts on the theories of the mechanism of action of fluoride. Acta Odontol Scand 1999;57:325–9.
[10] Zhu QY, Zhang A, Tsang D, Huang Y, Chen Z. Stability of green tea catechins. J Agric Food Chem 1997;45:4624–8.
[11] Friedman M, Jürgens HS. Effect of pH on the stability of plant phenolic compounds. J Agric Food Chem 2000;48:2101–10.
[12] Deng M, Wen H, Dong X, Li F, Xu X, Li H, et al. Effects of 45S5 bioglass on surface properties of dental enamel subjected to 35% hydrogen peroxide. Int J Oral Sci 2013;5:103–10.
[13] Li JY, Zhan L, Barlow J, Lynch RJ, Zhou XD, Liu TJ. Effect of tea polyphenol on the demineralization and remineralization of enamel in vitro. Sichuan Da Xue Xue Bao Yi Xue Ban 2004;35:364–6.
[14] Huang S, Gao S, Cheng L, Yu H. Remineralization potential of nano-hydroxyapatite on initial enamel lesions: an in vitro study. Caries Res 2011;45:460–8.
[15] ISO. ISO 11609. Dentistry-toothpastes-requirements, test methods and marking. Geneva: International Organisation for Standardisation; 1995. p 1–10.
[16] Shi B, Di Y. Plant polyphenol. Beijing: Science Press; 2000.
[17] Snyder LR, Dolan JW. High-performance gradient elution: the practical application of the linear-solvent-strength model. Hoboken: John Wiley & Sons; 2007.
[18] Yamazaki H, Margolis HC. Enhanced enamel remineralization under acidic conditions in vitro. J Dent Res 2008;87:569–74.
[19] Kanekanian AD, Williams R, Brownsell VL, Andrews AT. Caseinophosphopeptides and dental protection: concentration and pH studies. Food Chem 2008;107:1015–21.

[20] Travis DF, Glimcher MJ. The structure and organization of, and the relationship between the organic matrix and the inorganic crystals of embryonic bovine enamel. J Cell Biol 1964;23:447–97.
[21] Zhang L, Xue J, Li J, Zou L, Hao Y, Zhou X, et al. Effects of Galla chinensis on inhibition of demineralization of regular bovine enamel or enamel disposed of organic matrix. Arch Oral Biol 2009;54:817–22.
[22] Deng M, Dong X, Zhou X, Wang L, Li H, Xu X. Characterization of Dentin Matrix Biomodified by Galla Chinensis Extract. J Endod 2013;39:542–7.
[23] Yu S. Oral histology and pathology. 6th ed. Beijing: People's Medical Publishing House; 2007.
[24] Xiao J, Mao F, Yang F, Zhao Y, Zhang C, Yamamoto K. Interaction of dietary polyphenols with bovine milk proteins: molecular structure–affinity relationship and influencing bioactivity aspects. Mol Nutr Food Res 2011;55:1637–45.
[25] Fang Z, Zhang M, Tao G, Sun Y, Sun J. Chemical composition of clarified bayberry (Myrica rubra Sieb. et Zucc.) juice sediment. J Agric Food Chem 2006;54:7710–16.
[26] Haslam E. Plant polyphenols: vegetable tannins revisited. Cambridge: CUP Archive; 1989.