

1 **Di-calcium-phosphate and phytosphingosine as an innovative acid**
2 **resistant treatment to occlude dentine tubules**

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16 **Key words:** Acid attack; Artificial saliva; Brushite; Dentine permeability; FTIR;
17 tubule occlusion, Phytosphingosine; SEM

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24 **ABSTRACT.** This study evaluated the ability of an experimental di-calcium-
25 phosphate desensitising agent (DCP) used alone or combined with
26 phytosphingosine (PHS) to occlude dentine tubules and resist a citric acid (CA)
27 or artificial saliva (AS) challenge. Three groups of human dentine specimens (DS)
28 were treated with 1) PHS alone, 2) DCP or 3) a combination of PHS and DCP.
29 Dentine hydraulic conductance was evaluated using a digital flow sensor at 6.9
30 kPa. The fluid volume average of each treated-DS was used to calculate the total
31 dentine permeability reduction (P%) prior to and following CA immersion for 1
32 min or 4 weeks in AS. Treated-DS were submitted to SEM and FTIR
33 spectroscopy analysis. Statistically significant differences (P%) were identified
34 between the groups by ANOVA and Fisher’s multiple comparison test (P < 0.05).
35 Interestingly, PHS and DCP appeared to work synergistically. DS treated with

1 DCP or PHS/DCP demonstrated a significant reduction (P%) prior to and
2 following CA or AS challenge ($P < 0.05$). SEM and FTIR analysis showed
3 consistent brushite crystals occluding the dentine tubules. Conversely, the
4 application of PHS alone failed to demonstrate any significant reduction of
5 dentine permeability ($P > 0.05$) or show any evidence of occlusion of the dentine
6 tubules. DCP can however, be used alone or combined with PHS to decrease the
7 dentine permeability as well as resisting an acid and artificial saliva challenge.
8 This may therefore represent a suitable treatment for dentine hypersensitivity.

9 **INTRODUCTION**

10 Dentine hypersensitivity (DH) represents a common clinical condition within the
11 young and adult population in western countries [West *et al.*, 2013] mainly due to
12 gastric and dietary acids revealing underlying dentine [Lussi *et al.*, 2004]. DH
13 develops in two phases [Dowell and Addy, 1983]: i) lesion localisation:
14 subsequent loss of enamel caused by tooth wear or to gingival recession; ii) lesion
15 initiation and DH symptomatology, which occurs after the protective smear layer
16 is removed and the underlying dentine tubules are exposed. According to the
17 hydrodynamic theory, the movement of fluid within the dentine tubules following
18 either physical or osmotic stimulation may cause pain [Brannstrom *et al.*, 1968].
19 The main treatment for DH is based on the reduction of the fluid flow through the
20 physical occlusion of the dentine tubules [Pashley, 1986]. Although several
21 products are currently available, there is still the need to develop innovative acid
22 resistant desensitising agents. Acidic di-calcium phosphates (e.g., brushite) have
23 been widely used as a hard tissue substitute due to their bioactivity and
24 biocompatibility [Cama *et al.* 2009]. Moreover, it has been recently reported that
25 pre-treatment of experimental hydroxyapatite discs (HAp) with sphingoid bases
26 such as sphingosine, phytosphingosine (PHS), PHS phosphate and sphinganine
27 significantly protected HAp against acid demineralisation *in vitro* [Valentijn-
28 Benz *et al.*, 2015]. Cukkeman *et al.*, (2015) revealed using atomic force
29 measurement that PHS and other sphingoid bases can form diffusion barriers
30 against H^+ ions and bacteria. In principle, the reported anti-erosive properties
31 would suggest that PHS could be included in oral care products for DH treatment.
32 The aim of the present study was to evaluate the ability of experimental
33 desensitising agents based on an acid di-calcium-phosphate (DCP) alone or in
34 combination with PHS to occlude exposed dentine tubules. This aim was
35 accomplished by quantitatively evaluating the reduction of the hydraulic
36 conductance following the application of the tested materials and a subsequent

1 citric acid (CA) or artificial saliva (AS) challenge. SEM and FTIR spectroscopy
2 analysis were also conducted. The null hypotheses tested were: 1) the application
3 of DCP onto exposed dentine when used alone or in combination with PHS would
4 not reduce the hydraulic conductance of EDTA-treated dentine; 2) the citric acid
5 (CA) or artificial saliva (AS) challenge would reduce their ability to maintain the
6 occlusion of the dentine tubules (longevity of treatment).

7

8 **MATERIALS AND METHODS.**

9 ***Preparation of specimens.*** Thirty sound human molars were extracted for surgical
10 reasons under institutional ethical approval (granted by the research ethics
11 committee) and used to create mid coronal dentine discs (DS) as described by
12 Sauro *et al.*, [2006]. In brief, occlusal enamel was removed using a slow-speed,
13 water-cooled diamond saw (RS-70300; Struers, Copenhagen, Denmark). A
14 second parallel cut was performed 1.5 mm beneath the cementum-enamel junction
15 in order to remove the roots. A standard smear layer was created using a 180-grit
16 silicon-carbide paper (30 s) and subsequently removed using 17% EDTA (pH 7.4)
17 for 1 min followed by ultrasonic bath containing distilled water (5 min). DS were
18 randomly divided into two main groups based on the challenge storage (n=15/
19 group): i) CA: citric acid; ii) AS: artificial saliva. Each main group was then
20 divided in three sub groups (n=5/sub-group) based on the desensitising treatment:
21 A) PHS: 4-hydroxysphinganine; B) DCP: Di-calcium-Phosphate (Brushite); C)
22 PHS/DCP: phytosphingosine + Brushite. A Tris-Tween/ethanol solution (5
23 mg/ml) of PHS was prepared as described by Valentijn-Benz *et al.*, [2015].

24 ***Desensitising dentine treatment.*** Specimens were rinsed with deionised water
25 prior to the pre-treatment with PHS. PHS (0.1 ml) was gently brushed onto the
26 dentine surface of all the specimens in Group A using a micro-brush (20 s), in
27 triplicate (60 s; 0.3 ml) and then rinsed with deionised water (10s). The DCP was
28 prepared as described by Cama *et al.*, [2009] by mixing equimolar quantities of
29 β -tricalcium phosphate (β -TCP, Sigma-Aldrich, Gillingham, UK) and
30 monocalcium phosphate monohydrate (MCPM, Sigma-Aldrich) in deionised
31 water (R= 3 g/ml). The DCP specimens (Group B) were treated by an application
32 of DCP (0.3 g) on the EDTA-treated dentine. Two consecutive layers of a semi-
33 fluid paste (30s each; ~0.15 g) were gently brushed onto the dentine surface using
34 a micro-brush (60s) and left undisturbed for a further 30 s. Finally, the specimens
35 were rinsed with deionised water (10s) and the excess of DCP was removed from

1 the dentine surface using the tip of a soft paint brush. The PHS/DCP specimens
2 (Group C) also received the same PHS treatment, immediately followed by
3 application of DCP as described above.

4 ***Dentine permeability evaluation.*** All DS were cemented (ROCKET Heavy DVA,
5 USA) to Plexiglass blocks penetrated with an 18 Gauge stainless steel tube. Each
6 specimen was finally connected to a hydraulic pressure device (Fig. 1) under a
7 constant hydraulic pressure of 6.9 kPa (Sauro *et al.*, 2007; Pashley *at al.*, 1986)
8 for the measurement of the fluid volume (FV) through a digital sensor with a
9 resolutions of ~100 nl/min and a response reading frequency of 1.56 Hz (ASL
10 1600, Sensirion, Staefa, Switzerland). The highest hydraulic conductance of each
11 specimen was recorded (Lp-max = 100% was arbitrarily assigned); specimens
12 with a fluid flow rate less than 3µl/min were excluded and replaced with discs
13 with a higher flow rate. Lp-max permits an evaluation of the changes in dentine
14 permeability following the application of the test treatments. Each specimen was
15 treated with the test materials as described above, and based on observations
16 obtained during a pilot study, five FV readings were performed every 3 minutes
17 for 15 minutes. These readings were then averaged and used to calculate the
18 permeability reduction (P %) of each specimen using the following equation:

$$19 \quad \%P = \frac{\text{fluid filtration rate of the treated dentine}}{\text{fluid filtration rate of EDTA-etched dentine}} \times 100$$

20 The specimens were subsequently tested according to two different ageing
21 protocols (CA or AS). DS were immersed in CA (6 wt%; pH 1.5) and then left
22 undisturbed for 60 s or in AS for 4 weeks (37°C). The composition of the AS was
23 1.5 mmol/L CaCl₂, 50 mmol/L KCl, 0.9 mmol/L KH₂PO₄, 20 mmol/L Tris, pH
24 7.4. This solution (25 ml) was replaced every 72 h. The means (P %) and standard
25 deviations of each group were calculated and any significant differences were
26 observed between the groups by One-way ANOVA and Fisher's least test (P <
27 0.05).

28 ***ATR/FTIR Spectroscopy and SEM evaluation.*** Two further DS were prepared
29 for each sub-group and subsequently treated and challenged as previously
30 described. These were analysed using a ATR/FTIR Spectrometer (Perkin-Elmer,
31 Beaconsfield, UK) with a resolution of 4 cm⁻¹ to characterise the chemical
32 composition of the dentine prior to and following each product application and
33 challenge protocol (*i.e.* CA or AS). The same specimens were then dried overnight
34 in a silica-containing desiccator at 37°C, gold sputter-coated (SCD004 Bal- Tec,

1 Vaduz, Liechtenstein) and examined using SEM (S-3500; Hitachi, Wokingham,
2 UK).

3 **RESULTS.**

4 The results of dentine permeability reduction (P%) are illustrated in Figure 2. The
5 application of DCP or PHS/DCP onto the EDTA-etched dentine significantly
6 reduced dentine permeability ($P < 0.05$). However, the specimens treated with
7 PHS/DCP demonstrated an ability to reduce dentine permeability by 92.2% after
8 CA attack (Fig. 2A) and 83.1% after AS immersion (Fig. 2B). There was no
9 significance reduction ($P > 0.05$) prior to and following CA or AS challenge in
10 any group. PHS induced the lowest Lp reduction (10%) and no significant change
11 ($P > 0.05$) was observed following CA or AS challenge. These results were
12 confirmed by the SEM analysis, which showed a demineralised dentine surface
13 with patent dentine tubules and exposure of collagen fibrils following EDTA
14 etching (Fig. 3A), PHS application (Fig. 3B) and after CA attack (Fig. 3C). The
15 FTIR analysis showed demineralised dentine (Amide I and II) both after PHS
16 application (Fig. 3D) and after AS aging (Fig. 3E).

17 Conversely, dentine treated with DCP or PHS/DCP showed dentine tubules that
18 remained occluded following CA (Fig. 4A and 4B, respectively) or AS challenge
19 (Fig. 4C). Conversely, the EDTA-etched specimens treated with PHS alone and
20 subsequently immersed in AS presented only very few mineral deposits on the
21 outer surface and patent dentine tubules (Fig. 4D). The FTIR analysis revealed
22 that the mineral crystallites precipitated on the dentine surface following DCP or
23 PHS/DCP application was brushite (Fig. 4E). The brushite's crystals (size $< 2\mu\text{m}$)
24 that precipitated within the tubules and on the dentine surface (Fig. 4A and 4B),
25 converted into a more complex apatite-like calcium phosphate following AS
26 immersion (Fig. 4F), although the size and the morphology of such latter crystals
27 presented no clear change over time (Fig. 4C). Conversely, the EDTA-etched
28 specimens treated with PHS and immersed in AS presented a very low PO peak
29 at 1019 cm^{-1} and a clear demineralised dentine surface (Amide I and II), (Fig.
30 4G).

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33 **DISCUSSION**

34 An ideal dentine desensitiser should be easy to apply, act rapidly, cause no
35 alteration to the tooth structure and/or irritation to pulp, and last as long as
36 possible [Grossman, 1935].

1 However, in order to reduce the symptomatology of DH, it is of key importance
2 to decrease dentine permeability (Lp), but also maintain the occlusion of the
3 dentine tubules following subsequent acid and saliva challenges [Wang *et al.*,
4 2010]. The risk for DH may increase with the presence of dietary acids, as these
5 remove the smear layer and open the underlying dentine tubules [Sauro *et al.*,
6 2007]. Citric acid is a common component of both fruit and soft drinks, and it is
7 widely used in *in vitro* studies to simulate the oral environment and test the
8 resistance of desensitisers to an acid challenge [Wiegand *et al.*, 2007]. Saliva can
9 also solubilise materials adhering to teeth and contains calcium and phosphate
10 ions that can interact with surfaces [Arrais *et al.*, 2003]. Therefore, it is essential
11 to evaluate whether novel desensitising agents have the potential to effectively
12 occlude the dentine tubules under circumstances similar to the oral environment.
13 The results of the present study would therefore appear to reject both of the two
14 null hypotheses since the DCP paste alone or in combination with PHS caused a
15 significant ($P < 0.05$) permeability reduction before and after a CA challenge due
16 to the precipitation of brushite both within the dentine tubules and on the dentine
17 surface (Fig. 4A, 4B and 4E) or after AS storage, where this brushite converted
18 to a different and probably more complex calcium-phosphate (Fig. 4 F) thereby
19 maintaining the status of tubular occlusion over a period of 4 weeks (Fig. 4C).
20 Indeed, Jiang *et al.*, (2009) demonstrated that brushite may convert to stable
21 hydroxyapatite when immersed in a calcium-rich solution at a slightly alkaline
22 pH.

23 Similarly, a novel calcium phosphate desensitising agent (TEETHMATE™,
24 Kuraray corp., Japan), consisting of tetracalcium phosphate and di-calcium
25 phosphate anhydrous (i.e. Monetite) has been demonstrated both in clinical [
26 Mehta *et al.*, 2014] and *in vitro* [Thanatvarakorn *et al.*, 2013] studies to be
27 efficacious as dentine desensitising agent. This product contains di-calcium
28 phosphate as one of the main constituent, whereas the DCP paste used in this
29 study was made of equimolar quantities of β -TCP and mono-calcium phosphate-
30 monohydrate that precipitate as brushite (Cama *et al.*, 2009) during application
31 (Fig. 4). Conversely, TEETHMATE appears to precipitate as an apatite-like
32 mineral [Brown and Chow, 1983]; its solubility in acid solutions [pH <5.0] may
33 be much lower than that of brushite [Jiang *et al.*, 2009]. The precipitation of
34 brushite however, is not a new issue in dental research. For instance, dentine acid-
35 etching induces the release of calcium and phosphate which may precipitate as
36 either brushite or octacalcium phosphate depending on the environmental pH.
37 However, Shellis *et al.*, (1997) demonstrated that at a pH below 4, as in the DCP

1 paste (Fig. 4E), brushite is mainly precipitated. Moreover, the acidic environment
2 created by the CA challenge induced further precipitation of monetite [Şahin and
3 Çiftçioğlu, 2014] and tubules occlusion (Fig. 4C). Indeed, crystals of di-calcium
4 phosphates may increase and create a mechanical interlocking in acidic pH,
5 thereby providing a more structural resistance to further hard tissue loss [Wang
6 and Nancollas, 2008].

7 Although PHS was not able to suitably occlude the dentine tubules (Fig. 3B) even
8 after prolonged AS immersion (Fig. 4D), it appears to work synergistically in
9 combination with DCP, forming an effective DH desensitiser. These specific
10 results were probably due to the anti-erosive characteristic of PHS. Indeed, PHS
11 may capture ionised phosphate and have a protection effect against
12 demineralisation by binding any remaining HAp crystals [Kosoric *et al.*, 2007].
13 However, due to its amphipathic character, in solution, PHS has the tendency to
14 assemble into highly positively-charged aggregates or micelles, with the fatty
15 acid tails buried inside and the positively charged head groups exposed to the
16 bulk of the solution. Hence, the high density of positive charges on such
17 aggregates will more likely produce a high avidity for negatively charged
18 phosphate-rich surfaces such as HAp [Valentijn-Benz *et al.*, 2015].

19 It is also acknowledged that erosion initiated by dietary acids may exacerbate DH
20 and cause demineralisation of the collagen matrix. Demineralised dentine is
21 characterised by unprotected collagen fibrils (Fig. 3A) that can be degraded by
22 endogenous enzymes e.g., metalloproteases and cysteine cathepsins [Zarella *et*
23 *al.*, 2015]. Moreover, further collagen degradation can be also induced by
24 salivary esterases and/or bacteria proteases [Park *et al.*, 2008]. However, it has
25 been demonstrated that mineral precipitation induced by bioactive substances
26 e.g., calcium-phosphates and bioactive glasses may also reduce the enzymatic-
27 mediated collagen degradation [Tezvergil-Mutluay *et al.*, 2014] and the risk for
28 further wear of hard tissues (e.g. dentine) [Zarella *et al.*, 2015].

29 In conclusion, this experimental *in vitro* study demonstrated that the use of DCP
30 paste alone or in combination with PHS may represent a suitable treatment for
31 DH. The formation of acid resistant crystals within the dentine tubules produced
32 by the experimental materials evaluated in the present study would suggest that
33 they may be useful as potential long-term desensitisers for the treatment of DH.
34 Further evaluation however, would be required in order to define and create more
35 suitable clinical formulations for commercial products and their subsequent
36 application *in vivo*.

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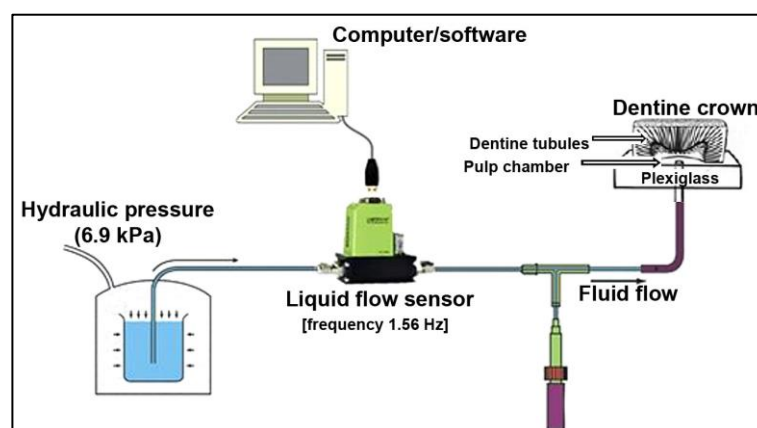
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35 FIGURES AND CAPTIONS

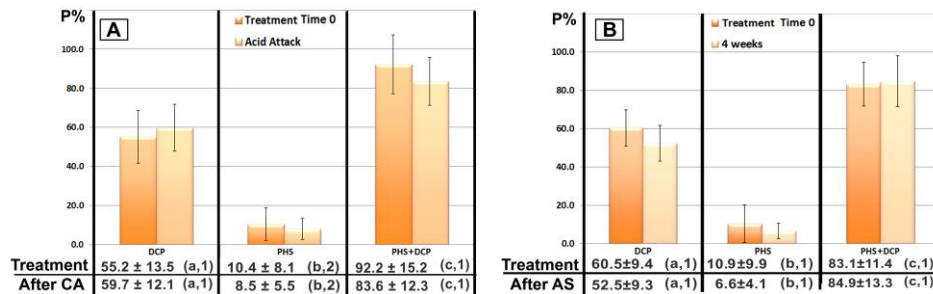
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6 **Figure 1.** Schematic illustration of how the dentine specimens were connected to a
7 hydraulic pressure device under a constant hydraulic pressure (6.9 KPa) and the
8 measurements of the fluid volume (FV) were attained via a digital sensor.

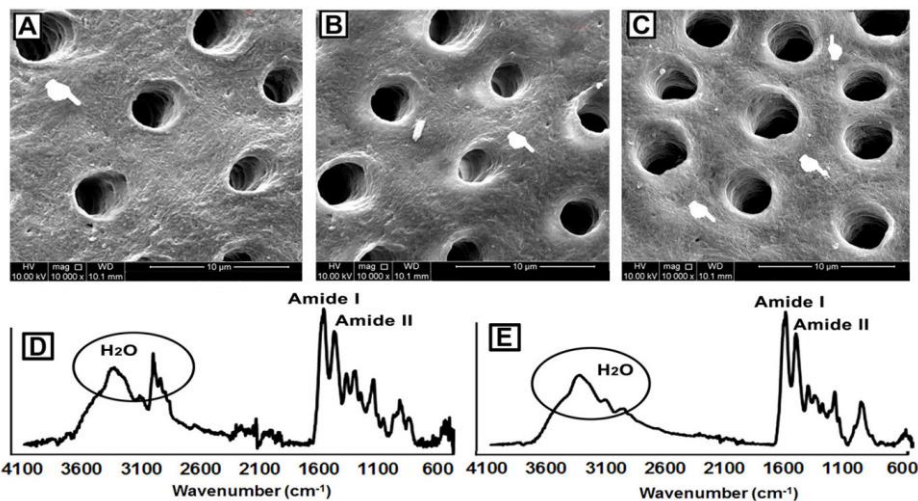
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18 **Figure 2.** Mean and standard deviations of %P (dentine permeability reduction) values
19 before and after a citric acid **A**) and AS **B**) challenge. In rows, different superscript letters
20 indicate significant differences between the three experimental desensitising agents
21 following application or following a CA or AS challenge ($P < 0.05$). In columns, different
22 superscript numbers indicate significant differences in the same desensitising agent,
23 between application and CA or AS challenge ($P < 0.05$).

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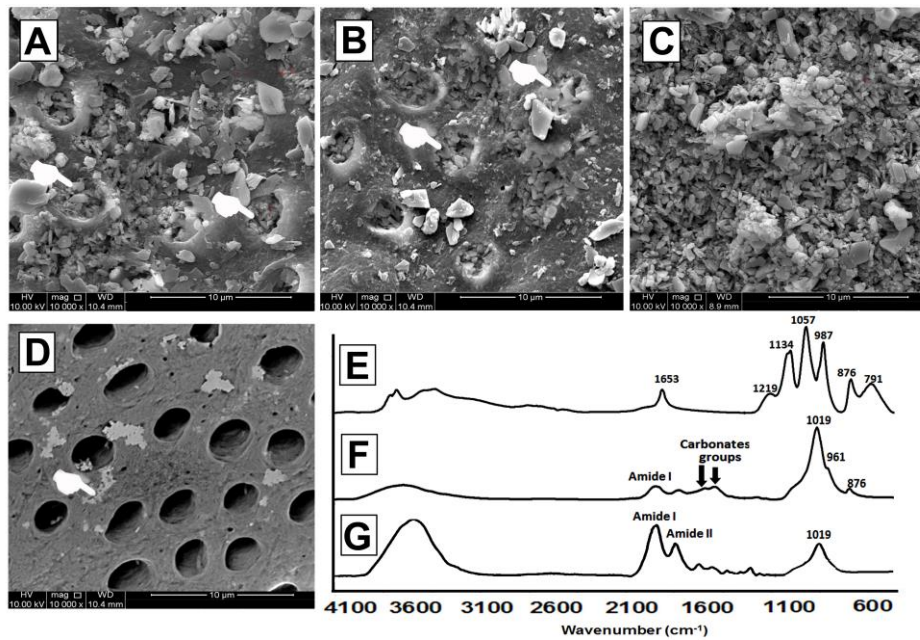


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41 **Figure 3.** SEM micrograph of EDTA-etched dentine showing several patent tubules and
42 collapsed collagen fibrils (pointer). **B**: EDTA-etched dentine following application of
43 PHS showing no tubules occlusion, but only collapsed collagen fibrils (pointer). **C**:
44 EDTA-etched dentine surface following application of PHS and subsequent CA attack.
45 Note the presence of patent tubules and collapsed collagen fibrils (pointer). **D**: Spectra of
46 EDTA-etched dentine treated with PHS. Note the bands at 3200–3400 cm^{-1} due to the O–
47 H stretching of water (H_2O) and amide bands of collagen (1200–1725 cm^{-1}) in dentine.
48 The same spectra was also observed following PHS application and after CA attack (**E**).
49 EDTA-etched dentine that received no desensitising treatment shows the same FTIR

1 features observed in figure-(E).

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21 **Figure 4. A:** SEM micrograph of the EDTA-etched dentine following application of DCP
22 and subsequent CA attack. Note the presence of mineral crystals (size <2 μm) inside the
23 dentine tubules (pointer). **B:** EDTA-etched dentine treated with PHS/DCP and exposed
24 to CA; mineral crystals are still present inside dentine tubules (pointer). **C:** EDTA-etched
25 dentine following application of PHS/DCP and AS immersion. Note the greater amount
26 of crystals covering the dentine surface; similar features were also observed in the
27 specimens treated with DCP and immersed in AS. **D:** SEM micrograph of EDTA-etched
28 dentine treated with PHS and immersed in AS. Note the presence of very few mineral
29 deposits on the dentine surface (pointer) **E:** FTIR spectra obtained from EDTA-etched
30 dentine treated with DCP and submitted to CA attack. Note bands at 3200–3400 cm⁻¹ (O–
31 H stretching of water in dentine). Water in brushite can be observed at 1653 cm⁻¹ (bending
32 mode), O–H in-plane bending at 1219 cm⁻¹ and H₂O oscillating motion at 791 cm⁻¹. The
33 PO stretching peaks of the brushite is observed at 1134, 1057, and 987 cm⁻¹. The same
34 spectra was attained after application of PHS/DCP on EDTA-treated dentine and
35 subsequent CA attack. **F:** FTIR spectra specimens treated with PHS/DCP and immersed
36 in AS. Note the PO peaks at 961 cm⁻¹ (ν₁), 1019 cm⁻¹ (ν₃ – asymmetric stretching mode
37 of hydroxyapatite) and carbonate bands at 1400–1500 cm⁻¹. These peaks were also present
38 in mineralised dentine and in the specimens treated with DCP and submitted to AS ageing.
39 **G:** FTIR spectra of EDTA-etched dentine treated with PHS and immersed in AS. In this
40 case, only a low PO peak at 1019 cm⁻¹ could be detected. Whereas, amide bands from
41 organic components (1200–1725 cm⁻¹) were clearly visible, indicating that dentine was
42 still demineralised.

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45 **Role of all authors:**

46 **Sauro S:** Corresponding author. He wrote the entire manuscript. He elaborated of the
47 experimental project and performed part of the dentine permeability assessment.

48 **Lin CY:** He contributed to perform the experimental project, specifically in the
49 preparation of specimens and dentine permeability assessment.

50 **Bikker FJ:** He contributed to revise the manuscript. He was also involved in the
51 elaboration of the experimental project. He formulated the experimental
52 phytosphingosine used in this study.

1 **Cama G:** He contributed to revise the manuscript. He was also involved in the elaboration
2 of the experimental project. He formulated the experimental Di-calcium phosphate used
3 in this study.
4 **Dubruel P:** He performed the entire experimental part about FTIR analysis and he was
5 involved in the interpretation of the results.
6 **Soria JM:** He was involved in the interpretation of the statistical analysis.
7 **D'Onofrio A:** She performed the SEM analysis and interpretation the results.
8 **Gillam D:** Head of the group. He contributed to write and revise the entire manuscript.
9 He was also involved in the elaboration of the experimental project and interpretation of
10 results.