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Setting and incubation environments affect the physicochemical evaluation of two hydraulic calcium silicate-based endodontic materials

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This study evaluated how commonly used setting and incubation conditions influence the properties of hydraulic calcium silicate-based materials, and whether changes observed during prolonged laboratory incubation reflect intrinsic material behaviour or protocol-dependent surface interactions. Setting times of BioRoot RCS and Biodentine were measured using Gillmore needles; crystalline phase changes during setting were assessed by X-ray diffraction (XRD) under dry and humid conditions. Solubility (mass changes), porosity (micro-CT), ion release (inductively coupled plasma optical emission spectrometry), pH, surface morphology (scanning electron microscopy), and chemical composition (XRD, Fourier transform infrared spectroscopy, Raman spectroscopy) changes were measured at 24 h, 7 days, and 28 days. BioRoot RCS was incubated in 0.73 mL (surface area-to-volume ratio of 3 cm^2/mL) or 15.22 mL (surface area-to-volume ratio of 0.145 cm^2/mL) of Hank's balanced salt solution (HBSS). Biodentine was incubated in 0.73 mL HBSS or 0.73 mL human plasma-like medium, both at a surface area-to-volume ratio of 3 cm^2/mL. Results showed that changes in crystalline phases continued beyond the Gillmore setting endpoints. Under dry conditions, both materials reached the Gillmore setting endpoints but no changes in the tricalcium silicate peaks were observed during the first 24 h. BioRoot RCS showed greater mass loss in the larger HBSS volume; Biodentine showed progressive mass gain in HPLM and significant mass gain only at 28 days in HBSS. Carbonation of the material surfaces increased over time, whereas internal porosity remained relatively stable. These findings highlight the strong influence of moisture availability, immersion volume, medium composition, and incubation time on physicochemical evaluation, with prolonged humid incubation promoting surface-localized dissolution and precipitation.

Keywords Hydraulic calcium silicate, Calcium carbonate, Endodontic materials, Setting time, Solubility

Hydraulic calcium silicate-based (HCS) materials are widely used in endodontics as root canal sealers, repair cements, and materials for vital pulp therapy. Available materials vary substantially in composition, physicochemical and biological characteristics, and clinical indications. The composition and properties of HCS products affect the quality and outcome of endodontic treatment[1]. Therefore, laboratory characterization is essential for understanding and comparing different commercial formulations. Bibliometric data reveals that biological (63.7%), mechanical (59.0%), and physical (53.0%) properties are frequently evaluated in studies of endodontic materials[2], underscoring the important role of physicochemical testing in this field. However, because HCS materials interact with water and the surrounding environment, the measured properties may be particularly sensitive to the experimental conditions under which they are assessed.

Physicochemical assessment using ex vivo tooth models is challenging due to multiple confounding factors, including variations in root canal anatomy, dentin composition, moisture within dentinal tubules, and residual tissue or debris. Therefore, simplified in vitro models using standardized material specimens comprise the largest proportion of investigations of HCS materials[3] and are commonly employed to isolate material-related

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properties, improve reproducibility, facilitate product benchmarking, and investigate material behavior under selected environmental conditions. Consequently, methodological variables introduced into these protocols should themselves be considered when interpreting physicochemical results and extrapolating them to clinically relevant material behavior.

ISO 6876:2012 has been widely used as the reference standard for the physicochemical assessment of root canal sealing materials, specifying requirements and test methods for flow, working and setting times, film thickness, radiopacity, and solubility. However, the solubility protocol specified by ISO 6876:2012 has been criticized for the relatively low surface area-to-volume ratio and the use of distilled water, both of which may poorly reflect the amount and composition of biological fluids under clinical conditions[4,5]. Consequently, modified solubility-testing protocols have increasingly been used to investigate HCS materials under alternative immersion conditions. Although the revised ISO 6876:2025 introduced improvements to certain aspects of the solubility-testing protocol, the specimen size and immersion volume remain unchanged. Therefore, the continued use of alternative immersion volumes can be expected. However, differences in specimen surface area-to-volume ratios and medium compositions introduce methodological heterogeneity and complicate direct comparisons of results across studies.

Despite methodological variations, the general framework of commonly used solubility-testing protocols remains similar. Samples of sealing materials are typically molded and allowed to set under humid conditions at 37 °C for 24 h[1]. Although water availability is essential for the hydration of HCS materials, the amount of moisture required to reproduce the clinical conditions for each material is unknown. Varying humidity during setting is therefore commonly used to support hydration while limiting both desiccation and material washout. After the setting period, specimens are immersed in an aqueous medium, such as distilled water, Hank's balanced salt solution (HBSS), simulated body fluid (SBF), Dulbecco's modified Eagle medium (DMEM), or phosphate-buffered saline (PBS)[1,6]. Many published studies have used immersion volumes based on ISO 6876, although lower volumes and alternative specimen surface area-to-volume ratios have increasingly been investigated. In particular, ISO 10993-12:2021, originally developed for the biological evaluation of medical devices, has been proposed as an alternative framework for defining specimen surface area-to-volume ratios[4]. Thus, both the setting humidity conditions and the subsequent immersion volume and composition represent methodological variables that may influence the measured physicochemical properties of HCS materials.

Although the ISO 6876 protocol uses 24 h of soaking in water to determine solubility, extended testing over 4 weeks has become increasingly common, as physicochemical changes in HCS materials continue beyond the initial period[7]. Longer immersion is considered valuable for assessing material stability[8]. However, prolonged aqueous incubation can itself modify the measured properties through dissolution, ion exchange, and secondary precipitation. Atmospheric carbon dioxide dissolved in the incubation medium, and bicarbonate-components in the immersion media such as HBSS, SBF or DMEM, may contribute to the formation of calcium carbonate on the surface of the HCS materials[9,10]. Although carbonation may also occur under physiological conditions, its extent under laboratory immersion may differ from that in contact with tissue fluids[11,12]. Moreover, under clinical conditions, the amount of fluid in contact with the material is limited and continuously exchanged, whereas laboratory protocols commonly employ static immersion in predefined surface area-to-volume ratios[11,12]. Differences in immersion volume may affect ion accumulation and solution saturation in ion measurements or solubility tests. The ionic composition of the medium may alter material–solution interactions and surface transformation[4,6,10]. Therefore, it remains uncertain whether prolonged incubation provides increasingly meaningful information on intrinsic material stability and properties or progressively reflects protocol-dependent material-medium interactions.

The present study evaluated how commonly used setting and incubation protocols influenced the properties of two HCS materials: the root canal sealer BioRoot RCS (Septodont, France) and the restorative/repair cement Biodentine (Septodont, France). The setting times were assessed under dry and humid conditions using the Gillmore needle test; crystalline phases during setting were characterized by X-ray diffraction. Solubility, porosity, ion release, pH, surface morphology, and chemical composition were assessed at time points up to 28 days and at various immersion volumes and media. The null hypotheses were that (1) setting moisture conditions do not influence setting time and phase-related changes, (2) incubation time, immersion volume, and medium composition do not influence the measured physicochemical properties.

Methods

Setting time

The setting times of BioRoot RCS and Biodentine were measured using the Gillmore needle apparatus (Controls, Italy). BioRoot RCS and Biodentine were mixed according to the manufacturers' instructions and placed into non-reactive cylindrical plastic molds with an internal diameter of 10 mm and a depth of 1 ± 0.1 mm specified in ISO 6876:2012. Biodentine pre-dosed capsules were mixed with 5 drops of the supplied liquid, whereas 0.5 g of BioRoot RCS powder was weighed (equivalent to one level manufacturer-provided scoop) and mixed with 5 drops of the supplied liquid. The molded specimens were incubated at 37 °C under either humid or dry conditions ($n = 8$/group). Under dry conditions, specimens were stored individually in clean, empty, 30-mm-diameter Petri dishes and sealed. Under humid conditions, specimens were individually sealed in Petri dishes on top of gauze moistened with 2 mL of HBSS without calcium and magnesium (Gibco™, Thermo Fisher Scientific, USA). Specimens were briefly removed from the respective incubation conditions, and tested with the initial Gillmore needle (100 ± 1 g weight, 2 ± 0.1 mm diameter), vertically applied to the specimen surface for 5 s at 60-second intervals. When no impression was visible, the time was recorded as the initial setting time, after which the specimens were immediately returned to the respective conditions for further setting. The final setting time was determined using the second Gillmore needle (400 ± 1 g weight, 1 ± 0.1 mm diameter) using the same method.

X-ray diffraction (XRD) patterns were recorded to monitor the chemical setting of the samples over 24 h. The newly mixed and molded materials (10 × 1 mm) were stored at 37 °C under either humid or dry conditions, as described above. At 1-hour intervals, three specimens of each material were removed from the incubation conditions, immediately ground to a fine powder using a mortar and pestle without prior drying or desiccation, and analyzed using a Rigaku SmartLab X-ray diffractometer (Rigaku, Japan) with Cu Kα radiation (λ = 1.5418 Å) in the 2θ range of 10–80°, a step size of 0.02°, and a scanning rate of 5.0°/min. An additional set of BioRoot RCS and Biodentine specimens was prepared, sealed in empty Petri dishes without an external moisture source, and incubated at 37 °C. These specimens were also scanned at 1-hour intervals using the same XRD parameters. The XRD patterns of BioRoot RCS and Biodentine powders were recorded as the control.

Solubility

BioRoot RCS and Biodentine powders were mixed as described above, placed into molds (10 mm diameter × 2 mm thickness, $n = 20$) with a slight excess, and incubated in Petri dishes with gauze soaked in 2 mL HBSS (without calcium and magnesium). The dishes were sealed with Parafilm and incubated at 37 °C for 24 h. The specimens were then polished with fine polishing discs (Sof-Lex, 3 M ESPE, Germany), removed from the molds, rinsed with distilled water for 30 s, dried at 80 °C for 1 h, and weighed to an accuracy of 0.0001 g. The BioRoot RCS specimens were immersed in closed tubes containing either 0.73 mL or 15.22 mL of HBSS (without calcium and magnesium). The lower volume corresponded to a specimen surface area-to-medium-volume ratio of 3 cm^2/mL, as described in ISO 10993-12:2021, whereas the larger volume (surface area-to-medium-volume ratio of 0.145 cm^2/mL) was included as an experimental condition to assess the influence of immersion-medium volume. Biodentine, which often comes into direct contact with blood, was immersed in a closed tube containing 0.73 mL HBSS (without calcium and magnesium) or 0.73 mL human plasma-like medium (HPLM; Gibco™, Thermo Fisher Scientific, USA). The immersed specimens were stored at 37 °C for 24 h, then rinsed with distilled water, dried at 80 °C for 1 h, and re-weighed. The solubility test was repeated after 7 and 28 days of immersion, using the same specimens and fresh immersion medium that was changed weekly. The solubility was calculated as the percentage difference between the mass at the selected time point and the initial mass.

Chemical composition

BioRoot RCS and Biodentine were mixed, molded (10 × 2 mm), and incubated for 24 h, 7 days, or 28 days before XRD, Fourier transform infrared spectroscopy (FTIR), and Raman spectroscopy. For the 24-hour measurement, the BioRoot RCS specimens were incubated at 37 °C in sealed Petri dishes containing gauze soaked in HBSS (without calcium and magnesium), whereas the Biodentine specimens were incubated in sealed Petri dishes with gauze soaked in either HBSS (without calcium and magnesium) or HPLM. For the 7-day and 28-day measurements, the mixed and molded BioRoot RCS specimens were set for 24 h on moist gauze in a sealed Petri dish, then removed from the molds, immersed individually in closed microcentrifuge tubes containing 0.73 mL or 15.22 mL of HBSS, and incubated at 37 °C until tested. After setting and removal from the molds, Biodentine specimens were immersed in 0.73 mL of HBSS or 0.73 mL of HPLM and incubated at 37 °C until tested. The XRD and FTIR analyses were performed at each time point after rinsing specimens in distilled water for 30 s, gentle drying with absorbent paper to remove visible surface moisture, and grinding to a fine powder using a mortar and pestle. XRD patterns were recorded as described above, whereas FTIR spectra were recorded using an ALPHA spectrometer (Bruker, Germany) from 4000 to 400 cm^{-1} at a resolution of 2 cm^{-1}. Each spectrum was the average of 32 scans.

Raman spectra were recorded using a WiTec Alpha300R Raman microscope (Oxford Instruments, UK). Five locations on the surface of each specimen were analyzed using 532 nm (1800 lines/mm; 7.2 mW) laser excitation, 2-second acquisition time, and 40 averaged measurements. After Raman examination, the specimens were polished with a diamond bur to remove approximately 0.5 mm of the surface, rinsed in distilled water for 30 s, and re-analyzed by Raman to assess the composition of sub-surface layers. All measurements of XRD, FTIR, and Raman spectroscopy were made in triplicate. BioRoot RCS and Biodentine specimens incubated in sealed dry Petri dishes at 37 °C were used as controls and analyzed as described above without rinsing.

Microstructure

Materials were mixed, molded (10 × 2 mm), and incubated for 24 h, 7 days, and 28 days following the same protocol described for the assessment of the chemical composition. Surface morphology was examined using a Hitachi SU-70 scanning electron microscope (SEM) (Hitachi, Japan). Specimens ($n = 5$/group) were rinsed in distilled water, dried in the incubator at 80 °C for 1 h, sputter-coated with a thin layer of silver, and examined at 2.5k, 5.0k, and 10.0k magnifications for the three time poionts. Dry-condition specimens, without rinsing or any additional preparation, were directly sputter-coated with a thin layer of silver and examined by SEM for comparison.

Porosity analysis was performed using a high-resolution SkyScan 1273 micro-CT scanner (Bruker, Belgium). Specimens (10 × 2 mm, $n = 10$/group) were set at 37 °C for 24 h under humid and dry conditions, as described above. Micro-CT scanning was performed at 110 kV using a 10.0 µm isotropic voxel size, a 0.3 mm copper filter, a 0.3° rotation step, and a 360° rotation angle. Specimens from humid conditions were covered with moist gauze to prevent evaporation during the scan. The same specimens were rescanned after 7 and 28 days of incubation under their respective conditions. Images were reconstructed using NRecon v.1.7.1.0 software (Bruker, Belgium), with a beam-hardening correction of 35% and a ring-artifact reduction factor of 6. CTAn v.1.14.4.1 software (Bruker, Belgium) was used to analyze the reconstructed images and determine the percentage volumes of open and closed pores. The reported porosity values represent pores detectable within the spatial resolution (10 µm) of the applied micro-CT scanning protocol.

Calcium release and pH

BioRoot RCS and Biodentine specimens were mixed, molded (10 × 2 mm) and incubated at 37 °C for 24 h in sealed Petri dishes containing moist gauze, as described above ($n = 10$/material). Set specimens were then polished, removed from molds, rinsed in distilled water, and gently dried from visible moisture with absorbent paper. BioRoot RCS specimens were immersed in closed centrifuge tubes containing 15.22 mL of HBSS (without calcium and magnesium) for 24 h, 7 days, and 28 days. Biodentine specimens were immersed in 15.22 mL of either HBSS (without calcium and magnesium) or HPLM for 24 h, 7 days, and 28 days. At each time point, specimens were rinsed in distilled water, gently dried with absorbent paper, and transferred to new centrifuge tubes containing fresh HBSS or HPLM for further incubation. Separately collected immersion media from the incubation were manually agitated for 5 s and then divided into aliquots for pH and calcium-release measurements. A 7-mL aliquot was used for calcium ion quantitation using a PerkinElmer Optima 7000DV inductively coupled plasma optical emission spectrometer (ICP-OES) (PerkinElmer, USA), while the remaining aliquot was used for pH measurement using a Schott Lab 860 pH meter (SI Analytics, Germany). The pH meter was calibrated before use at pH 4.01, 7.01, and 10.01 using the standard calibration solutions. The electrode was rinsed with deionized water after each measurement to minimize contamination. The pH of fresh HBSS and HPLM was measured to establish baseline control values.

Statistical analysis

The minimum sample size was calculated using G*Power v.3.1 software (Heinrich-Heine-University, Germany), with α error probability of 0.05, 1-β error probability of 80% and effect size of 0.7. The minimum sample size was determined to be 8. Statistical analysis was performed using RStudio v.4.1.1 software (RStudio Inc., USA). Longitudinal quantitative data were analyzed separately for each material using linear mixed-effects models to account for repeated measurements over time, with specimen identity included as a random intercept. Time and the relevant experimental condition, together with their interaction, were included as fixed effects, where applicable. For solubility, the experimental condition was defined as the immersion volume for BioRoot RCS and immersion medium for Biodentine. For porosity, the experimental condition was defined as the incubation condition (HBSS or simulated dry conditions for BioRoot RCS, and HBSS, HPLM, or simulated dry conditions for Biodentine). For calcium release and pH, the incubation medium was included as the experimental condition for Biodentine, whereas BioRoot RCS was analyzed over time only because a single medium was used. For outcomes evaluated under a single experimental condition, time was included as the fixed effect. Pairwise comparisons of estimated marginal means were performed with Tukey adjustment for multiple comparisons. Model residuals were examined to assess normality and homoscedasticity. Statistical significance was set at $\alpha = 0.05$.

Results

Setting time

The setting times under dry conditions were 82 ± 5 min (initial) and 309 ± 8 min (final) for BioRoot RCS, and 16 ± 2 min (initial) and 25 ± 2 min (final) for Biodentine. Under humid conditions, BioRoot RCS had setting times of 162 ± 11 min (initial) and 286 ± 9 min (final), whereas those of Biodentine were 20 ± 2 min (initial) and 35 ± 5 min (final). The XRD analysis revealed that the main tricalcium silicate peaks did not completely disappear until the 24-hour measurement (Fig. 1A). The intensity of the main calcium hydroxide peak at 18.12° increased as the tricalcium silicate peaks decreased, confirming the calcium silicate hydration. The calcium silicate peaks for Biodentine also decreased but did not disappear at the 24-hour measurement (Fig. 1B). This indicates that the chemical setting of BioRoot RCS and Biodentine persisted beyond the time determined using the Gillmore needles. No substantial changes in the zirconium oxide (inert phase) and calcium carbonate peaks were observed throughout this incubation period.

BioRoot RCS set under dry conditions showed no significant reduction in calcium silicate peaks during the first 24 h (Fig. 1C), and calcium hydroxide peaks were absent. The amount of water provided by the mixing liquid alone appeared insufficient to induce substantial calcium silicate hydration detectable by XRD, despite the specimens reaching the Gillmore setting endpoint and exhibiting visible surface hardening. The same trend was observed for Biodentine setting under dry conditions (Fig. 1D).

Solubility

Incubation time and immersion conditions significantly impacted the solubility of both BioRoot RCS and Biodentine (both $p < 0.001$; Table 1). For BioRoot RCS, solubility increased over time under both immersion-volume conditions ($p < 0.001$) and was consistently higher in the larger HBSS volume at all time points ($p < 0.001$). For Biodentine in HBSS, solubility did not differ between 24 h and 7 days ($p = 0.425$), whereas at 28 days specimens gained weight and differed significantly from both earlier time points (both $p < 0.001$). In HPLM, solubility values were negative at all time points and decreased progressively over time ($p < 0.001$), indicating increasing mass gain. Solubility of Biodentine was significantly lower in HPLM than in HBSS at all time points ($p < 0.001$).

Chemical composition

FTIR analysis of BioRoot RCS showed characteristic bands associated with hydroxyl (~3640 cm^{-1}), water (~1650 cm^{-1}), carbonate (1500–1400 cm^{-1} and ~875 cm^{-1}), and silicate-containing phases (1000–970 cm^{-1} and ~810 cm^{-1}) (Fig. 2A). The band at 1650 cm^{-1} attributed to H–O–H bending of adsorbed water may also overlap with C = O stretching of povidone, which is present in BioRoot RCS powder. This band was more pronounced under dry conditions, which may reflect a greater relative contribution of the povidone-associated carbonyl signal in the absence of continuous hydration. Bands assigned to carbonate (CO_3^{2-}) vibrations at 1500–1400 cm^{-1} were detectable in all specimens, including those incubated under dry conditions, suggesting that the

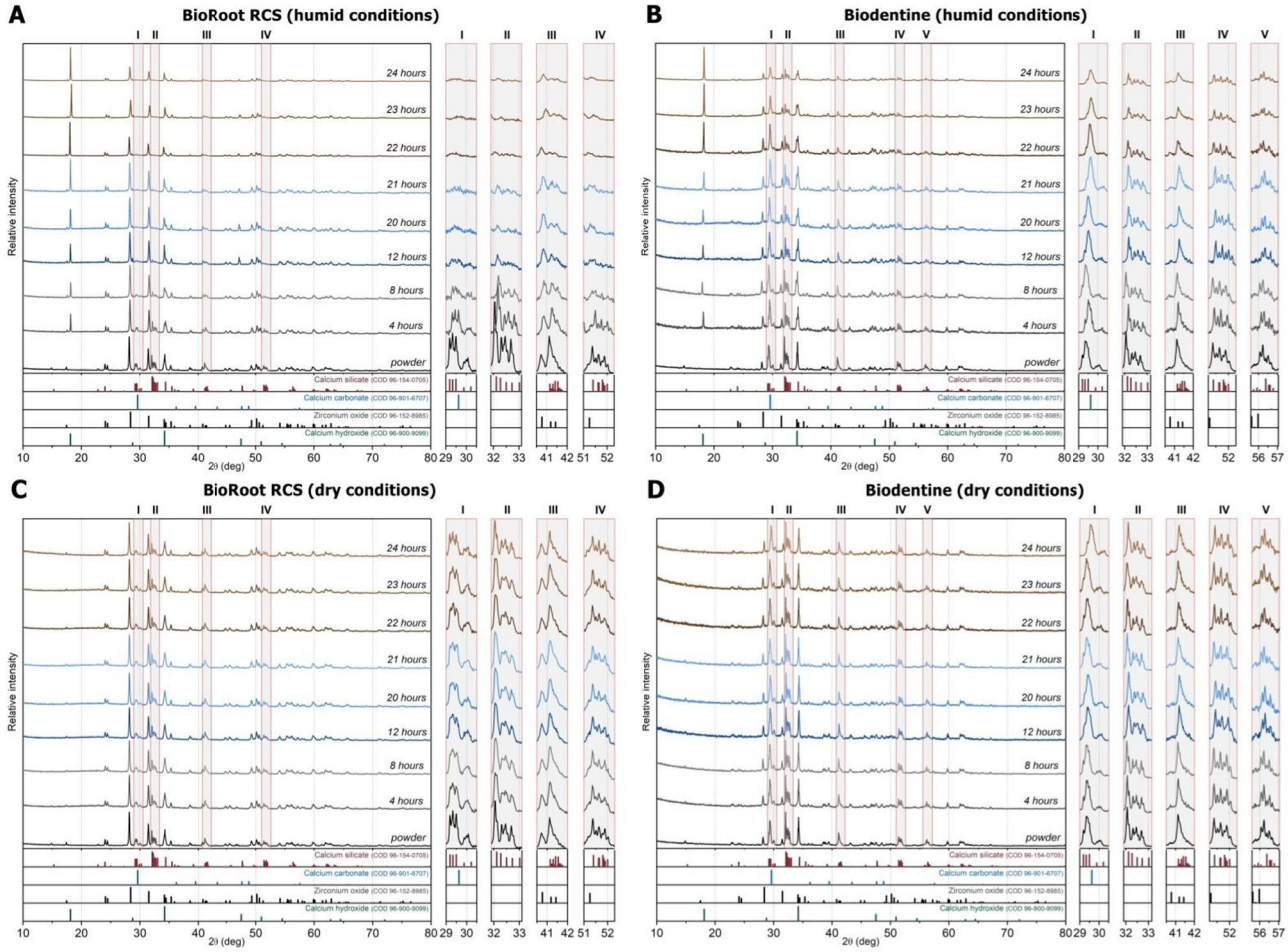

Fig. 1. XRD patterns of BioRoot RCS (**A,C**) and Biodentine (**B,D**) recorded during the first 24 h after material preparation and incubation under humid (**A,B**) and dry (**C,D**) conditions. Regions labeled I–V highlight the main calcium silicate diffraction peaks.

Material	Medium	Volume (mL)	Solubility (%)		
			24 h	7 days	28 days
BioRoot RCS	HBSS	15.22	2.93 ± 0.46^{aA} (2.45–3.53)	6.87 ± 0.77^{bA} (6.20–7.92)	11.71 ± 0.94^{cA} (10.94–13.01)
		0.73	1.43 ± 0.59^{aB} (0.92–2.45)	2.54 ± 0.80^{bB} (1.68–3.85)	3.26 ± 0.55^{cB} (2.75–4.20)
Biodentine	HBSS	0.73	0.62 ± 0.28^{aA} (0.21–0.98)	0.78 ± 0.58^{aA} (0.00–1.38)	-0.25 ± 0.67^{bA} (-1.26–0.60)
	HPLM		-0.78 ± 0.51^{aB} (-1.34 – -0.01)	-1.92 ± 0.69^{bB} (-2.79 – -0.88)	-3.19 ± 0.86^{cB} (-3.80 – -1.66)

Table 1. Mean solubility ± standard deviation, with the min – max range in parentheses, of BioRoot RCS and Biodentine at 24 h, 7 days, and 28 days. Positive solubility values indicate mass loss, whereas negative values indicate net mass gain. Lowercase superscript letters indicate comparisons among time points within the same immersion condition, whereas uppercase superscript letters indicate comparisons among immersion conditions within the same material at the same time point. Values sharing at least one superscript letter are not significantly different; values with no letter in common differ significantly (Tukey-adjusted; $p < 0.05$).

water provided by the mixing liquid initiated some degree of hydration and subsequent carbonation. However, these carbonate bands showed no increase in intensity with incubation time under dry conditions. In contrast, specimens incubated in HBSS showed a progressive increase in carbonate band intensity at ~ 1500–1400 cm^{-1}, particularly in specimens immersed in the larger HBSS volume (15.22 mL), and an increase in the ~ 875 cm^{-1} carbonate band.

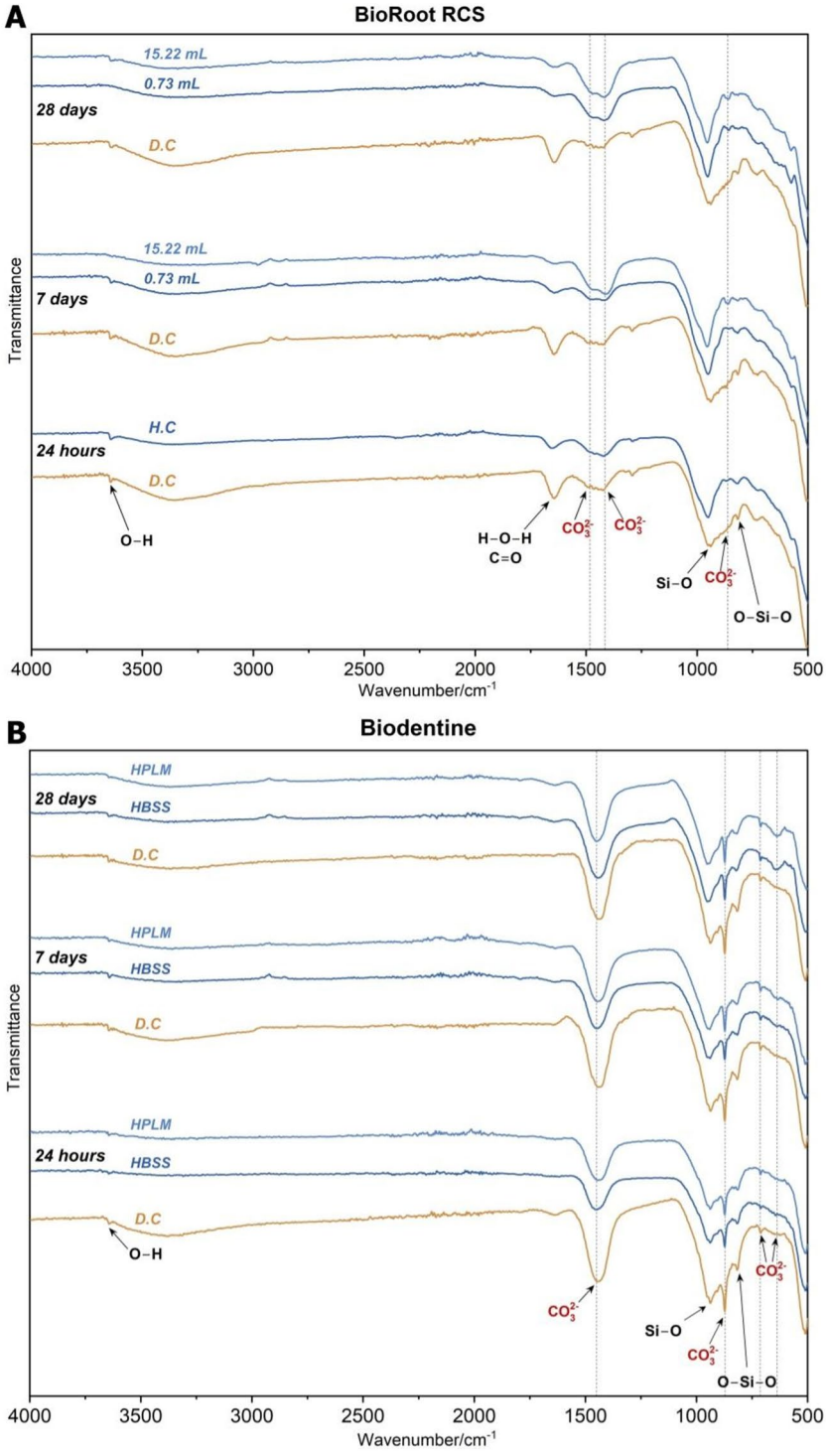

Fig. 2. FTIR spectra of BioRoot RCS (**A**) and Biodentine (**B**) incubated for 24 h, 7 days, and 28 days under dry and immersed experimental conditions. BioRoot RCS was incubated in 0.73 mL or 15.22 mL of HBSS, whereas Biodentine was incubated in 0.73 mL of HBSS or 0.73 mL of HPLM. The labels D.C. and H.C. represent dry and humid conditions, respectively.

FTIR spectra of Biodentine also showed bands assigned to hydroxyl groups (~3640 cm^{-1}), carbonate (~1450 cm^{-1}, ~875 cm^{-1}, ~710 cm^{-1}, and ~640 cm^{-1}), and silicate-containing phases (~940 cm^{-1} and ~810 cm^{-1}) (Fig. 2B). Characteristic carbonate bands were observed in Biodentine, consistent with the presence of calcium carbonate in the powder. Similar to BioRoot RCS, the intensity of the carbonate band at ~1450 cm^{-1} increased progressively over incubation time in specimens immersed in HBSS or HPLM. The carbonate peak at

~640 cm^{-1} also became clearly visible after 28 days of incubation in HBSS or HPLM. In contrast, no apparent changes in the intensity of the carbonate bands were observed in specimens incubated under dry conditions.

Raman spectroscopic analysis revealed progressive calcium carbonate deposition on the surface of both tested materials under humid conditions. No calcium carbonate peaks were detected in BioRoot RCS incubated under dry conditions (Fig. 3A), whereas Biodentine exhibited calcium carbonate peaks of very low intensity (Fig. 3B). Specimens of both materials exposed to HBSS or HPLM showed a progressive increase in the intensity of calcium carbonate peaks over time. At 28 days, these carbonate peaks dominated the surface spectra. After surface polishing, the deeper layers of BioRoot RCS resembled the 24-hour spectra, with no detectable calcium carbonate. The deeper layers of Biodentine were dominated by zirconium oxide, with weaker calcium carbonate peaks.

XRD patterns of BioRoot RCS incubated under dry conditions showed the persistence of the main tricalcium silicate peaks (29.19°, 29.33°, 29.49°, 29.88°, 30.22°, 32.27°, 32.49°, 32.72°, 33.01°, 34.42°, 41.65°, 51.65°, 51.84°, 56.38°, 59.98°, 62.09°, 62.18°, and 62.37°) throughout the 28-day testing period, with no detectable calcium hydroxide peaks at 18.12° and 49.47° (Fig. 4A). Under humid conditions, BioRoot RCS exhibited an intense calcium hydroxide peak at 18.12°, accompanied by the absence of the main tricalcium silicate peaks. No visible changes in the bulk phase composition of BioRoot RCS were observed under humid conditions between 24 h and 28 days.

For Biodentine, the main calcium silicate peaks (29.19°, 29.33°, 29.49°, 29.88°, 30.22°, 32.27°, 32.49°, 32.72°, 33.01°, 34.42°, 41.65°, 51.65°, 51.84°, 56.38°, 59.98°, 62.09°, 62.18°, and 62.37°) and calcium carbonate peaks (29.62°, 39.56°, 43.41°, 47.61°, 48.82°) were visible in specimens incubated for 24 h under both dry and humid conditions (Fig. 4B). However, no calcium hydroxide peak at 18.12° was detected, and no changes in the XRD patterns were observed in Biodentine specimens incubated under dry conditions over 28 days. In contrast, specimens exposed to HBSS or HPLM showed a distinct increase in the intensity of the calcium hydroxide peak (18.12°) at 7 and 28 days and less relative intensity of the other peaks. The calcium silicate peaks (32.27°, 32.49°, 32.72°, 33.01°, 34.42°, and 41.65°) remained detectable at 7 days but were no longer detected at 28 days, indicating that Biodentine setting reaction continued beyond one week, irrespective of the incubation medium. No increase in the calcium carbonate peak intensity was observed for Biodentine over time, and no differences were observed between specimens incubated in HBSS and HPLM.

Microstructure

No significant intra-group changes in micro-CT porosity occurred over time (Table 2), except for a decrease in closed porosity in Biodentine at 7 and 28 days compared with 24 h ($p < 0.019$). Open porosity dominated in all groups, regardless of material, immersion medium, or surface area–to–volume ratio. No statistically significant differences were found between Biodentine incubated in HBSS and HPLM ($p > 0.05$), whereas BioRoot RCS exhibited significantly higher porosity in HBSS at a surface area-to-volume ratio of 0.145 cm^2/mL compared with 3 cm^2/mL ($p < 0.042$).

The incubation conditions also affected the porosity. In BioRoot RCS, open porosity remained stable over time under dry conditions and in 15.22 mL HBSS, but decreased at 7 days in 0.73 mL HBSS. Open porosity was highest in 15.22 mL HBSS and lowest in 0.73 mL HBSS. Closed porosity remained stable over time, with a significant inter-group difference observed only at 7 days ($p < 0.05$).

For Biodentine, open porosity significantly increased over time in HBSS and under dry conditions ($p < 0.05$). Closed porosity significantly decreased in HBSS and HPLM ($p < 0.001$), but remained stable under dry conditions throughout the testing period ($p > 0.05$). Both open and closed porosity were higher under dry conditions than in HBSS or HPLM, although no significant differences in open porosity were observed between incubation conditions.

SEM analysis of specimens under dry conditions showed that both Biodentine and BioRoot RCS retained a granular surface morphology over 28 days, with particles of varying sizes resembling their powders and with no surface precipitates (Fig. 5A, B). The only morphological change observed for BioRoot RCS under longer dry incubation was crack formation (Fig. 5B). After 24 h of incubation under humid conditions, BioRoot RCS and Biodentine also exhibited a granular texture with irregular particle clusters (Fig. 5C, D), similar to that observed in specimens incubated under dry conditions. After 7 and 28 days of incubation in humidity, surface precipitates were observed on all BioRoot RCS and Biodentine specimens, regardless of the immersion medium or volume (Fig. 5E–H). The surface deposits displayed variable morphology, ranging from spherical, granular precipitates to larger, well-defined crystalline structures. Surface coverage by precipitates increased at 28 days.

Calcium release and pH

Both BioRoot RCS and Biodentine released calcium ions over 28 days (Table 3). BioRoot RCS showed a significant time-dependent increase in calcium ion release ($p < 0.001$). For Biodentine, the calcium concentration decreased at 7 days and subsequently increased at 28 days in HBSS, whereas no significant changes were observed in HPLM. Calcium concentrations were significantly lower in HPLM than in HBSS at 24 h and 28 days ($p < 0.001$).

The pH values of the immersion media (HBSS pH = 7.92; HPLM pH = 7.74) increased after the immersion of BioRoot RCS and Biodentine (Table 3). BioRoot RCS maintained a strongly alkaline environment, with a significant increase in pH at 28 days ($p < 0.001$). For Biodentine, the pH significantly increased over time in HBSS ($p < 0.001$), whereas in HPLM it decreased at 7 days and partially recovered by 28 days ($p < 0.05$). At all time points, the pH was significantly lower in HPLM than in HBSS ($p < 0.001$).

Discussion

The presented data provide important insights into the testing methodologies for HCS materials and highlight the limitations of interpreting and extrapolating laboratory results to clinical performance. Moisture availability

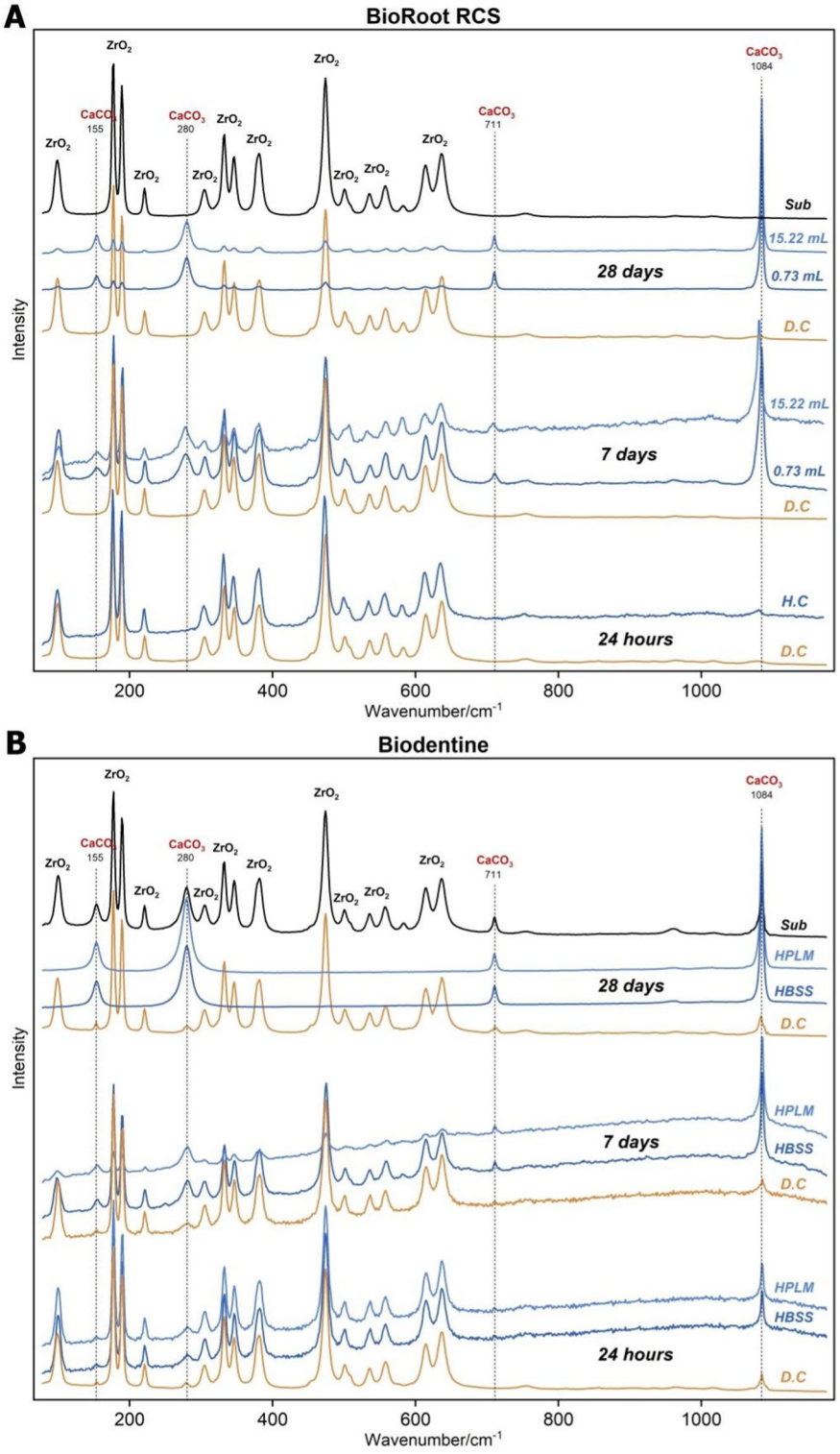

Fig. 3. Raman spectra of BioRoot RCS (**A**) and Biodentine (**B**) incubated for 24 h, 7 days, and 28 days under dry annd immersed experimental conditions. BioRoot RCS was incubated in 0.73 mL or 15.22 mL of HBSS, whereas Biodentine was incubated in 0.73 mL of HBSS or 0.73 mL of HPLM. The labels D.C. and H.C. represent dry and humid conditions, respectively. The 28-day Raman spectra labeled "Sub" represent the main peaks obtained from deeper layers of the specimens following surface polishing.

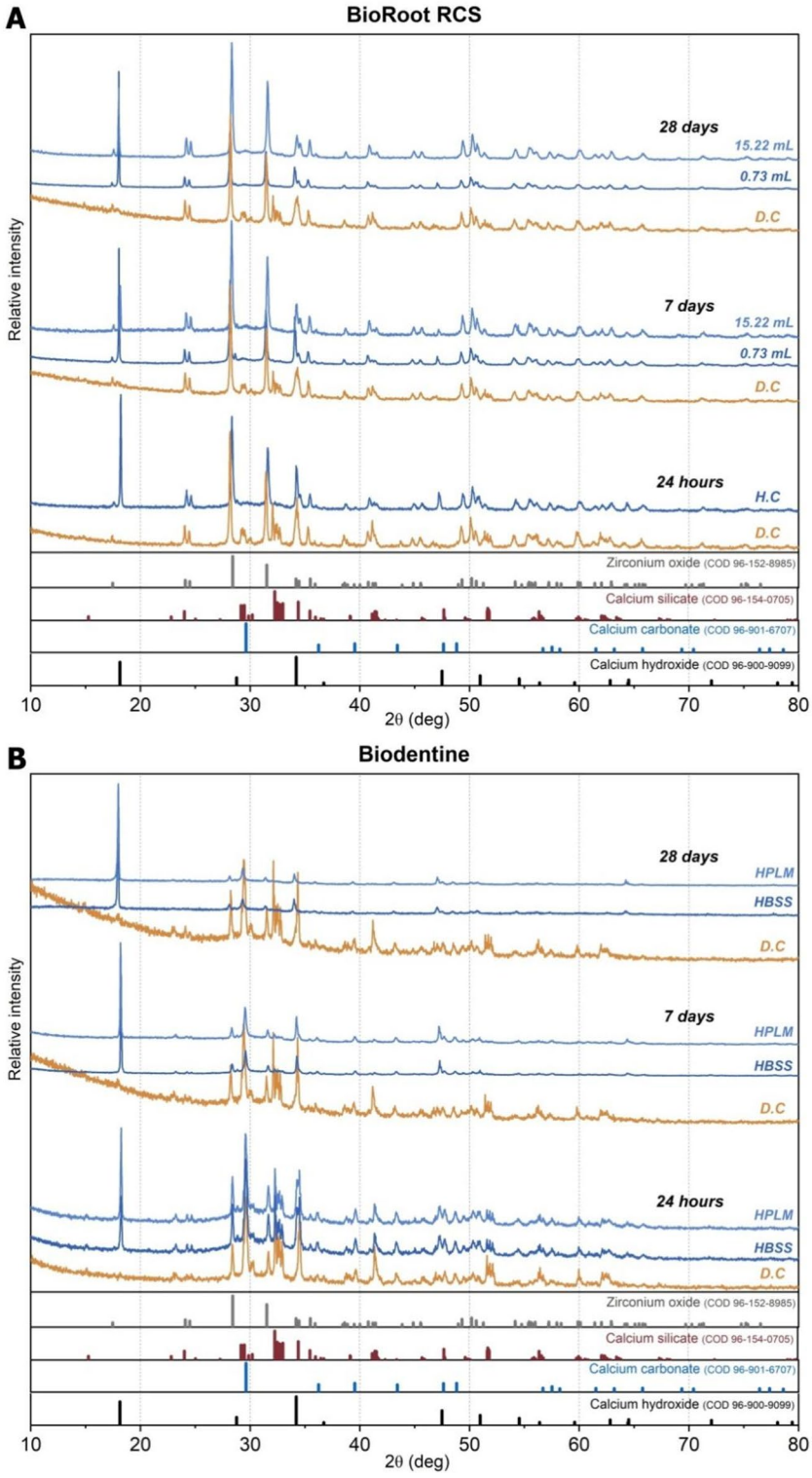

Fig. 4. XRD patterns of BioRoot RCS (**A**) and Biodentine (**B**) incubated for 24 h, 7 days, and 28 days under different experimental conditions. BioRoot RCS was incubated in 0.73 mL or 15.22 mL of HBSS, whereas Biodentine was incubated in 0.73 mL of HBSS or 0.73 mL of HPLM. The labels D.C. and H.C. represent dry and humid conditions, respectively. Reference spectra for zirconium oxide, tricalcium silicate, calcium carbonate, and calcium hydroxide are included for phase identification, with the corresponding COD identification numbers provided.

Material	Medium	Volume	Porosity (%)					
			24 h		7 days		28 days	
			Open	Closed	Open	Closed	Open	Closed
BioRoot RCS	HBSS	15.22 mL	14.02 ± 1.44^{aB} (9.97–15.74)	1.92 ± 0.15^{aA} (1.56–2.15)	14.91 ± 1.53^{aC} (10.23–16.34)	2.09 ± 0.18^{aB} (1.65–2.35)	13.98 ± 1.58^{aB} (9.54–15.84)	2.02 ± 0.23^{aA} (1.62–2.29)
		0.73 mL	11.19 ± 1.49^{aA} (9.50–13.56)	1.66 ± 0.28^{aA} (1.36–1.98)	9.60 ± 0.69^{bA} (8.90–10.83)	1.66 ± 0.21^{aA} (1.34–1.89)	10.16 ± 1.33^{abA} (9.03–12.71)	1.70 ± 0.20^{aA} (1.40–1.88)
	Dry		11.42 ± 0.57^{aA} (10.81–12.21)	1.68 ± 0.16^{aA} (1.43–1.89)	11.80 ± 0.56^{aB} (11.30–12.86)	1.70 ± 0.19^{aA} (1.46–1.97)	12.26 ± 0.57^{aB} (11.73–13.04)	1.74 ± 0.23^{aA} (1.45–2.02)
Biodentine	HBSS	0.73 mL	4.11 ± 0.74^{aA} (3.51–5.38)	0.20 ± 0.06^{aA} (0.14–0.29)	4.32 ± 1.04^{abA} (2.93–5.58)	0.10 ± 0.05^{bA} (0.06–0.17)	5.12 ± 0.65^{bA} (4.45–6.05)	0.10 ± 0.06^{bAB} (0.06–0.19)
	HPLM		3.91 ± 1.04^{aA} (2.73–5.48)	0.26 ± 0.05^{aA} (0.19–0.31)	4.34 ± 0.21^{aA} (4.08–4.66)	0.13 ± 0.04^{bAB} (0.07–0.19)	4.56 ± 0.55^{aA} (4.04–5.26)	0.08 ± 0.06^{bA} (0.01–0.17)
	Dry		4.40 ± 0.25^{aA} (4.04–4.73)	0.20 ± 0.06^{aA} (0.16–0.29)	4.72 ± 0.52^{abA} (4.11–5.42)	0.18 ± 0.04^{aB} (0.14–0.24)	5.34 ± 0.73^{bA} (4.59–6.52)	0.16 ± 0.04^{aB} (0.12–0.21)

Table 2. Mean percentage of open and closed pores ± standard deviation, with the min – max range in parentheses, of BioRoot RCS and Biodentine specimens at 24 h, 7 days, and 28 days. Lowercase superscript letters indicate comparisons among time points within the same incubation condition, whereas uppercase superscript letters indicate comparisons among incubation conditions within the same material at the same time point. Values sharing at least one superscript letter are not significantly different; values with no superscript letter in common differ significantly (Tukey-adjusted; $p < 0.05$).

influenced the setting-related processes, whereas incubation time and experimental conditions, including immersion volume and medium composition, affected the measured physicochemical properties. Accordingly, both null hypotheses were rejected.

First, the present study distinguished between surface hardening and chemical setting. The setting time determined by Gillmore needles, as specified in ISO 6876, detects the loss of specimen surface plasticity and the development of mechanical resistance rather than the completion of hydration reactions (chemical setting) in the bulk of the material. This distinction is sometimes overlooked, and the Gillmore setting endpoint is misinterpreted as indicating the full chemical setting of the material. Under humid conditions, the initial and final setting times of BioRoot RCS (162 and 286 min), determined by Gillmore needle, were within or close to the setting-time range reported by the manufacturer (45 min to 4 h). However, XRD analysis showed that BioRoot RCS reached chemical setting after approximately 24 h under humid conditions, as indicated by the near-complete disappearance of calcium silicate peaks and the concomitant increase in calcium hydroxide peaks. This finding confirms that the Gillmore needle reflects surface hardening rather than bulk hydration of the material.

Under humid conditions, Biodentine exhibited a substantially shorter final Gillmore setting time than BioRoot RCS (35 versus 286 min). However, its chemical setting continued for a substantially longer period than that of BioRoot RCS, with calcium silicate peaks remaining detectable at 7 days. Although the Gillmore needle results reinforce previous reports that putty formulation of HCS materials set faster than the paste/sealer formulation[13], the XRD findings challenge the assumption that rapid surface hardening reflects chemical setting. One possible explanation for this apparent contradiction is that faster surface hardening may restrict water ingress into the material from the surrounding environment[14], which is required to support further hydration of the remaining calcium silicate. This interpretation is supported by the present study, which showed that the water provided by the mixing liquid alone was insufficient to achieve complete hydration. As a result, surface densification may act as a physical barrier, slowing the progression of chemical reactions within the material core[15,16].

The differences in chemical setting between BioRoot RCS and Biodentine may be attributed to their chemical compositions. Although the manufacturer does not disclose the exact formulation of the materials, previous studies stated that Biodentine contains approximately 78–80 wt% calcium silicate[17,18], whereas the calcium silicate content of BioRoot RCS does not exceed 35%[19]. In addition to calcium silicate, Biodentine powder contains calcium carbonate and zirconium oxide, while its liquid includes calcium chloride and polycarboxylate. The incorporated calcium chloride may act as a hydration accelerator, while calcium carbonate and the relatively small particle size may further promote early hydration[17]. BioRoot RCS contains tricalcium silicate, zirconium oxide, and povidone in the powder, whereas its liquid consists of an aqueous solution of calcium chloride and polycarboxylate[5]. These compositional differences between BioRoot RCS and Biodentine are consistent with the XRD patterns obtained in the present study, which showed lower zirconium oxide and higher tricalcium silicate peak intensities for Biodentine. Higher calcium silicate content increases water demand and may prolong hydration, particularly when early surface hardening restricts water transport. Therefore, the tricalcium silicate peaks in Biodentine remained detectable even at 7 days in the present study. This finding is consistent with a previous study, which reported that Biodentine reached approximately 87% hydration at 6 h, 88.8% at 24 h, and 93.7% after 7 days[18].

Another critical observation was the necessity of moisture from the surrounding environment for chemical setting, as both HCS materials achieved surface hardening but showed no evidence of chemical setting during the first 24 h under dry conditions. Specimens incubated in the absence of humidity showed no reduction in

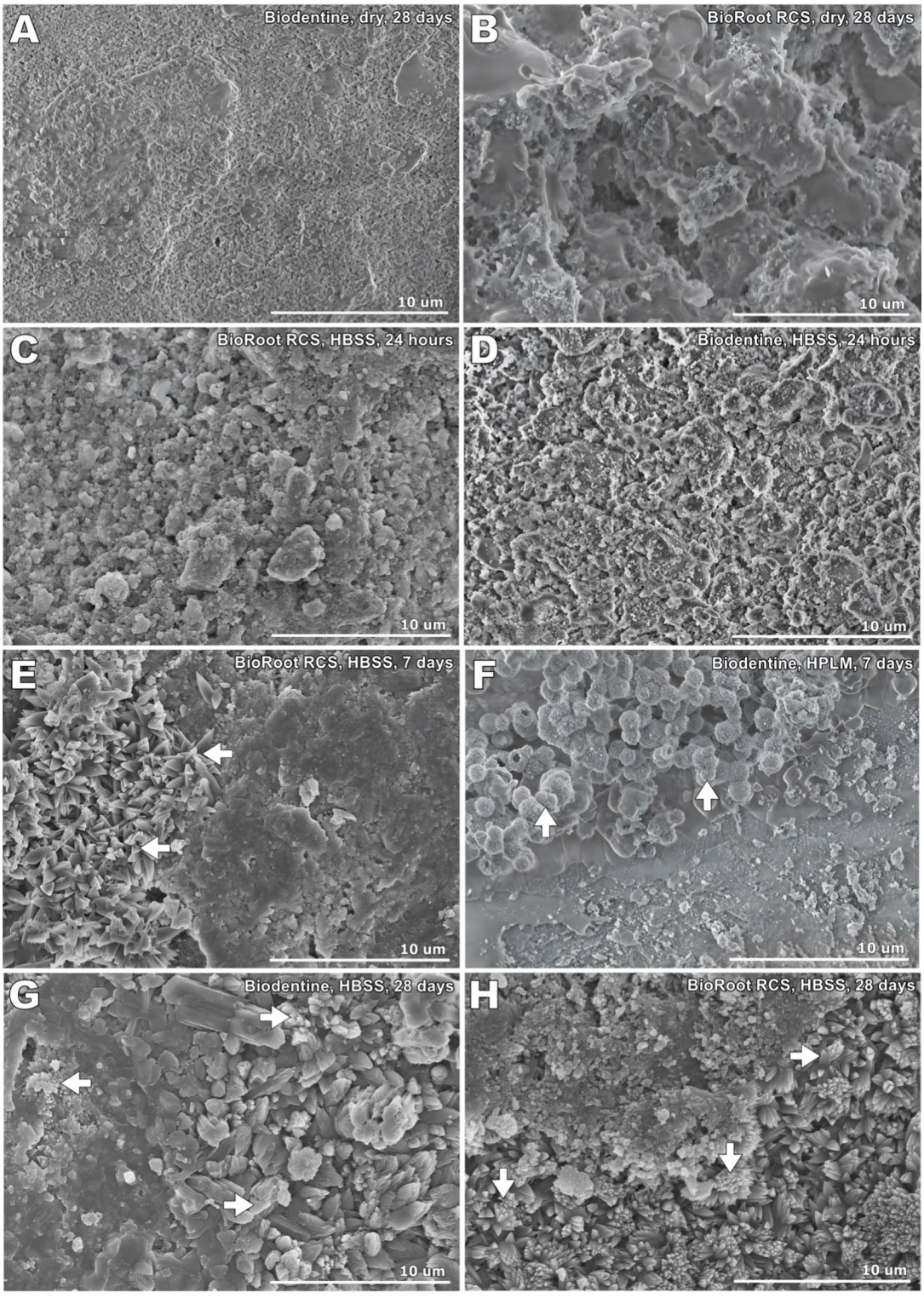

Fig. 5. Representative SEM images of (**A**) Biodentine and (**B**) BioRoot RCS after 28 days of incubation under dry conditions. (**C**) BioRoot RCS and (**D**) Biodentine after 24 h of incubation on gauze soaked in HBSS; (**E**) BioRoot RCS after incubation in 0.73 mL HBSS for 7 days; (**F**) Biodentine after incubation in 0.73 mL HPLM for 7 days; (**G**) Biodentine after incubation in 0.73 mL HBSS for 28 days; and (**H**) BioRoot RCS after incubation in 15.22 mL HBSS for 28 days. White arrows indicate precipitates of varying shapes and sizes.

Material	Medium	Calcium (mg/L)			pH		
		24 h	7 days	28 days	24 h	7 days	28 days
BioRoot RCS	HBSS	135.48 ± 46.67^{a} (99.4–188.2)	356.87 ± 52.18^{b} (320.8–416.7)	664.23 ± 119.87^{c} (531.1–763.6)	11.87 ± 0.05^{a} (11.82–11.93)	11.90 ± 0.16^{a} (11.68–12.05)	12.18 ± 0.07^{b} (12.08–12.28)
Biodentine	HBSS	278.33 ± 28.43^{aA} (255.9–310.3)	175.00 ± 19.80^{bA} (161.3–197.7)	353.07 ± 108.49^{cA} (238.0–453.5)	10.89 ± 0.20^{aA} (10.71–11.10)	11.84 ± 0.08^{bA} (11.78–11.93)	12.02 ± 0.22^{cA} (11.79–12.22)
	HPLM	74.11 ± 2.70^{aB} (71.1–76.2)	120.77 ± 5.71^{aA} (116.7–127.3)	95.17 ± 2.85^{aB} (92.0–97.5)	8.78 ± 0.21^{aB} (8.56–8.97)	7.58 ± 0.04^{bB} (7.55–7.62)	7.89 ± 0.03^{cB} (7.86–7.91)

Table 3. Mean pH and concentration of released calcium ions ± standard deviation, with the min – max range in parentheses, of BioRoot RCS and Biodentine. Lowercase superscript letters indicate comparisons among time points within the same medium and outcome; uppercase superscript letters compare HBSS and HPLM within Biodentine at the same time point and outcome. Values sharing at least one letter are not significantly different; values with no letter in common differ significantly (Tukey-adjusted; $p < 0.05$).

calcium silicate peaks and no formation of calcium hydroxide during the first 24 h. These findings demonstrate that the water provided by the mixing liquid alone is insufficient to sustain complete hydration of calcium silicate, thereby challenging the assumption that fourth-generation HCS materials, such as BioRoot RCS and Biodentine, are less dependent on moisture from dentinal tubules than ready-to-use formulations classified as fifth-generation materials[19,20]. This assumption is based on the fact that fourth-generation HCS materials contain a water-based liquid that is distributed throughout the powder during mixing and can initiate hydration immediately throughout the bulk of the material. In contrast, fifth-generation HCS materials contain a non-aqueous vehicle, typically water-soluble propylene glycol, which has to be replaced by tissue and dentinal fluids in the clinical environment to achieve chemical setting[19]. However, the results obtained under dry conditions indicate that the fourth-generation HCS materials evaluated in the present study remain dependent on moisture from the surrounding environment to set.

Although a completely dry root canal system is not expected in a clinical scenario[21], there is also no evidence-based information defining the minimum amount of moisture required for HCS materials to achieve complete chemical setting, nor it is known how much fluid can be absorbed from the surrounding pulpal or periodontal tissues. Therefore, the extent to which chemical setting is achieved after the placement of HCS materials into the root canal system remains uncertain. However, the level of chemical setting achieved is a highly important factor influencing the overall performance of HCS materials[20]. As demonstrated in the present study, insufficient moisture availability reduces calcium hydroxide formation, which is responsible for the alkaline pH and the antimicrobial effectiveness of HCS materials[22]. In addition, incomplete hydration results in unreacted particles, leading to inferior mechanical properties[20,23].

The longer time required for complete chemical setting could justify prolonged incubation of HCS specimens to evaluate the performance of fully set materials. However, prolonged incubation in HBSS or HPLM was accompanied by progressive calcium carbonate formation in the present study. Carbonation is a well-known process in cementitious materials, involving the reaction of dissolved CO_2 with alkaline phases, such as portlandite or CSH, to form calcium carbonate[24]. Importantly, carbonate formation should not be regarded as an inevitable consequence of prolonged incubation itself, as previous studies evaluating calcium silicate-based cements for up to 28 days in non-bicarbonate-containing media did not demonstrate carbonation[25]. Therefore, the selection of the incubation medium should be carefully considered. Although carbonation is generally considered a slow process, laboratory testing conditions commonly used for HCS materials in bicarbonate-containing media may accelerate it[24]. First, specimens are typically incubated at 37 °C, which corresponds to human body temperature. Previous study has shown that increasing the temperature to 40 °C results in a marked increase in both the initial reaction rate and the total CO_2 uptake capacity[26]. Second, the high pH generated during hydration enhances CO_2 dissolution in the aqueous medium[27], promoting carbonate formation. Accordingly, the FTIR, Raman spectroscopy, and SEM analyses in the present study revealed progressive deposition of calcium carbonate following incubation in HBSS and HPLM.

The absence of changes in calcium carbonate peaks in the XRD patterns over time further supports the surface-localized carbonation. Raman spectroscopic analysis revealed that after 28 days the superficial layers of the specimens were dominated by calcium carbonate peaks, whereas deeper layers exhibited no detectable calcium carbonate for BioRoot RCS and markedly lower carbonate peak intensities for Biodentine compared with the surface. The surface-localized calcium carbonate phases may therefore be difficult to detect by XRD because grinding the specimens can dilute the surface deposits within the bulk powder. In addition, XRD primarily detects well-crystallized bulk phases[28], and newly formed surface deposits may still contain poorly crystalline or partially amorphous calcium carbonate[23], which contributes minimally to XRD signals as non-stoichiometric CSH. Despite these XRD limitations, the collective findings indicate that, under the bicarbonate-containing incubation media used in the present study, carbonation occurred predominantly as a surface-dominated process. The persistence of intense calcium hydroxide peaks in XRD patterns, even after the disappearance of calcium silicate peaks, further suggests that carbonation consumes calcium hydroxide primarily at the surface, while the bulk material remains rich in crystalline portlandite.

The effect of immersion volume on BioRoot RCS further demonstrates the sensitivity of solubility measurements to the experimental protocol. Mass loss was consistently greater in 15.22 mL than in 0.73 mL HBSS, and the difference increased with incubation time. This finding is consistent with previous studies

showing that solubility values of HCS materials are strongly influenced by the testing methodology[6]. Ali et al.[4] reported significantly lower solubility of Biodentine when lower immersion volumes were used in modified ISO 6876 protocols. The larger immersion volume specified in ISO 6876 minimizes saturation or supersaturation of the solution, allowing greater dissolution and providing a standardized screening test for comparison among materials. In contrast, a smaller solution volume reaches saturation more rapidly and may limit further dissolution. Therefore, although the ISO 6876 protocol is useful for standardized comparison of material solubility, its results should not be interpreted as a direct simulation of clinical fluid availability or as a direct measure of intrinsic long-term material stability. As discussed above, the actual amount of moisture or fluid available clinically is not well defined, but is generally expected to be lower than the volume used in standardized ISO 6876 solubility testing. Consequently, the solubility measured under the ISO 6876 conditions may overestimate the extent of dissolution that would occur under clinical conditions, particularly where the available fluid volume is limited. Thus, a relatively high solubility value obtained under the standardized ISO conditions should not necessarily be interpreted as indicating that the same extent of material dissolution would occur clinically[4,6].

The need to avoid interpreting mass-loss-based solubility measurements as direct indicators of material stability was further highlighted by the calcium release and micro-CT findings of the present study. The continued mass changes observed for BioRoot RCS and Biodentine under the respective immersion conditions, together with sustained calcium release and the lack of a direct correspondence between mass changes and porosity changes over 28 days, suggest that the observed mass changes were predominantly associated with surface or near-surface processes rather than substantial alterations in the micro-CT-detectable pore structure of the bulk material. Differences between humid and dry conditions further indicate that material porosity was influenced by the incubation environment, with the pattern of changes varying according to material, pore type, condition, and time point. However, this interpretation should be considered within the limitations of micro-CT analysis. Porosity measurements are influenced by voxel size, image-processing parameters, and material radiopacity; thus, pores approaching or falling below the spatial resolution of the scan may not be reliably segmented because of partial-volume effects[29]. Consequently, the 10-µm voxel size used in the present study primarily reflects micro-CT-detectable porosity and does not exclude finer sub-resolution structural features.

The ongoing calcium release observed in laboratory studies is generally attributed to continued hydration of calcium silicates and is expected to decrease at the end of chemical setting[30]. However, calcium release may also continue after chemical setting through carbonation-related processes. Carbonation of calcium hydroxide may promote further dissolution of calcium-containing phases and release of calcium ions into the surrounding solution. In addition, the consumption of calcium ions from the pore solution during carbonation may trigger decalcification of CSH in the superficial layers[24]. This process reduces the Ca/Si ratio of CSH and can ultimately lead to its transformation into calcium carbonate and a highly polymerized silica gel, which retain morphology similar to that of the initial hydrate[24]. These carbonation-related processes may therefore sustain calcium release and apparent solubility even after calcium silicate phases are no longer detectable by XRD.

In contrast, Biodentine exhibited mass gain after prolonged incubation in HBSS and throughout incubation in HPLM. The persistence of calcium silicate peaks in Biodentine up to 7 days supports the notion that its chemical setting proceeds more slowly, allowing carbonation to occur concurrently with hydration. Interestingly, unreacted calcium silicate may also participate in carbonation processes, in which calcite and silica gel are ultimately formed instead of portlandite and CSH[24], resulting in increased calcium carbonate deposition. The presence of bicarbonate ions in HPLM may further promote carbonate precipitation[9] and, therefore, may also contribute to the continuous mass gain observed in this medium.

Although the present study did not quantify the exact amount of calcium carbonate formed under each experimental condition, the findings demonstrate progressive surface carbonation during incubation in bicarbonate-containing media. Surface carbonation should not itself be regarded as a non-physiological phenomenon, because bicarbonate and dissolved CO_2 are naturally present in biological fluids. Moinzadeh et al. demonstrated calcium carbonate deposition on the surface of a calcium silicate-based material retrieved after failed root-end surgery and suggested that the presence of CO_2 in vivo may favor calcium carbonate formation rather than the hydroxyapatite precipitation, which is commonly reported under in vitro conditions[31]. Thus, bicarbonate-containing media may reproduce an important aspect of the physiological environment, and carbonation itself should not necessarily be considered a laboratory artifact. However, the extent and kinetics of carbonation during static long-term laboratory incubation may differ from those occurring clinically, where fluid volume and composition, protein content, diffusion, and continuous fluid exchange are more complex.

These results raise important questions regarding the relevance of extended in vitro incubation protocols. While the long-term performance of HCS materials is important for clinical applications, widely used laboratory models inadequately represent root canal or vital pulp environments, compromising the clinical relevance of the obtained data. Simplified protocols may also affect how preclinical data are translated into clinical expectations. Materials appearing stable after prolonged immersion may have undergone substantial surface transformation, whereas higher apparent solubility may still be compatible with preserved bulk integrity if changes remain superficial. Likewise, fast needle-based setting does not indicate complete maturation at the time of clinical application, particularly in procedures such as pulp capping or pulpotomy. Therefore, future research should focus on developing testing models that simulate clinical environments, rather than relying solely on extended evaluation of HCS materials under simplified laboratory conditions. Approaches incorporating controlled moisture delivery, clinically relevant fluid volumes, fluid exchange, and dentin-containing models may be more informative for translational testing. Without such models, the prolonged in vitro testing is more likely to reflect laboratory-induced dissolution and precipitation processes instead of the long-term behavior of HCS materials expected in vivo.

Data availability

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

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Author contributions

G.B.: Conceptualization, Methodology, Investigation, Formal analysis, Visualization, Data curation, Project administration, Writing – original draft. S.D.: Conceptualization, Methodology, Visualization, Writing – review & editing. S.S.: Resources, Validation, Writing – review & editing.

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Declarations

Competing interests
The authors declare no competing interests.

Additional information
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