EFFECT OF ADDITIONS OF CHEMICALS ON PROPERTIES OF ENDODONTIC CEMENTS

by

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Abstract

Tricalcium silicate (TCS)-based materials are vastly applied in vital pulp therapy, mainly due to the calcium hydroxide release which is responsible for their biological and antimicrobial activity. Recently, alterations have been introduced in their formulation, namely incorporation of calcium phosphate, micro-silica or resins to improve potential limitations. The current study investigated whether different additives affect calcium ion release and in turn modify biological and antimicrobial properties by testing prototype and commercial TCS-based cements.

The water: powder ratio in the prototype formulations was determined with a rheological assessment. Hydration was monitored with scanning electron microscopy and x-ray diffraction analysis. Materials' eluates were evaluated for pH, calcium release, biocompatibility and antimicrobial potential.

Modifications altered the water demand, hydration and leaching profile of the prototype cements in different extent. Calcium phosphate did not alter calcium ion release significantly, albeit an initial stronger antibacterial effect was induced. Micro-silica replacement resulted in a decreased long-term calcium hydroxide formation, which was correlated with neutralised cytotoxicity and antibacterial activity. Hydration of commercial TCS-based materials was faster and calcium release was enhanced, except for a resin-modified cement.

Overall, incorporation of compounds in hydraulic cements can alter calcium ion release and consequently modify their biological and antimicrobial properties.

Dedicated to my nephew, Klearhos

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List of Abbreviations

%	Per cent
°C	Degrees Celsius
Activa	ACTIVA BioACTIVE Base/Liner
ADT	Agar Diffusion Test
ALP	Alkaline phosphatase
AmpB	Amphotericin B
ANOVA	Analysis of variance
ASTM	American Society for Testing and Materials
Bis-GMA	bisphenol A-glycidyl methacrylate
CH	Calcium hydroxide
cm	Centimetre
cm^2	Square centimetre
CP	Calcium phosphate
CW	Calcium tungstate
CSH	Calcium silicate hydrate
DC	Degree of conversion
DCL	Dental curing light

DCS	Dicalcium silicate
DMEM	Dulbecco's Modified Eagle Medium
DMEMsup	DMEM supplemented with 1% penicillin/streptomycin and 2 mM glutamine
DMEMsup-AmphB	DMEM supplemented with 1% penicillin/streptomycin, 2 mM glutamine and 2.5 mg/l Amphotericin B
DMEMsup-10FBS	DMEM supplemented with 1% penicillin/streptomycin, 2 mM glutamine and replaced by 10% heat inactivated foetal bovine serum
DMSO	Dimethyl sulfoxide
DW	Distilled water
EDS	Energy-dispersive X-ray spectroscopy
EDTA	Ethylenediaminetetraacetic acid
FBS	Heat inactivated foetal bovine serum
FT-IR	Fourier transform infrared
G	Gram
GIC	Glass ionomer cement
g/l	Grams per litre
h	Hour

HBSS	Hank’s balanced salt solution
HDPCs	Human dental pulp cells
ICP-OES	Inductively coupled plasma-optical emission spectrometry
ICDD	International Centre for Diffraction Data
ISO	International Organization for Standardization
kV	Kilovolt
l	Litre
mA	Milliampere
mg	Milligram
mg/l	Milligrams per litre
mg/ml	Milligrams per millilitre
min	Minutes
ml	Millilitre
mM	Millimolar
mm	Millimetre
mm^2	Square millimetre
MRS	Man, Rogosa & Sharpe
mS	Micro-silica

mS10	10% replacement of micro-silica
mS20	20% replacement of micro-silica
MTA	Mineral trioxide aggregate
MTT	3-(4,5 dimethylthiazolyl-2-yl)-2,5-diphenyltetrazolium bromide
NCTC	Natural Collection of Type Cultures
N	Newton
OD	Optical density
PBS	Phosphate Buffered Saline
PC	Portland cement
PI	Propidium iodide
rpm	Revolutions per minute
RRM	Root repair material
s	Second
SDS	Safety data sheet
SDW	Sterile distilled water
SEM	Scanning electron microscopy
TO	Tantalum oxide
TCS	Tricalcium silicate

TCS-CP/ZO	Tricalcium silicate with 15% replacement of calcium phosphate in its cementitious phase and 30% replacement of zirconium oxide
TCS-mS10/ZO	Tricalcium silicate with 10% replacement of micro-silica in its cementitious phase and 30% replacement of zirconium oxide
TCS-mS20/ZO	Tricalcium silicate with 20% replacement of micro-silica in its cementitious phase and 30% replacement of zirconium oxide
TCS/ZO	Tricalcium silicate with 30% replacement of zirconium oxide
TEGDMA	Triethylene glycol dimethacrylate
TGF-β1	Transforming Growth Factor β1
Theracal	TheraCal LC
TotalFill RRM	TotalFill Bioceramic Root Repair Material
TSA	Tryptic Soya Agar
TSB	Tryptonic Soya Broth
UV	Ultraviolet
ZO	Zirconium oxide
VPT	Vital pulp therapy
XRD	X-ray diffraction
w/p	Water: powder

wt%	Per cent by weight
µm	Micrometre
µg	Microgram
µg/g	Micrograms per gram

CHAPTER 1

INTRODUCTION

Parts of Sections 1.4.5, 1.7.1 and 1.8 are included in a manuscript published in Dental Materials (Koutroulis A, Batchelor H, Kuehne S, Cooper PR, Camilleri J).

The author has carried out the writing of the article.

1.1 Background to the study

During the past century, several advances have been made in the field of endodontic treatment. Further understanding of the pulp biology and evolution in the field of dental materials has created new perspectives for innovative less invasive treatment strategies [1].

Inflammation of the pulp of a tooth can be the result of exposure to traumatic, carious or iatrogenic injury [2]. Until recently, the dominant treatment option for the exposed dental pulp in such clinical cases was the complete removal of pulp tissue [2]. However, in several occasions, pulp vitality could be maintained should the correct treatment is provided and the infectious stimulus is adequately restrained [3].

Following thorough classification of the severity of the pulp inflammation and direct correlation to specific diagnostic signs and symptoms, new endodontic techniques have been introduced addressing the challenge of maintaining the vitality of the pulp tissue [3]. At the same time, the clinical success of these less destructive endodontic procedures is directly correlated with the development of biomaterials that can enable a healing response combined with the materials' sealing ability of the dental tissues [4]. Vital pulp therapy (VPT) is a term that encompasses all the procedures that include application of a biomaterial in close proximity or in contact with a reversibly affected vital pulp tissue to enable its viability and prevent further infection [1].

Introduction of hydraulic tricalcium silicate (TCS)-based cements in dentistry, initially with mineral trioxide aggregate (MTA), played a significant role in the increase

of clinical success of these procedures [1]. Today, these materials are regarded as the materials of choice for VPT due to their significant biological activity [3, 4]. TCS-based cements hydrate in contact with water and form calcium hydroxide as a by-product of the reaction [5]. Diffusion of calcium and hydroxyl ions in the substrate tissues has been reported to promote pulp tissue healing and regenerative procedures [6]. Elevation of pH assists in microbial elimination [7]. To date, despite the well-established beneficial role of calcium hydroxide in VPT, there is lack of knowledge on the association between the desirable dose-response effect of the ionic release from these pulp capping agents.

Furthermore, development of a new generation of materials based in hydraulic TCS cement that will present optimised physico-mechanical properties is a field of significant research focus. Studies are performed by inclusion of different fillers or additives in the conventional cement formulation. However, a significant challenge is imposed on how modifications applied in the chemical composition of these cements affect the hydration reaction and consequently the beneficial formation of calcium hydroxide. Thus, modified materials need to be thoroughly characterised to verify that efforts to overcome limitations of the traditional formulation of TCS-based cements do not diminish the calcium hydroxide formation which is strongly correlated with the biological and antimicrobial properties [6]. This study used a series of characterisation techniques to determine the impact of several alterations in the leaching profile of a new generation of hydraulic cements and correlate the calcium hydroxide leaching profile of these materials with their biological and antibacterial performance.

1.2 Vital pulp therapy (VPT)

The dental pulp can become exposed to several insults such as dental caries, traumatic injuries or iatrogenic events [2]. Potential loss of the integrity of the mineralised structure protecting the pulp can result in a series of inflammatory steps, the severity of which can vary as the infection progresses through the dental tissues [8, 9]. The pulp defence system can potentially shift the balance of inflammation towards healing if the infective stimuli are mainly controlled in severity [9] and do not result in chronicity [6].

A key rationale behind the success of techniques of VPT is the removal of the infected dental tissue and placement of a repair material in close proximity or even in direct contact with the pulp tissue. Pulp inflammation can therefore be subverted and healing can occur with deposition of a dentine-like barrier upon the surface of the pulp from the surviving odontoblasts or differentiated odontoblast-like cells [10, 11]. Despite poor initial findings in terms of the clinical success of these techniques in comparison with conventional pulpectomy and subsequent root canal therapy [12-14], more recently, VPT has been established as a reproducible technique with predictable outcomes and relatively high success rate up to 98% [15-19].

1.3 Pulp capping materials

Gold was the first material that was reportedly used for pulp capping back in 1756 [20]. It is well understood that the role of the material that will be placed in close proximity to the pulp tissue and potentially any enduring bacteria, should balance

between enabling a biological response from the pulp and preventing any further biofilm infection [21]. The ideal characteristics for a pulp capping material can be summarised below as:

1) Good handling properties for the operative,

2) Absence of induction of significant toxicity,

3) Facilitation of dentine-like barrier formation at the interface with the pulp,

4) Adherence to dentine, creating a firm seal at the pulp opening,

5) Antimicrobial activity,

6) Good mechanical properties with absence of solubility [7, 22, 23].

1.3.1 Calcium hydroxide

The behaviour of calcium hydroxide in proximity with the pulp tissue has been proposed sufficient to meet the desirable biological and antimicrobial properties of an ideal pulp capping agent [6, 24-26]. Notably the material dissociates in the field of placement into calcium and hydroxyl ions.

Specifically, hydroxyl ions that leach in the environment raise the pH and have been suggested to induce a necrotic zone in the pulp opening [20] that can stimulate dental pulp defence mechanisms and repair, acting as a substrate for initial connective tissue formation and subsequent mineralisation [27]. An odontoblast differentiation potential has also been reported at this site [28]. The mineralised dentine-like tissue which is formed as an outcome of the healing procedures at the exposure site of the pulp-material interface has been described as a “bridge” [29] or “hard-tissue barrier” [30].

In addition, the strong alkaline pH assists in the elimination of residual or invading bacteria [7, 31]. The bactericidal potential of hydroxyl ions has been attributed to 3 mechanisms:

1) Lysis of the bacterial cellular membrane through degradation of their phospholipid bilayer (lipid peroxidation). Phospholipids are the main constituents of the cytoplasmic membrane. The hydroxyl ions attract hydrogens from the lipids and initiate the process of their destruction [32, 33].

2) Inhibition of the replication of bacterial DNA through a direct reaction [34].

3) Damage to the tertiary structure of bacterial proteins by compromising their ionic bonds [32].

At the same time, release of calcium ions is also considered to act synergistically with the alkaline pH during the odontoblast-like cell differentiation of dental pulp stem cells and deposition of mineralised matrix nodules by increasing the secretion of mineralisation-related genes, contributing significantly to the reparative dentine formation [25, 35-37].

Abundance of calcium ions in the extracellular area induces a mineralising cell differentiation phenotype and proliferation potential through specific calcium recognition receptors in the cells [36]. However, varying amounts of calcium can affect differently the differentiation procedures [36, 38], while the exact desirable factors have not been fully elucidated yet [39]. It is also not clear whether a dose-dependent cytotoxic effect is induced following exposure to relatively high calcium concentrations [36, 40, 41].

Despite the beneficial effects, calcium hydroxide paste formulations that were initially considered as the ideal materials for VPT present significant limitations. Notably, a high dissolution effect reported in these formulations results in poor mechanical properties and unacceptable sealing in the long-term, compromising thus the final treatment outcome [22, 42]. Additionally, histological observations of the dentine-like barrier formed in the interface with the pulp orifice showed anatomical irregularities that render the structure permeable, facilitating further bacterial contamination [43-45] (Table 1.1).

1.3.2 Glass ionomer cements

Another category of materials used in VPT is glass ionomer cements (GICs). The chemical composition of these materials is different to calcium hydroxide, consisting mainly of an acid-decomposable fluoroaluminosilicate glass powder mixed with an acidic polymer liquid [46]. The cement which is consequently formed consists of polyalkenoate salts and exhibits adhesive properties [47]. A main advantage of GICs is the cariostatic effect which has been attributed to fluoride release [46]. However, an acidic environment is induced by their application, as a result of their setting reaction, which is not capable of stimulating a similar pulp healing response as the caustic effect caused by the alkalinity of calcium hydroxide [48]. Additionally, findings regarding cytotoxicity of GICs have restricted their use only in indirect pulp capping procedures [1, 3, 48] (Table 1.1).

1.3.3 Resin modifications

Resin-based formulations were introduced both in calcium hydroxide pastes and in GICs to minimise the material solubility and improve the sealing adaptation to dentinal walls and the overlying resin restoration [2]. Several of these formulations were reported as cytotoxic, mainly due to leaching out of monomers to the underlying pulp, namely triethylene glycol dimethacrylate (TEGDMA) or bisphenol A-glycidyl methacrylate (Bis-GMA) [49, 50]. The hydrophobic nature of the resin–based materials imposes also one additional contraindication of these materials for placement in direct contact with the wet environment of the pulp tissue [22]. Overall, resin formulations have shown less favourable results in moderating pulp inflammation and stimulating a reparative dentine-like barrier formation [51-53] (Table 1.1).

1.3.4 Tricalcium silicate (TCS)-based materials

TCS-based materials were introduced in dentistry through the use of Portland cement (PC). PC is a material used in the construction industry for production of concrete. Interestingly, it was introduced in dentistry as a root canal filling material in a case report published in the end of 19th century [54]. Approximately one century later, Professor Torabinejad proposed the use of a PC-based material for endodontic procedures and patented his invention under the name mineral trioxide aggregate (MTA) [55, 56]. Today, materials based in TCS are considered the gold standard for a variety of endodontic applications, mainly due to their hydraulic characteristics and the calcium hydroxide formation [5]. They can serve as root repair materials (RRMs) in

cases of perforation, tooth resorption or root facture, providing a seal between the root canal and the periodontal tissues. Additionally, they are indicated for cases of VPT (direct or indirect pulp capping and pulpotomy), endodontic surgery and treatment of immature teeth with incomplete root formation [57] (Table 1.1).

Table 1.1 Presentation of the main advantages and limitations of conventional pulp capping agents.

Pulp capping materials	Advantages	Disadvantages
Calcium hydroxide	+ Dentine bridge formation [25, 35-37] + Antimicrobial activity [7, 31]	- Solubility [22, 42] - Permeable hard tissue formation [43-45] - Non-adhesive to dentine [22, 42, 58]
Glass ionomer cements	+ Adhesiveness [47] + Cariostatic activity [46]	- Cytotoxicity [1, 3, 48] - Absence of induction of a biological healing response [48]
Resin-based formulations	+ Micromechanical bonding to dentine [2] + Minimised solubility [2]	- Cytotoxicity [49, 50] - Hydrophobic nature [22]
TCS-based cements (MTA)	+ Acceptable solubility [31, 59-61] + Superior biological performance [45, 62-65] + Moderate antimicrobial potential [66, 67] mainly in fresh samples [68-70]	- Difficulties in handling [31, 71, 72] - Absence of adherence to dentine [73]

1.3.4.1 Chemical composition and hydration of Portland cement

The manufacture process of PC includes crushing and consequently firing of limestone and shale in a rotary kiln [74]. These raw materials are used as a source of calcium, silicon, aluminium and iron oxides [57]. The main products of the burning procedure are tricalcium silicate (45-70%), dicalcium silicate [5-30%], tricalcium aluminate (<10%) and calcium aluminoferrite (<10%) [5, 57]. Inclusion of trace oxides which derive from the raw materials are also evident, based on magnesium, sodium, phosphorous, potassium and other metals (<0.5%) [5, 75]. Consequently, calcium sulphate is further added and the product is mixed by grinding, creating the final powder of PC [57].

The powder of PC hydrates in contact with water to produce a mixture that will harden through time. The water to powder ratio that it is used varies from 0.3 to 0.7 [57]. Following mixing, the reaction commences immediately, leading to formation of two main phases [74]. Tricalcium aluminate reacts initially with water and in the presence of calcium sulphate, ettringite is formed. Calcium sulphate prevents the rapid reaction of tricalcium aluminate, which would have a negative effect in the workability of the cement [57]. Consequently, calcium silicates (tri- and di-) react to a high extent with water leading to the formation of amorphous calcium silicate hydrate and calcium hydroxide [76], as shown in Figure 1.1.

The by-products of hydration are spread in the bulk of the cement surrounding non-hydrated particles [76]. These reaction series lead to the gradual hardening of the cement. Hydration continues for a significant time period but in a much slower pattern [57].

Figure 1.1 Illustration of the main reaction taking place during hydration of PC. DCS: Dicalcium silicate, CSH: Calcium silicate hydrate, CH: Calcium hydroxide.

1.3.4.2 Introduction of PC-based materials in dentistry: MTA

MTA consists of a relatively simple blend of 80% PC mixed with 20% bismuth oxide, which serves as a radiopacifier in the formulation [55, 56, 77]. The material has been thoroughly investigated using *in vitro* studies [31, 78, 79], research in animals [62, 80-82] and application in human patients [83] before being commercialised as ProRoot MTA (Dentsply, Tulsa Dental, Johnson City, TN, USA) in 1998 for use as a root repair material [84]. Apart from its hydraulic nature, MTA presents desirable physico-chemical and biological properties [85] which are attributed mainly to the PC; the calcium hydroxide by-product is particularly responsible for its chemical profile [86].

Several commercial MTA formulations are today available on the market. The presence of iron was initially minimised, thus changing the initial grey colour of the material [87]. Overall, few differences exist between the commercially available MTA products, in particular concerning the fineness of the PC, the particle size, and the depletion of the calcium sulphate and tricalcium aluminate such as in MTA Angelus (Angelus, Londrina, Brazil) or the percentage of bismuth oxide [85]. The beneficial biological and antimicrobial properties in commercial materials reportedly all derive from the calcium hydroxide release. In this overview, the term MTA is used both to

refer to the original formulation of MTA as it was initially patented and to the most popular and extensively studied commercial products available in the market.

1.4 Review of the properties of MTA

1.4.1 Physico-chemical characteristics

MTA powder is mixed with water in a 0.33 water: powder (w/p) ratio [88]. The material is hand spatulated and applied in the operative field with appropriate carriers. The cement phase reacts with water as described previously, while bismuth oxide remains unhydrated. A matrix of amorphous calcium silicate hydrate gel interrupted by un-hydrated cement particles and large white bismuth oxide particles comprise the typical microstructure of the bulk of a set material [76].

Calcium hydroxide is one of the main products of the hydration process. Hydroxyl ions that dissolve in the surrounding environment raise significantly the pH [89, 90] in values close to 13 at only a few hours after mixing [31, 66]. Overall, reports for pH values of MTA in the literature vary depending on the methodological conditions used for assessment, namely immersion in different solutions or direct measurements upon the material's surface and time period of measurement [66, 89, 91]. Calcium leaching is also relatively high in all the commercial formulations [92, 93] and significantly higher in comparison with conventional calcium hydroxide pastes [94].

Notably the material can set in humidity. The setting time, however is prolonged and lasts several hours [5]. Depletion of calcium sulphate in some

formulations has been reported to decrease the setting time as it allows tricalcium aluminate to react faster [95, 96]. In terms of solubility, most studies have reported that MTA exhibits minor changes in its mass [31, 59-61], however, differences in the period of assessment appear to affect the results, as an increase has been found in the longer term observations [97]. The w/p ratio employed to mix the cement is also positively correlated with material solubility [98]. Even though lack of high solubility values is an undisputed desirable property in all endodontic materials, it is worth noting that elements leached from the material are responsible for the antimicrobial properties [99] and the biological response from the host tissue [100]; therefore, potential minimal material solubility can affect ion release [101, 102].

1.4.2 Biological properties

One of the main advantages of MTA over materials of a different chemical composition is its biocompatibility in addition to its ability to induce a healing-related biological response. The beneficial effect has been mainly attributed to the calcium hydroxide by-product [6]. The mechanism of action is similar, as described in Section 1.3.1, to that of calcium hydroxide formulations. In addition to the superiority of MTA in terms of its physicomechanical properties over calcium hydroxide formulations, it has been also shown that MTA is capable of inducing an enhanced biological effect, in terms of a more sustainable initial degree of inflammation and local necrosis, as well as formation of a thicker and less porous reparative dentine bridge [45, 62-65] (Table 1.1).

The biological properties of MTA have been studied extensively in terms of biocompatibility, induction of cellular responses associated with healing and bioactivity in a series of *in vitro* studies, animal models and clinical case observations. The effect of MTA in the proliferation and morphology of various cell lines has been evaluated through direct or indirect contact assays with overall favourable results. Specifically, most studies report absence of cytotoxicity or induction of an initial cytotoxic effect followed by cell recovery and even enhancement of cell growth in combination with acceptable cellular attachment upon the material surface in direct contact assays [103-110]. Additionally, MTA has been found to possess a mild stimulatory effect in terms of the expression of cytokines and other proteins which are regulators of inflammation, as well as in the secretion of bioactive markers and growth factors associated with cell differentiation and mineralisation [111-115].

The inflammatory response induced by MTA has been also evaluated histologically following subcutaneous or intraosseous implantation in animals. Overall, most studies reported the induction of an initial inflammatory phase of varying degree in the first days, which decreased over time and was accompanied with a favourable tissue response [81, 116-126].

Beyond biocompatibility, MTA has been also characterised as 'bioactive', due to the formation of calcium phosphate precipitates upon its surface following immersion in simulated body fluids. This property has been shown in several studies and is considered beneficial as this mineralised layer can potentially act as a barrier for the underlying pulp tissue and as an induction site for hard tissue deposition [127-131].

In a similar pattern, studies regarding the application of MTA in animal teeth are overall in accordance with the *in vitro* findings. Notably, dentine bridge formation has been reported histologically following pulp capping in healthy animal teeth [62, 63, 65, 132-135]. Clinical trials or case studies of application of MTA as a pulp capping agent in human patients showed a high success rate in terms of formation of a calcified barrier and maintenance of pulp vitality, as well as providing an adequate seal between the material and the surrounding tissues [64, 134, 136].

1.4.3 Antimicrobial activity

Despite the extensive assessment of the antimicrobial properties of MTA formulations, studies often conclude contradictory findings due to differences in methodological procedures, bacterial species tested and type of bacterial growth studied (planktonic or biofilms) [85]. The antimicrobial potential of MTA formulations has been tested against a plethora of caries-associated bacteria, such as *Streptococci* and *Staphylococci*, as well as *Fungi* and species associated with refractory endodontic cases, namely *Enterococcus faecalis*. Overall, MTA has been proposed to possess a moderate antimicrobial effect due to its highly alkaline pH [66, 67] (Table 1.1). However, other studies have questioned this effect, particularly against anaerobic bacteria, where no impact in their growth has been observed [79, 137]. It has been also suggested that the antimicrobial effect is stronger in fresh samples and deteriorates in those which have set [68-70].

1.4.4 Potential limitations of MTA

Despite several beneficial physicochemical characteristics and the reported success in clinical cases, the original formulation of MTA also exhibits limitations. Trace elements such as lead, chromium and arsenic are present in the powder phase of MTA, deriving from the unprocessed materials used for manufacture of PC [138-140]. The latter is produced in large amounts for construction purposes and it is therefore difficult to accurately control the raw materials used in the procedure. Despite the very low concentrations of these traces reported, concerns have been raised for their potential leaching in human tissue [5].

Additionally, the mixture of MTA presents a grainy consistency and prolonged setting time which compromise the delivery efficiency of the material in the operating field [31, 71, 72]. The long setting period has been attributed to the calcium sulphate added in the PC to control the speed of the reaction [57].

The literature has also questioned the suitability of bismuth oxide as an acceptable radiopacifier for use in dental materials [5]. Even though it renders the material adequately radiopaque depending on the amount incorporated in each commercial formulation [57, 141], several studies have reported that it does not remain inert in the formulation, interfering with the hydration of tricalcium silicate by replacing silicon in the calcium silicate hydrate gel [76] and leaching out from the cement [92]. A tooth discoloration potential has been associated with the latter [142], while a cytotoxic effect has been also suggested [143, 144]. Finally, a reduction in the

radio-opacity of the material can be observed in long term clinical use as a consequence of the leaching from the cement [130].

1.4.5 Alterations in the formulation

Today, several modifications have been introduced in an effort to overcome some of the significant limitations of the initial formulation of MTA and to optimise its existing properties. Towards this, pure calcium silicate manufactured with finer laboratory procedures has replaced PC [130, 145]. With this process, not only inclusion of trace elements can be prevented, but the particle size of the constituents can be also monitored, which can have a significant effect in the control of the speed of the hydration reaction [146].

Additionally, inert materials with high molecular weight and which do not leach out have been incorporated as radiopacifiers in the cement replacing bismuth oxide [147]. Notably, zirconium oxide, tantalum oxide and calcium tungstate are being used in commercial materials for this purpose amongst others [148-150].

Issues with the consistency and the prolonged setting time of MTA have been mainly addressed with the inclusion of water-soluble polymers or hydration accelerators in the liquid phase respectively. Propylene glycol is a polymer that was introduced to improve the workability [151] and the mechanical properties of the material by decreasing the water demand in the cementitious formulations [130]. Additionally, calcium chloride is an accelerant that has been incorporated with the rationale to effectively decrease the setting time [152-154]. At the same time, the

elimination of calcium sulphate from the cementitious phase results also in a more rapid setting reaction [155].

Further research for enhancement of the physicochemical properties of monophasic TCS-based materials has been directed towards the incorporation of compounds into the cementitious phase of the material. Experimental and commercial materials have been introduced including additions of another cementitious phase, such as tricalcium aluminate [156], calcium sulphoaluminate or calcium fluoraluminate cement [157, 158] and calcium phosphate (CP) [159-161].

Notably, CP cements encompass a wide variety of salts of orthophosphoric acid, which were initially introduced as bone cements for repair of associated defects [162]. CP sets also in the presence of a liquid vehicle, with a process of dissolution and precipitation [163]. The main rationale for its use in TCS-based formulation was the enhancement of the biological activity of the material, based on the formation of mineral structures of set CP, that closely resemble the mineralised structure of bone tissue [164].

Additions of methylcellulose [152], calcium carbonate [165], silicon oxide [128, 166] or a resin component [7, 167] in calcium silicate-based formulations have been also used to improve the cement's physical properties. In particular, inclusion of silicon oxide in PC was initially exploited in the construction industry in an effort to increase the mechanical strength of concrete [168]. Micro-silica (mS), which consists mainly of amorphous silicon oxide, increases the compressive strength and reduces the porosity and setting time of the concrete, reacting with the calcium hydroxide that is produced during hydration of the cement [74]. Thus, more calcium silicate hydrate is formed.

Additionally, the high reactivity of this component accelerates the hydration, resulting in a decrease in the setting time [74, 166].

Furthermore, using a similar rationale as described in Section 1.3.3, inclusion of light-curable monomers has been proposed to address mainly the poor bonding properties of MTA with the dentine walls and the overlying composite restoration materials [73]. At the same time, the deterioration of the physicochemical characteristics of hydraulic cements that has been reported following acid etching procedures for placement of composite restorations [169] could potentially be overcome.

1.5 The new generation of commercial TCS-based materials

Overall, a plethora of chemical compounds has been used in experimental studies and has been incorporated in the new generation of TCS-based materials. Modified materials with chemical additions, could potentially present significant differences from the conventional hydraulic cement formulation, in an effort to develop the ideal materials for use in endodontic applications. As a result, the hydration reaction and the calcium releasing ability have the potential to be altered.

A series of significant modifications to the initial chemical formulation of MTA will be further examined following reviewing of representative commercial materials suitable for pulp capping and respective prototype formulations studied in the literature with a similar chemical composition. Particular focus will be given to the

chemical properties of these cements, and specifically how modifications applied affect their solubility and calcium release, which in turn can affect their biological and antimicrobial properties. At the same time, as several modifications are carried out with the rationale to improve specific physico-chemical properties (setting time, compressive strength, workability, bonding), it would be interesting to determine their potential effect on the materials' biological and antimicrobial activity. A summary of this review is presented in Table 1.2.

1.5.1 Chemical formulations

One of the first materials of the new generation of TCS-based pulp capping cements introduced clinically, Biodentine (Septodont, Saint Maur-des-Fosses, France), utilised pure calcium silicates (tri- and di-) in the cementitious phase instead of PC. More specifically, its powder phase consists of TCS, dicalcium silicate, calcium carbonate and zirconium oxide as a radiopacifier. The liquid contains water, with calcium chloride as a setting accelerator along with a water-soluble polymer which improves the consistency and handling properties of the material [130, 170].

A recently introduced material, Bio-C Pulpo (Angelus, Londrina, PR, Brazil) presents a similar liquid vehicle to Biodentine, consisting of distilled water, calcium chloride, a plasticiser and metilparaben. Its powder phase however, is comprised of calcium silicate, tricalcium aluminate, calcium hydroxide, calcium fluoride, silicon oxide, iron oxide and zirconium oxide as radiopacifier [171] and therefore presents a typical formulation of a PC-based material rather than the pure calcium silicate used in Biodentine. As the material has not been chemical and physical characterised so far, it

would be interesting to identify the rationale behind the addition of several compounds. Specifically, the presence of calcium fluoride and silicon oxide enhance the material's mechanical strength [158, 168], however they can also alter significantly the hydration of the cement [172]. Bio-C Pulpo is hand spatulated in contrast with Biodentine which is mixed on a capsule mixing device.

Difficulties in handling and delivery efficiency of the hydraulic TCS-based cements have been addressed with the introduction of premixed materials. TotalFill Bioceramic Root Repair Material (TotalFill RRM; FKG, La Chaux-de-Fonds, Switzerland) was introduced together with a series of materials with similar chemical composition and slight modifications depending on their consistency and endodontic application. TotalFill RRM paste, TotalFill RRM Putty and TotalFill RRM Fast Set Putty differ mainly in the setting time, with the putty forms exhibiting a thicker consistency and a significantly lower setting time period [173]. The same materials are also commercialised as Endosequense by Brasseler in USA and iRoot BP by Innovative BioCeramix in Canada [133]. TotalFill RRM is delivered using an injectable syringe. All TotalFill RRM products have the same safety data sheet (SDS) [174], which provides an indication that these materials present minor differences in chemical composition, potentially due to the inclusion of a setting accelerator.

The chemical composition of TotalFill RRM differs mainly from the other TCS-based materials due to the incorporation of CP monobasic. The material consists additionally of calcium silicate, calcium sulphate, filler agents along with zirconium oxide and tantalum oxide as radiopacifiers [57, 174]. Interestingly, the CP is not reported in the SDS, although its presence in the material's formulation is stated in the

“instructions for use” information provided for Endosequence RRM [175]. The amount of CP incorporation in the cementitious phase is therefore unknown. The hydration of these materials depends on the humidity absorbed from the dentinal tubules in the field of application [173], which is also a significant parameter that separates TotalFill materials from conventional cements mixed with water.

Another premixed material, TheraCal LC (Theracal; Bisco, Schaumburg, IL, USA) introduced the inclusion of a resin matrix in the composition of hydraulic TCS-based cements with the rationale to improve handling characteristics as well as setting and mechanical properties. The resin formulation addressed also the negative effect of acid etching procedures in the physicochemical characteristics of TCS-based cements during placement of composite restorations and improved the sealing with the dentine walls [7]. Theracal consists of a similar amount of a Portland cement type III and resins, namely rethane dimethacrylate, bis-GMA, TEGDMA and hydroxyethyl methacrylate [176]. Polyethylene glycol dimethacrylate is used to promote miscibility of the cement with the resin; barium zirconate acts as radiopacifier, while a small amount of hydrophilic thickening agent (fumed silica) is also added [176-178]. A glass phase has been identified in Theracal’s matrix as well, which is not specified by the manufacturer, consisting of aluminium, silicon and strontium [179]. The material is available in a light-curable paste and is indicated for use in pulp capping procedures. As no liquid vehicle is used to hydrate the cementitious phase of Theracal, hydration is highly dependent on the absorbed moisture in the field of application, which can be further compromised by the hydrophobic nature of the resin vehicle [5].

Table 1.2 Characteristics of selected pulp capping materials from new generation of TCS-based cements.

Material	Composition	Calcium release - solubility	Biological activity	Antimicrobial properties
Biodentine	- Powder: Calcium silicates, calcium carbonate and zirconium oxide - Liquid: water, calcium chloride, water-soluble polymer [130, 170]	- High leaching [180]- escalating over 1-week period [181] - Negligible solubility [181-185]	- Acceptable biocompatibility [6] - Induces odontoblastic differentiation, mineralisation [186-191] and dentine bridge formation [192-195] - Bioactive [47, 90, 130, 196]	- Effective against *E. faecalis, Candida albicans* and *Escerichia Coli* [68, 197, 198] - Effective only after direct contact against a *Streptococci* suspension [7, 199]
Bio-C Pulpo	- Powder: Calcium silicate, tricalcium aluminate, calcium hydroxide, calcium fluoride, silicon oxide, iron oxide and zirconium oxide - Liquid: distilled water, calcium chloride, plasticiser, metilparaben [171]	- Not yet studied	- Acceptable biocompatibility, osteogenic potential and induction of a moderate time-diminishing inflammation [200]	- Not yet studied

TotalFill RRM	- Calcium silicate, calcium phosphate monobasic, calcium sulphate, filler agents along with zirconium oxide and tantalum oxide [57, 174]	- Relatively high and escalating leaching during the 1st week - Significant dissolution [161]	- Good overall biocompatibility values [108, 109, 201-207] - Odontoblastic and osteoblastic cell differentiation potential -Induction of dentine barrier [132, 208] - Adequately bioactive [209, 210]	- Time-diminishing activity against *E. faecalis* [211, 212] - Effective against *C. albicans* [67]
Theracal	- PC type III, resins, strontium glass, barium zirconate, fumed silica [176-178]	- Compromised calcium release [7, 179, 180, 213] - Absence of solubility [181, 214]	- Initial cytotoxic effect [215]- controversial findings in the long-term [7, 176, 216, 217] - Failure to adequately control inflammation and promote healing [6] - Bioactive, albeit exhibiting different characteristics in the CP layer in comparison with the other TCS-based cements [179, 181, 213]	- Significantly effective following direct contact against a *Streptococci* inoculum [199] - The 1-day leachate of Theracal was not found antibacterial against a *Streptococci* suspension [7]

Table 1.2 (Continued)

1.5.2 Solubility and calcium release

Several studies have assessed the chemical profile of the commercial materials presented above. Depending on the methodological standards used to test Biodentine's dissolution, the material has been shown to exhibit acceptable solubility values [181-185] or even negative numbers due to calcium phosphate deposition upon its surface [165]. The improved calcium leaching profile of Biodentine has been attributed to its optimised formulation [180]. Specifically, calcium release is relatively high from the first day [7] and escalates after a week of immersion in simulated body fluids [181]. Despite the high initial calcium release, controversial results have been reported in the literature as to whether leaching plateaus following longer incubation periods [101, 170, 218, 219]. Overall, the majority of studies concluded that Biodentine releases significantly higher amounts of calcium comparing with MTA formulations [90, 181, 184, 220, 221], while a few other studies have reported a similar [218] or even lower leaching than MTA following a 28-day period [101].

Findings from comparisons with experimental materials with a similar composition to Biodentine are controversial. Li and co-workers showed that TCS with various percentages of radiopacifiers released higher amounts of calcium after 1-week and 1-month immersion periods in a buffered medium [219], while Camilleri *et al.* found that Biodentine released more calcium than a radiopacified TCS prototype cement after 2 weeks [180]. In the first study, calcium chloride was used in the liquid phase of the prototype material, which can also increase calcium release [154], while several materials were developed with varying percentages of zirconium oxide.

Differences in results for calcium release can be also attributed to the experimental design and sensitivity of the technical equipment used in each study.

Leaching properties of Bio-C Pulpo have not been assessed so far. However, there is evidence from another commercial TCS-based material which contains silicon oxide that a potential deterioration of calcium release can occur due to the reaction taking place between silicon oxide and the calcium hydroxide by-product [128]. These data indicates the need for further investigation of the recently introduced formulation.

A report of solubility for TotalFill RRM was recently published, showing that the material presents significant dissolution in water, while values in the putty form are moderately lower than the paste [161]. In terms of calcium release, the material appears to leach consistently increasing amounts from the first 3 h and over a 7-day period, which consequently decrease significantly in a 14- and a 28-day period [161]. The calcium leaching profile of the putty formulation is overall less aggressive than the TotalFill RRM paste and MTA during the first 3 and 5 days respectively, however more calcium has been reported to be released than the TotalFill RRM paste over the 14-day period [102, 161].

Additionally, no clear insight is provided following assessment of prototype formulations in the literature regarding the effect of the incorporation of CP in the calcium release of biphasic cements. Two studies reported a more aggressive release in experimental TCS-based materials containing CP [159, 222]; depending on the type of the CP inclusion (alpha tricalcium phosphate or dicalcium dehydrate), materials also presented a different leaching profile throughout a 28-day period [159]. Another study

showed significantly lower calcium release in Hank's balanced salt solution (HBSS) in the biphasic prototype material containing CP monobasic after a 28-day immersion period [223]. Differences in results could also derive from the different assessment techniques.

Conversely, the effect of the resin inclusion on hydration and leaching behaviour is more profound. Specifically, the presence of a resin matrix renders the material significantly less soluble than MTA as evidenced in Theracal [181, 214]. Solubility values remain relatively low even after a 2-month period [224]. In terms of calcium release, it has been shown that the resin incorporation compromises calcium leaching in comparison with non-modified TCS-based formulations [7, 179, 180, 213]. However, one recent study did not identify any differences in calcium release compared with Biodentine and the authors even reported a better leaching profile for Theracal than MTA [221]. Overall, deterioration of the calcium leaching ability has been also shown in prototype materials incorporating light-curable resins within MTA formulation or TCS [7, 225-227], albeit one report finding higher amounts of calcium than MTA in one experimental resin-modified TCS [228]. Variations in the results could be explained by differences in the permeability of the polymeric resin matrix which can allow the water diffusion throughout their bulk to a different extent and thus modulating the degree of hydration [167]. Solubility of the experimental resin-modified cements was also significantly lower than the conventional TCS-based cements [225, 228].

1.5.3 Biological properties

Overall, it is clear that a similar pattern in the behaviour of the TCS-based materials is evident with an initial stimulatory effect that is consequently replaced by a mild response and induction of reparative procedures. However, this behaviour could potentially be masked by several alterations in the chemical profile and leaching of the modified cements.

Biodentine is the second most extensively studied TCS-based material with regards to its biological properties following MTA [229]. In general, it is non-cytotoxic and non-genotoxic [230]. A series of studies have shown that Biodentine induces a similar mild cytotoxic effect in various cell types compared with MTA [185, 187, 189, 216, 217, 231-239] and this toxicity decreases over time [7]. Other studies, however, have reported contradictory results in comparison with MTA, concluding that Biodentine presents a better [240] or a lower proliferative effect [109, 241]. Cell adhesion assays have shown results comparable with MTA, or even improved performance [232, 237, 242, 243]. Differences in the susceptibility of cell types used as well as variations in the experimental protocols are the main explanations for these few contradictory reports. Despite the relatively positive overall biocompatibility, Biodentine appeared moderately more cytotoxic than an experimental pure TCS cement [219].

The CP-containing TotalFill RRM has exhibited relatively good biocompatibility for both fresh and set samples evaluated, which are similar to MTA and Biodentine [108, 109, 201-207] or possibly even better [191, 244]. Conversely, two studies

reported decreased cell viability compared with MTA [245, 246]. Indeed an improvement in the proliferation index has been reported with prolonged incubation periods [202, 207, 244, 247] until cell cultures reached a confluent state [201, 203]. However, inhibition in cell growth has been reported in other studies evaluating fresh samples for longer incubation periods [206, 245]. The biocompatibility of TotalFill RRM formulations has been also verified following observations of acceptable cellular adhesion after seeding, as morphologically healthy cells which increase throughout the incubation period have been observed [201, 202, 207, 248].

The resin-based Theracal induces an initial cytotoxic effect [215], while results for longer incubation periods are controversial, as two studies suggested that cytotoxicity diminished with time [7, 216], while two other reports indicated an increased cytotoxicity over a 3- and 7-day culture period [176, 217]. This detrimental effect on proliferation has been mainly attributed to leaching of potentially cytotoxic monomers [6]. Interestingly, studies on cytotoxicity of experimental light-curable hydraulic cements have showed good biocompatibility performance [7, 228].

Beyond cytotoxicity and in terms of assessment of the regenerative potential, Biodentine has been characterised as capable of shifting the balance of inflammation towards healing [6]. Specifically, it has been shown to induce a desirable moderate production of cytokines and other inflammatory mediators similar to MTA [187, 237, 249] which is also evident for TotalFill RRM [250]. Additionally, a recruitment of dental pulp cells to the injury site has been linked with Biodentine [188, 251]. Consequently, an odontoblastic differentiation potential in murine and human dental pulp stem cells [186-191] as well as an osteogenic potential in human dental pulp and bone marrow

stem cells has been well described for Biodentine, following observations of enhanced extracellular mineralisation and increased expression of related gene markers or growth factors, such as Transforming Growth Factor β1 (TGF-β1) [113, 188, 207, 216]. TotalFill RRM can also induce odontoblastic and osteoblastic cell differentiation, which was shown by the expression of relevant genes in stem cells [114, 191, 206, 207, 252] and differentiation markers [207, 244, 253] following exposure to the material. Contradictory results however, have been found with regards to the potential of expression of alkaline phosphatase (ALP), a protein which is associated with the mineralisation process. Overall, an upregulation in secretion of ALP has been reported, in values comparable with MTA [203, 206], although one study observed a decrease in the expression of this protein [246]. Results using experimental TCS cements which incorporate CP suggested that the biphasic cements behave similarly in cell proliferation assays in a time-dependent rate [159, 223], while one study reported enhanced cell viability as the percentage of CP incorporation increased in the formulations [254]. A significant upregulation in expression of osteoblastic-related markers was reported in one biphasic formulation [159].

For Bio-C Pulpo, one recently published study assessed the material's inflammatory effect and potential to stimulate expression of osteogenic markers following implantation in rats, with findings of acceptable biocompatibility, a time-dependent decrease of inflammation and a similar osteogenic potential as MTA [200]. The leaching profile of the material has not been assessed so far so no further correlation with the material's biological properties can be performed.

In terms of modulating the inflammatory reaction, a recent review by Giraud *et al.* concluded that Theracal and all the modified TCS-based materials including a resin matrix in general, could not promote pulp tissue regenerative procedures in contrast with the conventional TCS-based cements, such as Biodentine and MTA which exhibited excellent biological properties [6]. Similarly, despite the material's ability to promote extracellular mineralisation, Theracal exhibited a significantly lower osteogenic potential than Biodentine [216].

The degree of the inflammatory response and the potential to induce healing events have been also assessed using animal subcutaneous tissue implantation studies for some materials. Biodentine has shown acceptable responses, by promoting the formation of collagenous capsules following induction of an initial inflammatory reaction [255]. This response was significantly higher than that observed for MTA Angelus during the first 2 weeks [119, 120, 124]. Conversely, the severity of inflammation provoked by TotalFill RRM during a 21- and 30-day subcutaneous implantation period in rats has exhibited contradictory results compared with MTA [126, 256].

In terms of bioactivity, the potential of Biodentine to produce a calcium phosphate layer both in simulated body fluids and in dentine in a similar pattern as MTA, has been well described [47, 90, 130, 196]. A significant amount of crystal precipitates was observed also upon the surface of TotalFill RRM following immersion in water or physiological solutions [209, 210]. In experimental materials, the biphasic cements formed similar [159] or higher [254] amounts of crystal precipitates compared with pure TCS.

Interestingly, the precipitation of calcium phosphate upon Theracal's surface has been verified in several *in vitro* studies, but with occasionally different characteristics than the conventional TCS-based materials, namely the calcium to phosphorous ratio, thickness of the crystal layer and size of granular deposits [179, 181, 213]. Bioactivity of Theracal was also assessed following subcutaneous implantation in rats, observing the formation of a CP layer, the thickness of which was significantly lower than MTA and a prototype TCS cement tested [257].

Clinical cases following application of Biodentine in animals have shown stimulation of dentine bridge formation following pulpotomy or pulp capping in primary pig, rat and dog teeth [192-195] showing similar characteristics to that induced by MTA or even demonstrating a significantly greater thickness [194, 195]. Results of *in vivo* studies in human patients showed relatively high success rates when evaluated, with absence of clinical symptoms, acceptable regulation of pulp inflammation and preservation of pulp vitality and hard tissue barrier formation [45, 258, 259].

Similarly, the ability of TotalFill RRM to induce a reparative dentine bridge formation has also been observed in a rat model [132], while a better healing response than MTA was found in dog teeth after a 6-month evaluation of the outcome of root microsurgery [260]. Retrospective clinical trials of endodontic microsurgery in human teeth have shown comparable results with MTA [261] and overall acceptable success rates [262]. Finally, a randomised clinical trial of partial pulpotomy in human teeth showed a similar response to MTA, with formation of a complete dentine bridge [208].

Application of Theracal as a direct pulp capping agent in primate teeth following an infection model, showed induction of hard tissue and acceptable inflammation in the pulp [263]. However, the level of formation of a mineralised tissue barrier was significantly lower than that observed for MTA in dog teeth following partial pulpotomies [264]. Similarly, a clinical trial in human teeth showed significantly less favourable healing outcomes when treated with Theracal and in comparison with MTA and Biodentine, as only 11% of the cases showed normal pulp histology and dentine barrier formation [258].

1.5.4 Antimicrobial characteristics

The antimicrobial potential of the new pulp capping TCS-based cements has not been studied as thoroughly as their biological properties. Similarly with MTA, findings from the literature do not usually provide a clear insight into their true antimicrobial potential as they are not correlated with their leaching behaviour. Specifically, results for Biodentine from the Agar Diffusion Test (ADT) which have mainly evaluated its inhibitory effect against different *Streptococci* species using a nutrient agar plate are somewhat controversial [185, 198, 217, 265]. Interestingly, a bactericidal effect was found against a multispecies *Streptococci* suspension using a direct contact assay, which diminished over time [199]. However, the material's leachate did not exhibit any effect against the same bacterial suspension [7]. Finally, Biodentine has been found to exert antimicrobial effects against *E. faecalis* [68, 197, 198], *Candida albicans* [197, 198] and *Escerichia coli* [198].

TotalFill RRM was found to have comparable antimicrobial activity to MTA against *C. albicans* [67] and *E. faecalis* [211, 212]. However, this diminished over a 1-week period [211]. Similarly, the material was also effective against *Streptococcus mutans* and *Lactobacilli* species [266].

Few studies have assessed the antimicrobial properties of Theracal. The material's leachate and other experimental resin-modified TCS cements failed to inhibit the growth of a multispecies *Streptococci* suspension [7]. However, in direct contact with the same bacterial cocktail, the resin-based material presented a significant antibacterial activity over time, in contrast to Biodentine which was only antimicrobial during the initial contact period [199].

1.6 Pulp capping cements without TCS: ACTIVA BioACTIVE Base/Liner

It was previously reported that glass ionomers with or without inclusions of a resin component are indicated for use in indirect pulp capping procedures [3]. ACTIVA BioACTIVE Base/Liner (Activa; Pulpdent, Watertown, MA, USA) is a recently introduced light curable resin-modified glass ionomer cement which encompasses methacrylate monomers, such as diurethane in combination with polyacrylic acid in its formulation. Presence of amorphous silica and sodium fluoride are also specified in the material's SDS [267]. The material is available in a two-paste double-barrelled tube. Pastes are mixed through an auto-mix delivery syringe tip and consequently light-cured. Interestingly, there is a lack of studies investigating the chemical composition of Activa

as well as its properties such as solubility, calcium release, biological and antimicrobial activity. The manufacturer claims that the material can act as an ion depositor due to its resin matrix, allowing ionic diffusion namely calcium, phosphorous and fluoride in higher amounts than conventional glass ionomers, depending on the environmental conditions [268].

1.7 Effects of addition of chemicals in the conventional chemical composition

1.7.1 A potential change in the water demand

TCS-based materials are mixed with water to obtain a homogenous mixture. The w/p ratio of MTA is 0.33 [88]. A similar ratio is frequently used in studies to assess properties of experimental TCS-based materials [159, 166]. Notably when the w/p ratio is not sufficient to hydrate the mixture, alterations in the amount of water used are typically undertaken using visual inspection of the mixture by the operator. It has also been shown in experimental materials that the addition of calcium phosphate or silica-based compounds alters the hydration process of the cement [166, 223, 269]. Although a considerable amount of work is undertaken to create novel materials based on TCS for clinical use, to date, there are no studies reporting the exact alterations in the water demand of hydraulic cements following replacement by different compounds and radiopacifiers in their formulation to enable them to be adequately hydrated. Notably, it has been reported that modifications in the water amount used in the mixture can have a significant effect in the physicochemical properties of

cements [98, 270]. An accurate protocol for the determination of the w/p proportion in hydraulic cements is therefore still lacking.

Rheological properties of TCS-based materials are important clinically as they affect material manipulation, placement, and stability. So far the flowability of hydraulic cements has not been extensively studied [271-273]; however, flow properties of TCS-based endodontic sealers are more routinely reported in the literature [149, 274, 275]. This could be attributed to the lack of a standardised model for flowability tests on hydraulic cements.

1.7.2 Changes induced by incorporation of chemicals

In an effort to develop the ideal materials for use in endodontic application, particular care must be taken not to diminish any of the existing beneficial characteristics of TCS-based cements. From the materials that were previously presented, it is evident that the effects of different additives in the typical formulation of a radiopacified TCS cement are yet to be elucidated. Specifically, even though the beneficial effects of substitution of the PC with pure calcium silicates and the use of inert radiopacifiers instead of bismuth oxide are indisputable, there is still a great controversy in the literature regarding the effect of further alterations. So far, addition of a second cementitious phase, namely calcium phosphate to TCS, inclusion of a resin matrix or incorporation of silicon oxide have been found to affect the hydration of materials and the calcium hydroxide released to various extents [166, 179, 223]. Overall, it is evident that efforts directed in enhancement of physico-mechanical properties of the cements, might have an adverse effect on their chemical profile.

At the same time, a potential modification to the hydration process might have a significant effect on their biological and antibacterial properties as the hydration by-products have been directly linked to these processes [6]. A direct correlation between the different incorporations and their respective effects should be further explored, as the beneficial role of calcium hydroxide has been highlighted, although to date there is no data on the desirable amount [39]. Finally, only relatively few studies have assessed so far the biological and antimicrobial properties of these cements in respect to their calcium hydroxide leaching profile.

1.8 Aims of the study

The present study had the following goals:

- Develop a standardised model for the calculation of w/p ratio for hydraulic calcium silicate materials based on rheological properties.
- Investigate if the adjustment of the w/p ratio affects the physicochemical properties of the materials. The null hypothesis was that the various additions will not change the water demand of the hydraulic calcium silicate cements. A further hypothesis to be studied was that materials mixed at different w/p ratios will not present alterations in their physicochemical properties.
- Characterise a series of commercial pulp capping materials, namely Biodentine, TotalFill RRM, Theracal, Bio-C Pulpo, Activa and prototype TCS-based materials containing either CP monobasic or mS replacement.
- Investigate the effect of inclusion of CP, mS and a resin matrix on hydration, calcium release, biological and antimicrobial properties of TCS-based cements.

- Determine a potential ‘correlation path’ between the calcium hydroxide by-product release and biological and antimicrobial properties.

CHAPTER 2

MATERIALS AND METHODS

The experiments described in Section 2.2 have been publiced in Dental Materials (Koutroulis A, Batchelor H, Kuehne S, Cooper PR, Camilleri J). The author has carried out the execution of experiments.

Dr Batchelor, Senior Lecturer in Pharmaceutics, Formulation and Drug Delivery, Pharmacy and Therapeutics Section, Institute of Clinical Sciences, College of Medical and Dental Sciences, University of Birmingham supervised the study design described in Section 2.2.2.

2.1 Materials

Chemicals and equipment of the study were obtained from Sigma Aldrich, Gillingham, UK, unless stated otherwise.

The following commercial and prototype materials used or for prospective use as pulp protection materials were investigated:

- Bio-C Pulpo (Angelus, Batch No: 130917)
- Biodentine (Septodont)
- TotalFill RRM (FKG)
- Theracal (Bisco)
- Activa (Pulpdent)
- Tricalcium silicate cement (TCS; Mineral Research Processing, Meyzieu, France) replaced with 30 per cent (%) zirconium oxide (ZO) as radiopacifier (TCS/ZO)
- TCS/ZO with 15% replacement of calcium phosphate monobasic (CP) in the cementitious phase (TCS-CP/ZO)
- TCS/ZO with 10% or 20% micro-silica (mS, Meyco 610, MBT-FEB, Manchester, UK) replacement of the cement (TCS-mS10/ZO, TCS-mS20/ZO respectively)

The commercially available materials were mixed and employed according to manufacturers' instructions. Replacement of CP, mS and radiopacifiers in the prototype materials was performed by weight fractions.

Using a standard w/p ratio for the prototype cements resulted in having mixtures of different consistencies since the water demand of the additives varied resulting in excess or too little water in the final mixture. Thus, a series of experiments

were designed to determine the water demand of the prototype materials and thus establish the w/p ratio of these materials. The effect of the w/p ratio on the material chemistry and physical characteristics was also investigated.

2.2 Investigation of the effect of the w/p ratio on hydraulic cement properties

2.2.1 Test materials

The following prototype materials were studied:

- Tricalcium silicate (TCS) cement
- TCS and various radiopacifiers replacing 30% of the cement, namely zirconium oxide (ZO), tantalum oxide (TO), calcium tungstate (CW) and a mixture of zirconium oxide and tantalum oxide in 15:15% proportion (ZO-TO)
- Radiopacified TCS with 15% replacement of calcium phosphate monobasic (CP)
- Radiopacified TCS with 10% and 20% incorporation of micro-silica (mS10, mS20 respectively; Meyco 610, MBT-FEB, Manchester, UK)
- Portland cement (PC; Blue Circle Portland Cement, Tarmac, Wolverhampton, UK)

2.2.2 Rheological assessment

For the calculation of the water demand in the prototype materials, a rheological assay was designed. PC was used in this experimental model system as this material has been studied in the construction industry [276] and large quantities were available manufactured from a single batch. PC manufactured to BS EN 197-1: 2000, type CEMI 52.5 N was mixed with water at a w/p ratio of 0.35 (PC_0.35). This formulation was regarded as providing the ideal mixture with a clinically acceptable consistency and its hydration has been described thoroughly [145]. This w/p ratio was used as the baseline for all the experiments. The performance of PC mixed with water in the above ratio served as a control for the establishment of the optimum proportion of water in all the other cements with different inclusion of chemicals.

A shear rheometer with adjustable temperature (Discovery HR-1, TA instruments, New Castle, USA) equipped with stainless steel parallel plate geometry and a 40 mm diameter upper plate with crosshatched surfaces was used. Samples were prepared by weighing 2.5 g of each powder and vigorously mixing with the respective amount of water undertaken upon a glass plate with a metal spatula. An addition of 87.5µl of water per gram was transferred to the initial mixture to ensure reproducible sample loading and homogeneous contact surface with the upper plate. Samples were then loaded immediately upon the Peltier plate. The gap between the two plates was set at 700 µm and the temperature of the Peltier plate was established at 20^{o}C. Experiments were performed with a 2-min flow-sweep test, during which the shear rate applied escalated from 1 to 100 s^{-1}. The prototype materials were mixed

with different w/p ratios in order to obtain flowability values comparable with those of the control. Results of the values of viscosity per shear rate from 1 to 20 s^{-1} were plotted, as at higher shear rates the measurements were not always related to the samples as there was potential for separation of the upper plate from the sample. Tests were performed in triplicate for each material. An evaluation of the TCS was also performed and the values were translated to the TCS-based mixtures.

2.2.3 Flowability measurements

Flowability tests of the materials mixed either in a 0.35 or an adjusted w/p ratio established by the rheometer test were performed. The International Organization for Standardization (ISO) instructions for dental root canal sealing materials (ISO 6876:2012) for assessment of flowability were followed [277]. More specifically, immediately after mixing the prototype materials with the respective amount of water, they were transferred inside a 1 ml graduated syringe (BD Plastipak, Wokingham, UK) using a metal spatula. Consequently, 0.05 ml (± 0.005 ml) of each material was centrally delivered upon a 40 mm × 40 mm glass plate (5 mm thick). Two minutes after the start of mixing, a similar glass plate, weighing 20 g was centrally located upon the base plate, along with a 100 g weight. After a total of 10 min, the weight was removed. The minimum and maximum diameter of the material were assessed using a digital calliper with a 0.01 mm precision (Duratool, CPC Farnell, Preston, UK). If the variation of the measurements was within 1 mm, then the mean diameter was calculated; otherwise the experiment was repeated. Experiments were performed in triplicate. The TCS mixed at a w/p ratio of 0.35 served as a control.

2.2.4 Phase analyses

For phase determination, X-ray diffraction (XRD) analysis was performed. Materials mixed either with the 0.35 w/p ratio or the adjusted ratio as determined by the rheometer were compacted inside round rubber moulds (9 mm internal diameter, 1.2 ± 0.1 mm height) and immersed in HBSS. The chemical composition of HBSS was: KCl: 0.4 g/l, KH_2PO_4 anhydrous: 0.06 g/l, $NaHCO_3$: 0.35 g/l, NaCl: 8.0 g/L, Na_2HPO_4 anhydrous: 0.05 g/l, D-glucose: 1.0 g/l. Specimens were incubated for 7 days at 37^oC (Hybaid Shake n Stack, Thermo Scientific, Waltham, MA, USA). After this period, they were put in a glass desiccator. Silica gel was added at the bottom of the desiccator as a desiccant; a petri dish filled with soda lime without the lid was placed to prevent sample carbonation. Samples were vacuum desiccated (Dymax 5, Charles Austen Pumps Ltd., Byfleet, UK) for 30 min and consequently left inside the glass desiccator overnight. The next day they were crushed using an agate mortar and pestle and placed onto acrylic specimen holders (Bruker Corp., Billerica, MA, USA).

The diffractometer (Bruker D8 Advance, Bruker Corp., Billerica, MA, USA) with a CuKa radiation at 40 mA and 45 kV was set to rotate between 15-50^o with a 0.02^o 2θ step and a step time of 0.6 s. Phase identification was undertaken using a search-match software (DIFFRAC.EVA, Bruker Corp., Billerica, MA, USA) and the ICDD database (International Centre for Diffraction Data, Newtown Square, PA, USA).

2.2.5 Ion release assessment

Analysis of calcium leaching was performed using inductively coupled plasma-optical emission spectrometry (ICP-OES; Optima 8000 ICP-OES, Perkin Elmer, Waltham, MA, USA). Materials were prepared as described above, weighed (0.0001 g precision) and immersed in vials containing 5 ml HBSS at 37°C. After 7 days, pellets were retrieved from the vials and leachates were acidified to a final solution containing 2% HNO_3, by diluting them in a same volume solution of 4% HNO_3 (Fisher Scientific, Loughborough, UK). This process was performed to ensure that elements in the leachates remained stable during the assay period. Consequently, solutions were filtered through a 0.22 μm filter unit [Millipore Express PLUS (PES), Millipore Limited, Watford Hertfordshire, UK] and were analysed for calcium using HBSS acidified to 2% HNO_3 as the blank sample and 5 calibration solutions which were prepared by diluting a 10,000 mg/l Ca^{2+} calibration standard solution (Perkin Elmer, Waltham, MA, USA) to the blank. A standard curve was provided by these standards, the equation of which was used to determine the concentrations of the target ions of the test leachates. Results were expressed in respect to the materials pellets weight and the volume of the sample according to the following equation:

$$\text{Amount of element released per weight of pellet } (\mu g/g) = \frac{\text{amount of leachate per litre } (mg/l) \times \text{volume of solution } (l) \times 1{,}000}{\text{weight of pellet } (g)} \quad (2.1)$$

2.2.6 Scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDS)

Materials mixed with the adjusted w/p ratios were prepared in 15-mm rubber rings (3-mm thick), covered with a moist gauze and allowed to set for 24h at 37^{o}C in the incubator. Consequently, materials underwent vacuum desiccation for 30 min and left overnight in the glass desiccator. The following day, they were placed in cylindrical plastic moulds and embedded in resin (EpoFix, Struers Ltd., Catcliffe Rotherham, UK). On the fourth day, the resin blocks with the impregnated specimens were ground and subsequently polished with an automatic polisher (Phoenix Beta, Buehler, Coventry, UK). The grinding was accomplished with the use of progressively finer discs (MD-Piano, Struers Ltd., Catcliffe Rotherham, UK) under water irrigation, while polishing was carried out with 3 progressively finer cloths (MD-Largo, MD-Dac, MD-Nap respectively, Struers Ltd., Catcliffe Rotherham, UK) and diamond impregnated lubricants (DiaProDac 3 μm and OP-S, 0.04 μm, Struers Ltd., Catcliffe Rotherham, UK). Discs and cloths were set to rotate at approximately 300 revolutions per minute (rpm). The grinding and polishing period was approximately 2 and 4 min respectively for each disc/cloth. Consequently, specimens were cleaned from debris under warm water and were subsequently mounted on aluminium stubs and gold sputter coated (K550X Sputter Coater, Quorum Technologies Ltd., Kent, UK). Finally, they were viewed under the SEM (EVO MA10, Carl Zeiss Ltd., Cambridge, UK) in backscatter mode at a range of magnifications. EDS analysis was performed at determined points (particles) or bigger fields.

2.2.7 Radio-opacity evaluation

The prototype materials mixed with the adjusted w/p ratio were evaluated for radio-opacity values according to ISO 6876:2012 instructions [277]. Specimens were prepared in rubber discs measuring 10 ± 1 mm in diameter (3-mm thick) and allowed to set for 24 h at 37^{o}C in humidity. The next day, they were ground manually with silicon carbide discs (CarbiMet, Buehler, Coventry, UK) to a thickness of 1 ± 0.1 mm. Then, they were arranged on a photo-stimulable phosphor plate (DenOptix, Gendex Dental Systems, Hatfield, PA, USA) in proximity with a calibrated aluminium step wedge (Everything X-ray, HighWycombe, UK) with 3 mm increments. A standard X-ray machine (Progeny Dental, Midmark Corp, Kettering, OH, USA) was used to irradiate the specimens using an exposure time of 0.80 s at 10 mA, tube voltage at 65 ± 5 kV and a cathode–target film distance of 300 ± 10 mm. Consequently, radiographs were processed (Clarimat 300, Gendex Dental Systems, Hatfield, PA, USA) and a digital image for each was screened, as shown in Figure 2.1.

For interpretation of results, a method previously described by Tagger and Katz was used [278]. Briefly, an imaging programme, ImageJ (Rasband WS, ImageJ; US National Institute of Health, Bethesda, MD, USA) was utilised to calculate the grey pixel value on the radiograph of each increment in the step wedge, with the lowest value corresponding to pure black and the highest to pure white. Consequently, data for the thickness of the aluminium against the grey pixel value on the radiograph was plotted; the best-fit logarithmic trend line was then identified. With the use of the imaging programme, the grey pixel values of the specimens were obtained and loaded in the

equation, translating them in aluminium thickness in mm, which is the radio-opacity measurement unit.

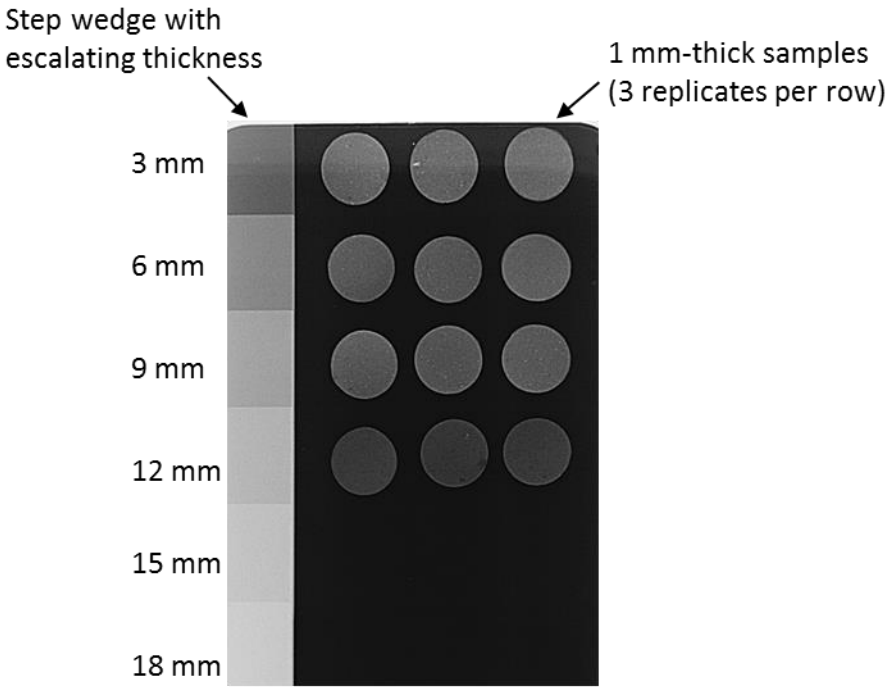


Figure 2.1 Indicative digital radiograph obtained for the radio-opacity evaluation. The step wedge was placed in the left side of the radiograph. Three replicates for each sample were used.

2.3 Assessment of chemical composition, leaching, biological and antimicrobial properties of pulp capping cements

2.3.1 Preparation of materials

Materials were compacted inside round rubber moulds (9 mm internal diameter, 1.2 ± 0.1 mm height). The commercial ones were manipulated according to

manufacturer's instructions. More specifically, the single dose powder of Bio-C Pulpo (0.33 g) was placed upon a glass plate and vigorously mixed with 3 droplets of the accompanying liquid. For Biodentine, 5 droplets of the liquid phase were transferred inside the powder-containing capsule and the material was vibrated for 30 s in an mixing device (R&S Vibracap 400 M, Dental Sky Wholesaler Ltd., Kent, UK). TotalFill RRM is a premixed paste and was therefore directly applied inside the moulds using syringe tips provided. Theracal and Activa were placed inside the moulds in two increments of less than 1 mm each; each increment was light cured for 20 s with a dental curing unit delivering 1600 mW/cm^2 blue LED (Ortholux Luminous curing light, 3M, Atherstone, UK) with a wavelength of 430–480 nm.

The prototype materials were all mixed with a metal spatula upon a glass plate with distilled water. The respective w/p ratios following the adjustment of the water demand with the rheological experiments described in Section 2.2.2 were used.

For the biocompatibility and microbiological assays described in Sections 2.3.5.2 and 2.3.6 respectively, the rubber moulds as well as the mixing and placement equipment were autoclaved. The powders of the prototype materials were sterilised using exposure to Ultraviolet (UV) irradiation at a wavelength of 254 μm for 60 min inside a UV box (BIO-LINK BLX, ALYS Labware, Lausanne, Switzerland) and were consequently mixed with sterile distilled water (SDW). All materials were prepared in the laminar flow cabinet (Guardian MSC T1200, Monmouth Scientific, Bridgwater, UK) under aseptic conditions.

Materials were immersed in vials containing 5 ml of HBSS and incubated at 37^{o}C for 1 or 28 days.

2.3.1.1 Determination of setting time

The extraction process in HBSS was scheduled to commence following initial setting time for the TCS-based materials (both commercial and prototypes) and immediately after photo-polymerisation for the light-curable ones (Theracal and Activa). Assessment of the setting time was carried out according to the American Society for Testing and Materials (ASTM) standards for setting time of hydraulic cements (ASTM C266-18) [279]. Briefly, materials were compacted inside the rubber moulds upon a glass microscope slide and incubated at 37^{o}C, 100% humidity. Specimens were covered with a wet gauze. A Gillmore-type needle with a mass of 110.4 g was carefully lowered vertically upon the surface of the material during specific time periods for each material and the formation of an impression was inspected. The time that no complete circular indentation was evident upon the horizontal surface of the material was recorded as the initial setting time. Bio-C Pulpo and Biodentine were inspected every 2 min, while TotalFill RRM and the prototype materials were inspected every 30 and 10 min respectively. This was decided using pilot data. Experiments were performed in triplicate.

2.3.2 Measuring the degree of conversion (DC) in the resin-based materials

A Fourier transform infrared (FT-IR) spectrometer (Nicolet 6700, Thermo Scientific, Waltham, MA, USA) coupled with micro-attenuated total reflectance crystal (Golden Gate-Diamond ATR, Specac, Kent, UK) was used to measure the attenuated

energy from evanescent wave as a result of infrared absorption by the sample. Real-time DC of Theracal and Activa were determined by applying a 1 mm-thick sample directly onto the ATR crystal and irradiating it from the top for 180 s using a dental curing unit. The distance of the dental curing light from the bulk of the material was standardised at 1 mm (Figure 2.2). The real-time DCs were measured during photo-polymerisation by monitoring the height of the 1640 cm^{-1} peak with respect to time with the following formula (Equation 2.2):

$$\mathrm{DC}\ (\%) = 100 \times \{1 - [\frac{(\mathrm{C{=}C_{Cured}/Aromatic_{Cured}})}{(\mathrm{C{=}C_{Unured}/Aromatic_{Uncured}})}] \quad (2.2)$$

The aforementioned peak corresponds to –CH=CH_2 (vinyl) stretching vibration. Decrease in the intensity corresponds to the conversion of monomers into a polymer. The peak that corresponds to the aromatic rings at 1608 cm^{-1} was used as an internal reference as it does not change in intensity during photo-polymerisation. Experiments were performed in triplicate.

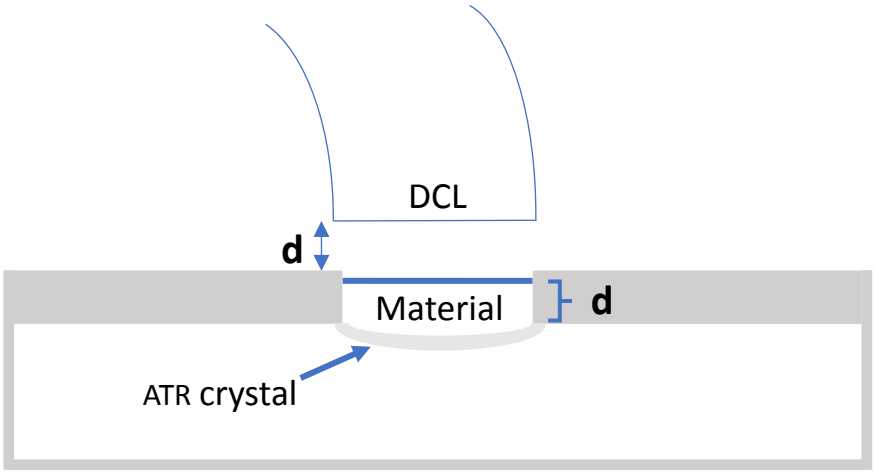


Figure 2.2 Schematic representation of the experimental FT-IR setup for assessment of the degree of polymerisation. DCL: Dental curing light, d: 1 mm.

2.3.3 Characterisation of set materials

Following a 28-day incubation period, pellets were retrieved from the HBSS solutions, vacuum desiccated and characterised using scanning electron microscopy (SEM), energy-dispersive spectroscopy (EDS) and X-ray diffraction (XRD) analysis as described below.

2.3.3.1 SEM and EDS analysis

Pellets of materials were manipulated and viewed under the SEM as described in Section 2.2.6. Briefly, the following steps were executed for sample preparation: a) overnight embedding in Epofix resin inside plastic cylindrical moulds, b) grinding and polishing of the moulds, c) mounting of the specimens on aluminium stubs and d) coating with gold. Finally, samples were viewed in backscatter mode in different magnifications, while EDS was carried out for identification of elemental composition of particles or bigger areas.

2.3.3.2 XRD analysis

Pellets were crushed to a fine powder with the use of an agate mortar and pestle. The settings which were described previously in Section 2.2.7 were used with the following modifications: analysis was carried out in a different diffractometer (Philips X'Pert 1 X-ray, Malvern Panalytical, Royston, UK) with a CoKa radiation that was set to rotate between 15-70^{o}. DIFFRAC.EVA software and ICDD database were used for phase identification.

2.3.4 Leachate analysis

2.3.4.1 Assessment of pH

Measurements of the pH of eluates were carried out 24 h after the immersion of materials and every 7 days subsequently for the 28-day period of immersion. A pH meter (Beckman Φ40 pH Meter, Beckman Coulter Inc, Brea CA, USA) with a suitable electrode (VWR International, Leicestershire, UK) was used for the assessment. Two standard solutions of pH 4 and pH 10 (Thermo Scientific, Waltham, MA, USA) served as calibration units for the analysis prior to measurements. Samples were assessed in triplicate.

2.3.4.2 Ion release analysis

One-day and 28-day leachates were assessed for concentration of calcium with ICP-OES. The same procedure as described in Section 2.2.5 was followed. Prior to assessment, leachates were acidified to a 2% HNO_3-containing final solution and filtered through a 0.22 μm filter. Five calibration solutions were used and HBSS acidified with 2% HNO_3 served as the blank sample. Calculation of results in μg/g was carried out using Equation 2.1.

2.3.5 Biocompatibility assays

The effect of 1-day, 28-day and fresh leachates on the metabolic activity of two different types of cell cultures was assessed with the 3-(4,5 dimethylthiazolyl-2-yl)-2,5-

diphenyltetrazolium bromide (MTT) assay [280]. Additionally, a method assessing the effect of eluates on cell confluency was used.

2.3.5.1 Cell cultures

ATCC CCL-92 mouse 3T3 fibroblasts deriving from a cell line [281-283] which were kept in frozen stock cultures and primary human dental pulp cells (HDPCs) harvested from healthy root canals were used. All experiments were conducted for passages 2-7 for both cell types. Leachates of materials were sterile filtered with a 0.22 µm filter unit prior to assessment.

2.3.5.1.1 ATCC CCL-92 mouse 3T3 fibroblast cell line culture

3T3 cells were cultured in a T75 culture flask (Thermo Scientific Nunc™, Waltham, MA, USA) with 15 ml Dulbecco's Modified Eagle Medium containing 4.5 g/l glucose, 1.5 g/l sodium bicarbonate (DMEM; Biosera, East Sussex, UK), supplemented with 1% penicillin/streptomycin, 2 mM glutamine (GlutaMAXTM-1; Thermo Scientific, Waltham, MA, USA) (DMEMsup) with 10% heat-inactivated foetal bovine serum (FBS; Biosera, East Sussex, UK) (DMEMsup-10FBS). Cell cultures were incubated inside a sterile culture box at 37^{o}C with 5% CO_2 supplemented atmosphere in a humidified incubator (Thermo Scientific, Waltham, MA, USA). Growth medium was changed every 2-3 days and cells were subcultured when 70-80% confluency was reached.

2.3.5.1.2 Extraction and cultivation of HDPCs

Ethical approval was obtained from the Birmingham Community Healthcare NHS Foundation Trust (REC Ref 14/EM/1128). For the extraction and culturing of HDPCs for the current experiments, two healthy intact maxillary first premolars extracted for orthodontic reasons from a male patient aged 15 were collected from the department of Oral Surgery, School of Dentistry, University of Birmingham after informed consent from the patient. A previously published protocol was followed [284] with minor modifications. The whole procedure was carried out in less than an hour after tooth extraction. Immediately after extraction, each tooth was immersed in a vial containing 5 ml Phosphate Buffer Saline (PBS). The procedure was initially performed on a benchtop in close proximity to a Bunsen Burner to ensure sterility. A series of washing and disinfecting steps were performed which included submerging the tooth in 5 ml of a 2% chlorhexidine solution (Gluco-Chex, Cerkamed, Stalowa Wola, Poland) for 2 min, then a 5 ml ethanol solution (70%) for 1 min and finally washing in 5 vials containing 5 ml of PBS for 30 s each.

The tooth was positioned in a precision cutting machine (Isomet Low speed saw, Buehler, Coventry, UK) in order to create a longitudinal cut, approximately 0.5 mm deep throughout its length using a sterile diamond disc. SDW was used as a cooling irrigant. This preparatory cut was not executed in the protocol that was followed [284]. Consequently, the tooth was wrapped in a sterile gauze and placed upon a metal tray. A chisel was inserted in the cut of the tooth and a metal hammer was used to bisect the tooth. The pulp tissue was carefully removed and transferred into a 35 mm^2 culture dish (Thermo Scientific Nunc™, Waltham, MA, USA) containing 2

ml of DMEMsup with 2.5 mg/l Amphotericin B (DMEMsup-AmphB). The culture dish was then transferred to the laminar flow hood and the pulp tissue was washed 3 times, by submerging it in different culture dishes containing 2 ml of DMEMsup-AmphB for 30 s in each. The pulp tissue was placed upon a microscope glass slide and 200 µl of DMEMsup with 20% FBS were added upon it to ensure it remained hydrated (Figure 2.3 A). A disposable scalpel (Swann-Morton Limited, Sheffield, UK) was used to cut the pulp tissue into 0.5-1 mm^2 pieces. Each section was carefully transferred and evenly placed upon the surface of a 25 cm^2 culture flask and further supplemented with 1 ml of DMEMsup-AmphB containing 20% FBS to aid attachment of the minced tissue upon the culture surface (Figure 2.3 B). The culture flask was placed inside a sterile culture box; the lid of the box was placed loosely upon the box to allow ventilation and incubated at 37^oC with 5% CO_2 supplemented atmosphere and relative humidity. The next day, the explant cultures were inspected for initial attachment and 4 ml of DMEMsup-AmphB with 20 % FBS were further added to the culture flask. The cultures were inspected daily for signs of infection with an inverted optical microscope and 10× objective lens (Primovert, Carl Zeiss Ltd., Cambridge, UK).

Approximately one week after undertaking the procedure, initial cell attachment was observed (Figure 2.3 C). Subsequently, the medium in the flasks was further supplemented with DMEMsup with 20% FBS and changed every 3 days. Once established cells were observed growing out from explanted tissues (day 14) (Figure 2.3 D), the pulp tissues were carefully transferred to a new culture flask for further culture. Cell confluency in the initial culture flasks was inspected for a period of approximately 20 days, until it reached 70-80%. Cells were then subcultured.

Consequently, the culture medium was aspirated and any minced tissue was washed away with 1 ml of PBS. One ml of a 0.25% Trypsin-EDTA solution was added to the flask and following a 5-min incubation period during which cells rounded up and lifted from the surface, 1 ml of DMEMsup-10FBS was further added in order to stop the trypsinisation. The cell inoculum was transferred to a falcon tube (Fisher Scientific, Loughborough, UK), centrifuged at 800 rpm for 4 min at room temperature (Jouan centrifuge B4i, Thermo Scientific, Waltham, MA, USA) and then re-immersed in 15 ml fresh DMEMsup-10FBS in a 75 cm^2 culture flask. Cells in this flask were marked as passage 1.

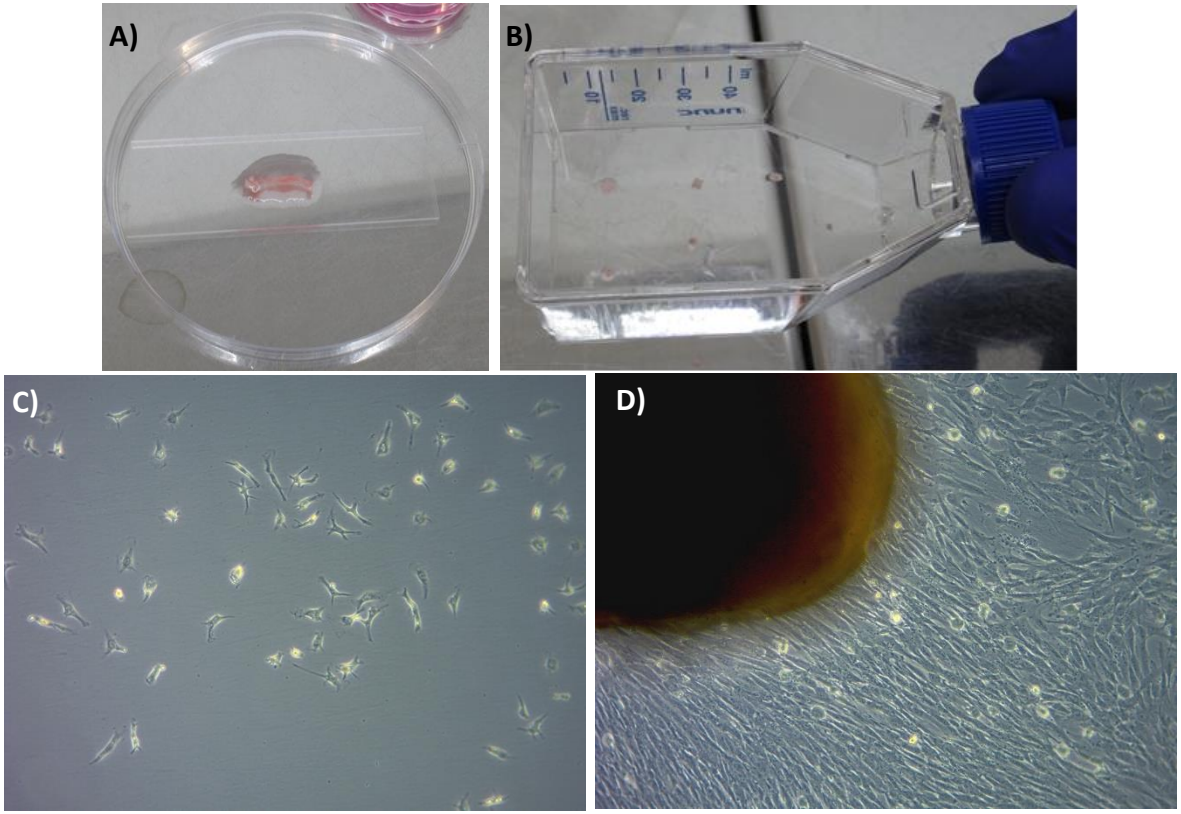


Figure 2.3 The pulp tissue was placed on a glass slide with a drop of medium (A). The minced pulp tissue was arranged throughout the surface of a T25 culture box (C). Dental pulp cells attached to the surface of the flask 1 week after initial seeding (C). Established cells inspected around a pulp tissue explant on the 14th day (D).

2.3.5.2 Assays on effect of leachates on cell proliferation

2.3.5.2.1 Cell growth curves

Prior to the commencement of the experiments, growth curves were established for both cell types, assessing the cell confluency during the experimental period. The procedure was carried out to determine the appropriate cell seeding densities for execution of the biocompatibility assays. Cells were seeded according to the ISO 10993-5:2009 specifications for the MTT assay [280]. Specifically, 10,000 cells were seeded per well of a 96-well plate (Thermo Nunc™ Scientific, Waltham, MA, USA) in 100 μl DMEMsup-10FBS; Formation of a half-confluent layer on the surface of the well after the first 24 h of incubation should be evident [280].

Cultures were incubated for a total period of 48 h at 37^{o}C, 5% CO_2 in the humidified incubator. This period corresponded to the experimental assay duration [280]. Cell confluency was assessed immediately after seeding where no adhesion had occurred and at 24 h and 48 h with a multi-mode microplate reader (Spark, Tecan Group Ltd., Männedorf, Switzerland).

For the 3T3 cell cultures, exponential growth of cells at the recommended by ISO cell seeding density was verified (Figure 2.4 A). Therefore, experiments were executed for the cell concentration specified above. On the contrary, seeding 10,000 HDPCs per well resulted in confluent cell culture after 24 h and a consequent plateau in growth (Figure 2.4 B). A range of cell densities were thus seeded per well in order to establish that HDPCs were growing exponentially throughout the assay duration and cells were half-confluent after overnight incubation. Finally, the aforementioned

criteria were met when 6,000 cells of HDPCs were seeded per 100 µl DMEMsup-10FBS in a 96-well plate (Figure 2.4 B).

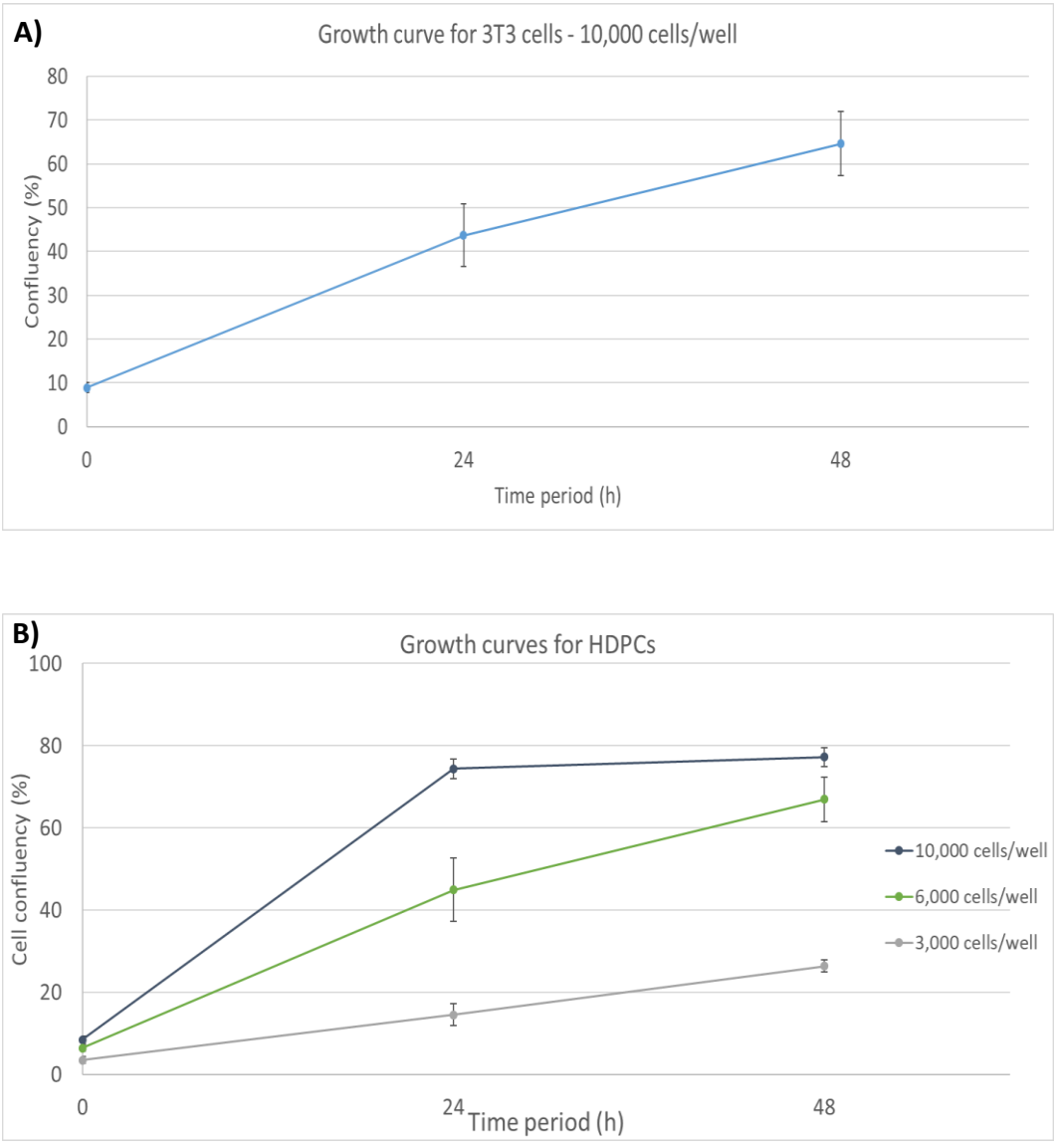


Figure 2.4 Growth curves for 3T3 cells (A) and HDPCs (B) during the 48-h period after cell seeding. The recommended cell density by ISO 10993-5:2009 met the specified criteria for 3T3 cells. For HDPCs, two additional cell densities were examined.

2.3.5.2.2 Assessment of cell metabolic activity after exposure to 1-day and 28-day leachates

The effect of eluates of materials in the mitochondrial activity of the 3T3 and HDPC cells was evaluated with the MTT assay. The ISO 10993-5:2009 instructions for indirect contact assays [280] were followed with minor modifications.

Specifically, cells were seeded in 96-well plates in the densities mentioned above; 10,000 for 3T3 and 6,000 for HDPCs in 100 µl of DMEMsup-10FBS without phenol red. These were then incubated for 24 h in appropriate conditions. The next day, 100 µl of the test eluate were added to the well. Four serial two-fold dilutions in HBSS of each leachate were also prepared and analysed, resulting in 4 additional concentrations of the initial eluate (undiluted, 1/2, 1/4, 1/8, 1/16). Pure HBSS added to the cell inoculum served as blank (control) group and DMEMsup without phenol red was the negative control. 5% NaOCl was used as a positive control. Test eluates and HBSS were also immersed in wells containing DMEMsup-10FBS without presence of cells for normalisation purposes. Plates were incubated for further 24 h.

The MTT solution was prepared by dissolving thiazolyl blue tetrazolium bromide powder in phenol red free DMEM at a concentration of 0.5 mg/ml. The solution was sterile filtered with a 0.22 µm filter as previously described.

The following day, the medium was aspirated from the wells and 100 µl of the MTT solution was added. Samples were incubated for a further 4 h. The MTT solution was then removed from the wells and 100 µl of dimethyl sulfoxide (DMSO; Fisher Scientific, Loughborough, UK) were added in order to dissolve any existing formazan crystals produced by the metabolic conversion of the MTT by the cells. Optical density

(OD) of the samples was read in a microplate reader (ELx800, BioTek Instruments, Swindon, UK) at 570 nm. Results were expressed as percentages in regards to the activity of cells in the blank group; background noise of the OD readings was normalised by subtracting the values of the groups of test eluates incubated with the DMEMsup-10FBS. Experiments were performed in triplicate with 3 parallel assays for each group.

2.3.5.2.3 Development of a transwell system to assess cell metabolic activity

In order to investigate the cytocompatibility of materials during the extraction procedure and immediately after material manipulation and placement, a transwell system was used. The model (Milicell-96, Merck KGaA, Darmstadt, Germany) consisted of a 96-well receiver plate, where the test material was placed and of a 96-well cell culture plate with a porous membrane (PCF membrane, 0.4 μm pore size), which was adjustable to the receiver plate (Figure 2.5).

Cells were seeded at the densities specified above (10,000 and 6,000 per 100 μl of medium for 3T3 cells and HDPCs respectively) upon the wells of the 96-well cell culture plate and incubated for 24 h at 37^{o}C, 5% CO_2, relative humidity. The following day, materials were prepared as described in Section 2.3.1 and placed upon the bottom of the 96-well receiver plate; 100 μl of HBSS were immediately transferred in each well. An equal amount of HBSS was also transferred to empty wells and served as the blank control group.

Consequently, the cell-culture plate was placed upon the receiver plate; each insert was therefore immersed in a solution which consisted of the 1-day's growth medium and the HBSS. Plates were incubated for 24 h; for assessment of the mitochondrial activity of cells, the cell-culture plate was removed from the receiver-plate and any excess of medium was aspirated. The MTT assay was then performed and analysed as is described in Section 2.3.5.2.2; the OD of the dissolved formazan crystals was assessed at 570 nm and expressed as a percentage of the blank control group.

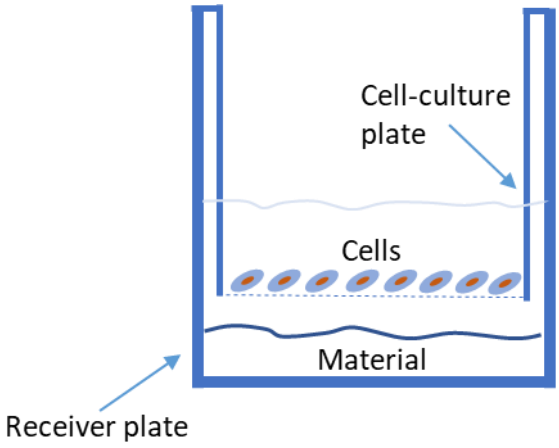


Figure 2.5 Schematic presentation of the transwell system. Cells were seeded onto the porous membrane of the cell-culture plate. The test material was placed into the bottom of the well of the receiver plate.

2.3.5.2.4 Effect of leachates on cell confluency and corresponding cell numbers

A model to investigate the effect of leachates on cell proliferation and subsequently on cell numbers was designed. Initially, an equation correlating the cell confluency values obtained by the multi-mode micro-plate reader to cell numbers was calculated. More specifically, cells were initially seeded in two different densities for both cell types; 3,000 and 6,000 cells per 100 µl DMEMsup-10FBS were incubated for either 24 or 48 h at 37^{o}C 5% CO_2, relative humidity. Cell confluency was assessed after the specified periods using the multi-mode microplate reader. Consequently, samples that exhibited the exact same confluency values (sample number ≥3) were selected to count their cell numbers. The medium was aspirated and 20 µl of a 0.25% Trypsin-EDTA solution was added. Following a 5-min incubation period, 20 µl of a 0.4% Trypan Blue Solution were transferred to each well and mixed thoroughly.

Cell counts were assessed using a haemocytometer. A glass coverslip which was previously moistened was placed upon the counting grid of the haemocytometer. Consequently, 10 µl of the cell suspension were transferred onto the counting grid. The number of cells on the designated area was counted using a 10× objective lens upon the inverted optical microscope. Results of cell confluency per cell numbers were then plotted in graphs. An equation of the corresponding cell numbers to the respective confluency was provided for each cell type (Figure 2.6).

Following the calculation of the standard curves, experiments were performed using a similar model as described in Section 2.3.5.2.2 for the MTT assay. Cell seeding was carried out at the same densities used for the MTT experiments and test leachates

(1- and 28-day) were immersed in the wells following the 24-h cell growth period. After an exposure period to leachates for 24 h, cell confluency of the wells was assessed with the multi-mode micro-plate reader with the rationale to convert the confluency values to number of cells with the equations obtained from the standard curve.

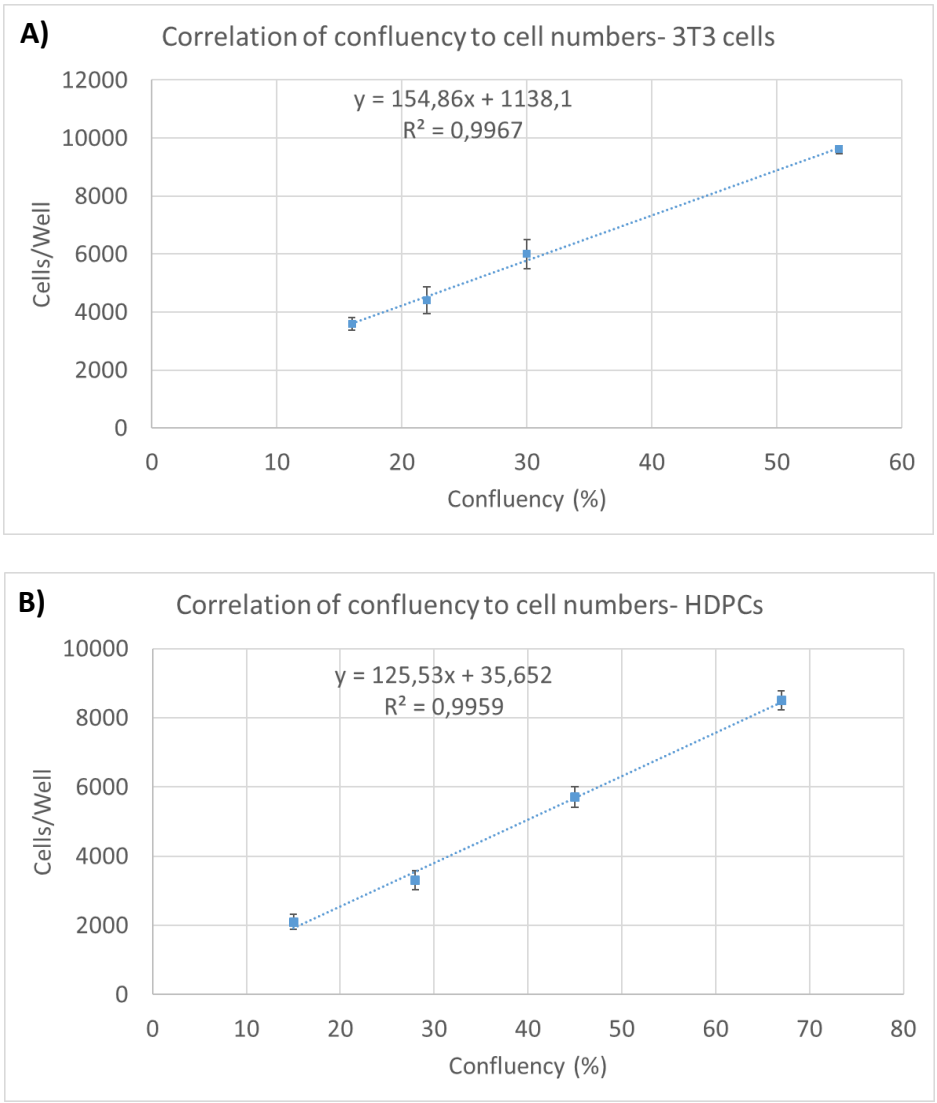


Figure 2.6 Standard curves correlating the cell confluency to numbers of cells for 3T3 cells (A) and HDPCs (B). Cell confluency was assessed with a multi-mode microplate reader and cell counting was carried out with an optical microscope following trypsinisation, staining with trypan blue and suspending in a haemocytometer. R^2= Coefficient of determination.

2.3.6 Microbiological assays

2.3.6.1 Minimum inhibitory concentration (MIC) assay

The potential of leachates to inhibit bacterial growth was assessed using two caries associated bacterial species, *Streptococcus mutans* Strain N3209 and *Lactobacillus casei* Natural Collection of Type Cultures (NCTC) 16341 [285, 286]. All procedures were carried out in the laminar flow hood.

S. mutans was grown on Tryptic Soya Agar (TSA) plate from frozen stock cultures stored at -80^{o}C overnight at 37^{o}C, 5% CO_2, supplemented atmosphere (Thermo Scientific, Waltham, MA, USA); subsequently, two colonies from the TSA plate were inoculated in 10 ml Tryptonic Soya Broth (TSB) and incubated in a shaking incubator (NB-205, N-Biotek, Inc, Bucheon, South Korea) at 100 rpm, 37^{o}C for further 18 h. *L. casei* was streaked from a glass ampoule (Public Health England, Salisbury, UK) onto Man, Rogosa & Sharpe (MRS) agar plates and grown overnight at 37^{o}C; two-three colonies were transferred in 10 ml MRS broth the following day for further overnight incubation in a shaking incubator at 100 rpm, 37^{o}C.

For subsequent studies, the overnight grown bacterial cultures were initially centrifuged at 4,000 g for 5 min at room temperature (IEC Centra CL2) and the pellets were then resuspended in fresh broth (TSB and MRS broth for *S. mutans* and *L. casei* respectively). The OD of the bacterial cultures was standardised to 1.0 at 600 nm using a spectrophotometer (Jenway 7315, Cole-Parmer, Staffordshire, UK) and then further diluted in fresh medium in a 1:4 ratio to ensure exponential growth of the bacterial species throughout the experimental period.

Experiments were performed in 96-well plates. One hundred fifty microliters of the final bacterial inoculum were added to each well and an equal amount of the test leachate was then transferred. The test leachates were analysed either undiluted or following 4 two-fold serial dilutions in HBSS (1/2, 1/4, 1/8, 1/16). The extract medium (HBSS) served as a positive control in the experiments; independent negative controls were carried out for each group, containing sterile broth and the respective test leachate or HBSS. Culture plates were incubated overnight for 18 h at 37^{o}C, 5% CO_2, supplemented atmosphere; subsequently, the OD of each well was determined using a microplate reader (ELx800, BioTek Instruments, Swindon, UK) at 600 nm. Experiments were performed in triplicate and in three independent wells for each group. Results were normalised by subtracting the values of the negative control from each group, in order to eliminate false positive results from increased OD values obtained due to potential sedimentation effect from the leachates.

2.4 Statistical analyses

All results were processed statistically with the use of the Statistical Package for the Social Sciences software version 24 (IBM SPSS Statistics, IBM Corp, Armonk, NY, USA). Prior to each statistical analysis, data were assessed for normality with the Shapiro–Wilk test. Microsoft Excel (Microsoft Corporation, Redmond, WA, USA) was used for data handling and the expression of results in graphical formats. Mean values and standard deviations were expressed. The significance level of p=0.05 was used for all analyses.

2.4.1 Studies on the effect of w/p ratio on hydraulic cement properties

For the rheological assessment, Dunnett's test using the PC_0.35 as a control was conducted for viscosity values with similar shear rate applied (1 decimal precision) between 1-20 s^{-1}. The mean values of viscosity of each material were also compared. Independent sample t-tests between materials of the same components and with different w/p ratios were conducted; for the flowability tests and calcium leaching, Dunnett's test using the TCS_0.35 as a control was conducted additionally. For the radio-opacity assessment, one-way analysis of variance (ANOVA) and Tukey's multiple comparison tests were performed.

2.4.2 Studies on chemical, biological and antimicrobial properties of eluates

All results were processed with one-way ANOVA and Tukey post-hoc tests for multiple comparisons. Additionally, independent sample t-tests were performed to compare the effect of same leachates between different cell types in the biocompatibility assays. Finally, Pearson correlation coefficient (r) was calculated to evaluate the relationship between the mean values of calcium ion release and the respective biological and antimicrobial performance of the eluates.

CHAPTER 3

RESULTS

Data presented in Section 3.1 have been published in Dental Materials (Koutroulis A, Batchelor H, Kuehne SA, Cooper PR, Camilleri J).

The author has carried out the analysis and presentation of the results.

3.1 Determination of the water demand and its effect on properties of hydraulic cements

3.1.1 Rheological adjustment

The w/p ratios of the different formulations in comparison to the one established for Portland cement mixed at a w/p ratio of 0.35 are shown in Table 3.1. Both calcium phosphate and the micro-silica increased the water demand of the mixture. The replacement of the different radiopacifiers did not have the same effect. The zirconium oxide did not affect the w/p ratio required while the tantalum oxide and calcium tungstate increased and decreased the w/p ratios respectively. Figure 3.1 shows the viscosity values per shear rate for each material after the adjustment of the water volume added. It was also shown that replacement of each cement by inclusion of 30% radiopacifier resulted in a standard change in the water amount needed to hydrate the material, regardless of the composition of the cement, except for the zirconium oxide which had no effect in the w/p ratio. These data are shown in Table 3.2 where the percentage increase or decrease in the water demand is provided.

Table 3.1 Adjusted w/p ratio for Portland cement (PC) with different compounds or radiopacifiers as calculated by the rheological assessment. The numerical scale has been set in two decimal digits.

PC	*No radiopacifier*	*30% radiopacifier*			
		ZO	**TO**	**ZO-TO**	**CW**
No addition	0.35 *(control)*	0.35	0.42	0.37	0.26
15% CP	0.50	0.50	0.60	0.53	0.37
10% mS	0.40	0.40	0.48	0.42	0.30
20% mS	0.50	0.50	0.60	0.53	0.37

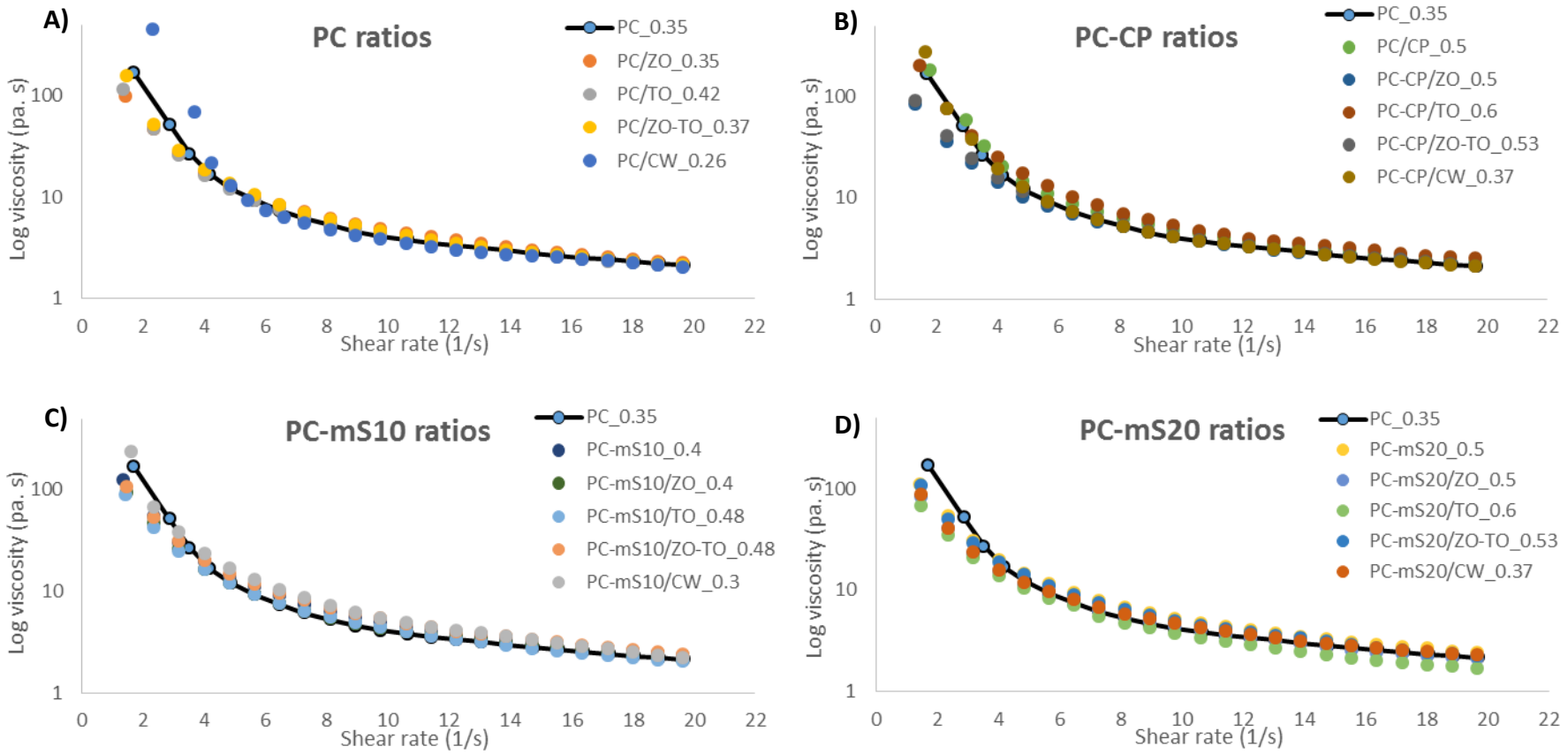


Figure 3.1 Mean apparent viscosity (log scale) per shear rate of hydraulic cements following the adjustment of w/p ratio in comparison with the performance of the control (PC_0.35).

Table 3.2 Standard percentage change in the water demand of hydraulic cements after 30% replacement of radiopacifier.

30% radiopacifier	***Modification in the w/p ratio***
ZO	Same
TO	20% ↑
ZO-TO	5.71% ↑
CW	25.71% ↓

3.1.2 Flowability tests

Table 3.3 shows results and comparisons between flowability of materials with the same components but different w/p ratios, as well as comparisons with the TCS mixed with the 0.35 w/p ratio. The inclusion of calcium phosphate increased significantly the flowability of the materials in all the material combinations tested following the w/p ratio adjustment, regardless of the radiopacifier used ($p<0.05$). Calcium phosphate incorporation increased the water demand substantially as indicated in the rheology testing. Consequently, flowability was reduced for the CP-containing materials mixed with the 0.35 w/p ratio in comparison with the pure TCS

($p<0.05$), except for the TCS-CP/CW. The incorporation of calcium tungstate resulted in a relatively low water demand as indicated in the rheology testing (Table 3.2). Thus it compensated for the high water demand of the calcium phosphate in the mixture.

The micro-silica altered the flowability characteristics of the materials when the adjusted w/p ratio was applied ($p<0.05$), except for the TCS-mS10/ZO-TO and TCS-mS20/CW, where no significant change was reported ($p>0.05$).

The different radiopacifiers incorporated in the pure tricalcium silicate did not affect the flowability values of cements in comparison with the non-radiopacified control (TCS_0.35) at both w/p ratios tested ($p>0.05$). The incorporation of zirconium oxide and calcium tungstate did not change the flowability characteristics following the adjustment of the water amount used to mix them, however tantalum oxide incorporation increased the material flowability at the adjusted w/p ratio indicated comparing with the standard 0.35 ratio. The tantalum oxide replacement increased the water demand substantially as shown in Table 3.2 for the rheology study.

Table 3.3 Mean flow values and standard deviation of tested prototype materials mixed with different ratios according to ISO 6876:2012. A = adjusted. The latin letter '*a*' indicates significant difference in flowability values of materials with the same components after mixing with different water amounts ($p<0.05$); the asterisk indicates significant difference in flowability values comparing to the TCS mixed at a 0.35 w/p ratio (TCS_0.35) ($p<0.05$).

Material	***Ratio***	*30% radiopacifier*			
		ZO	**TO**	**ZO-TO**	**CW**
TCS	0.35	9.22 ± 0.1	8.63 ± 0.5	9.99 ± 0.9	10.8 ± 1.1
	A	-	10.91 ± 0.3^{a}	10.09 ± 0.6	9.38 ± 1
TCS-CP	0.35	8.14 ± 0.04*	6.84 ± 0.3*	7.06 ± 0.5*	8.34 ± 0.4
	A	10.45 ± 0.3^{a}	10.3 ± 0.5^{a}	10.12 ± 0.2^{a}	9.17 ± 0.2^{a}
TCS-mS10	0.35	8.65 ± 0.3	8.34 ± 0.5	8.35 ± 0.4	10.75 ± 0.2^{a}
	A	$11.36 \pm 0.3^{*a}$	10.72 ± 1.4^{a}	9.31 ± 0.5	9.37 ± 0.6
TCS-mS20	0.35	8.41 ± 0.2	8.42 ± 0.3	8.47 ± 0.4	10.57 ± 0.3
	A	10.57 ± 0.5^{a}	$11.11 \pm 0.4^{*a}$	$13.97 \pm 0.3^{*a}$	10.34 ± 0.4
TCS_0.35 (*control*)		9.65 ± 0.6			

3.1.3 X-ray diffraction analysis

Results from the XRD analysis are presented in Figures 3.2-3.5. All materials exhibited peaks for calcium hydroxide (ICDD: 01-078-0315). The tricalcium silicate had completely reacted after 7 days and the only peaks visible in the trace were those of the calcium hydroxide at 18 and 34° 2θ. The micro-silica was amorphous and no peaks were detected in the respective materials (Figures 3.4, 3.5). The radiopacified materials exhibited peaks for zirconium oxide (ICDD: 00-037-1484) (Figures 3.2-3.5 A, C) tantalum oxide (ICDD: 01-081-8067) (Figures 3.2-3.5 B, C) and calcium tungstate (ICDD: 04-007-2671) (Figures 3.2-3.5 D) depending on the respective radiopacifiers used.

The use of tantalum oxide as radiopacifier enhanced the formation of crystalline calcium hydroxide in the materials with adjusted w/p ratios (Figure 3.2 B). This increase was also evident when calcium phosphate was included (Figure 3.3 B) but not in the 10% micro-silica addition (Figure 3.4 B). When 20% micro-silica was added the formation of crystalline calcium hydroxide was enhanced following the water adjustment with the mixtures of zirconium oxide and the calcium tungstate radiopacifiers (Figure 3.5 A, C, D).

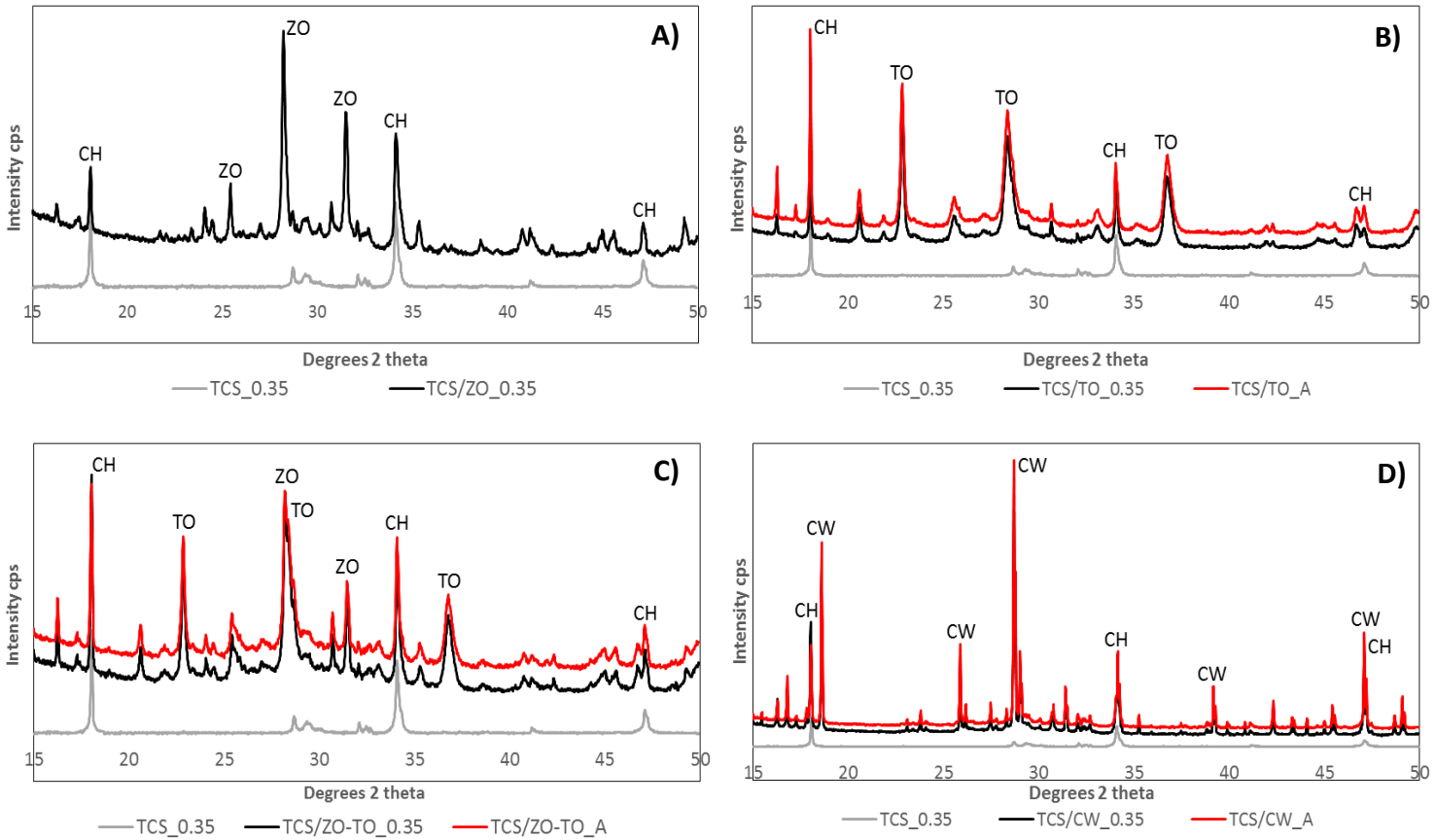


Figure 3.2 X-ray diffraction plots of tricalcium silicate cement and test prototype materials replaced with zirconium oxide, tantalum oxide, a mixture of zirconium oxide and tantalum oxide and calcium tungstate mixed in a 0.35 w/p ratio (_0.35) or an adjusted ratio (_A) after immersion in HBSS for 1 week. CH: Calcium hydroxide.

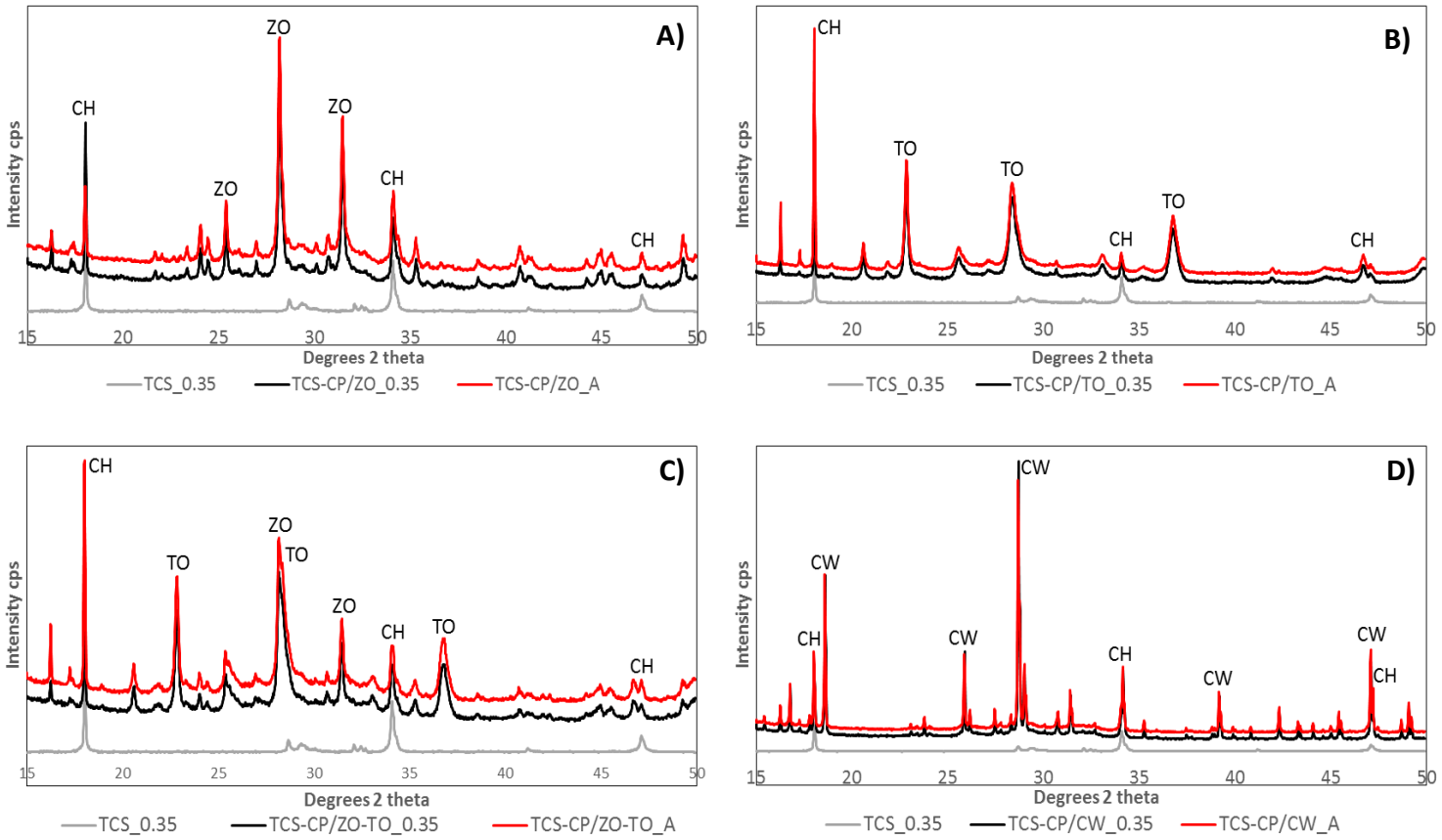


Figure 3.3 X-ray diffraction plots of tricalcium silicate cement and test prototype materials incorporating calcium phosphate (15%) and radiopacifiers mixed in a 0.35 w/p ratio (_0.35) or an adjusted ratio (_A) after immersion in HBSS for 1 week.

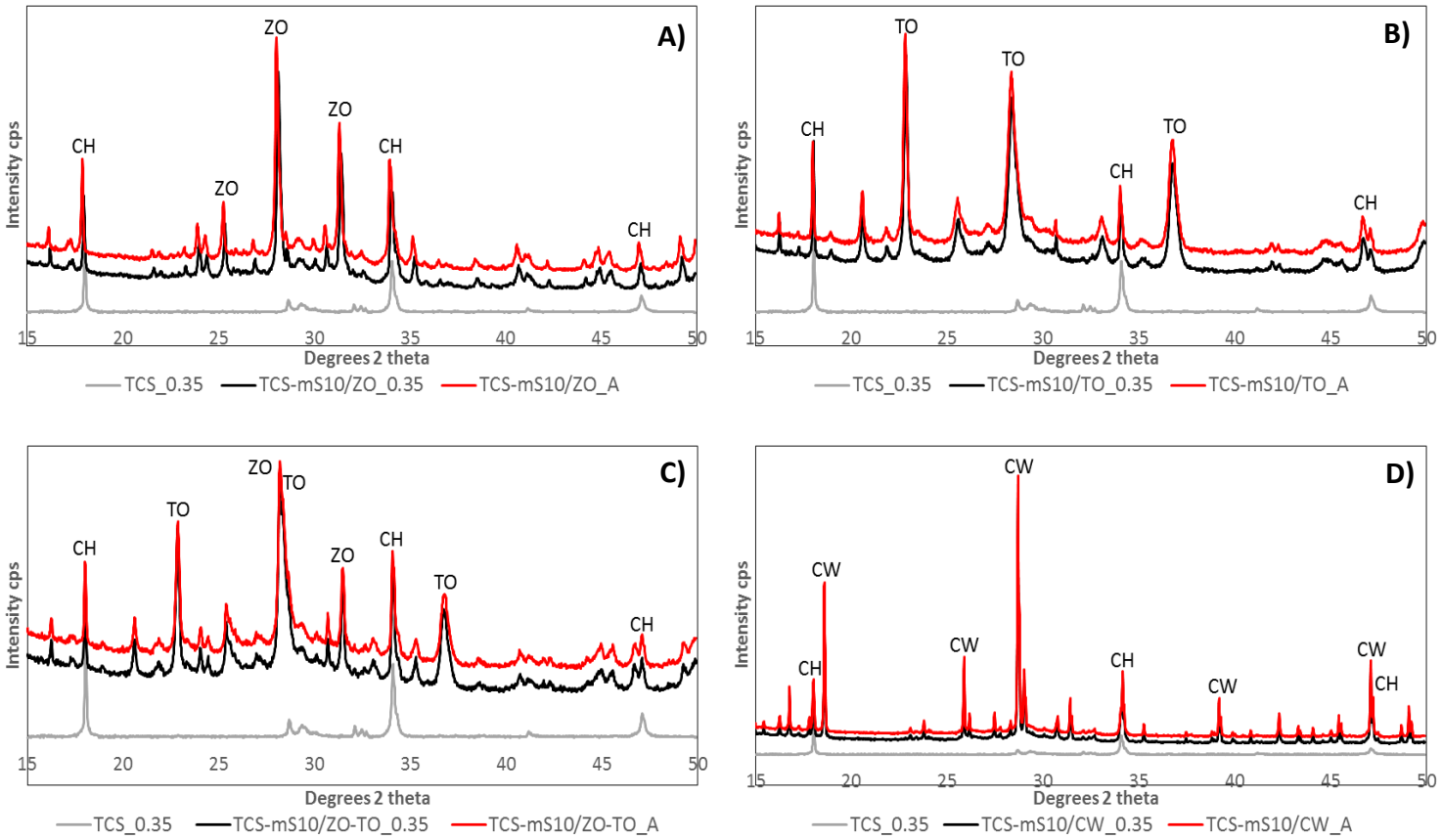


Figure 3.4 X-ray diffraction plots of tricalcium silicate cement and test prototype materials following replacement of micro-silica (10%) and different radiopacifiers mixed in a 0.35 w/p ratio (_0.35) or an adjusted ratio (_A) after a 1-week immersion period in HBSS.

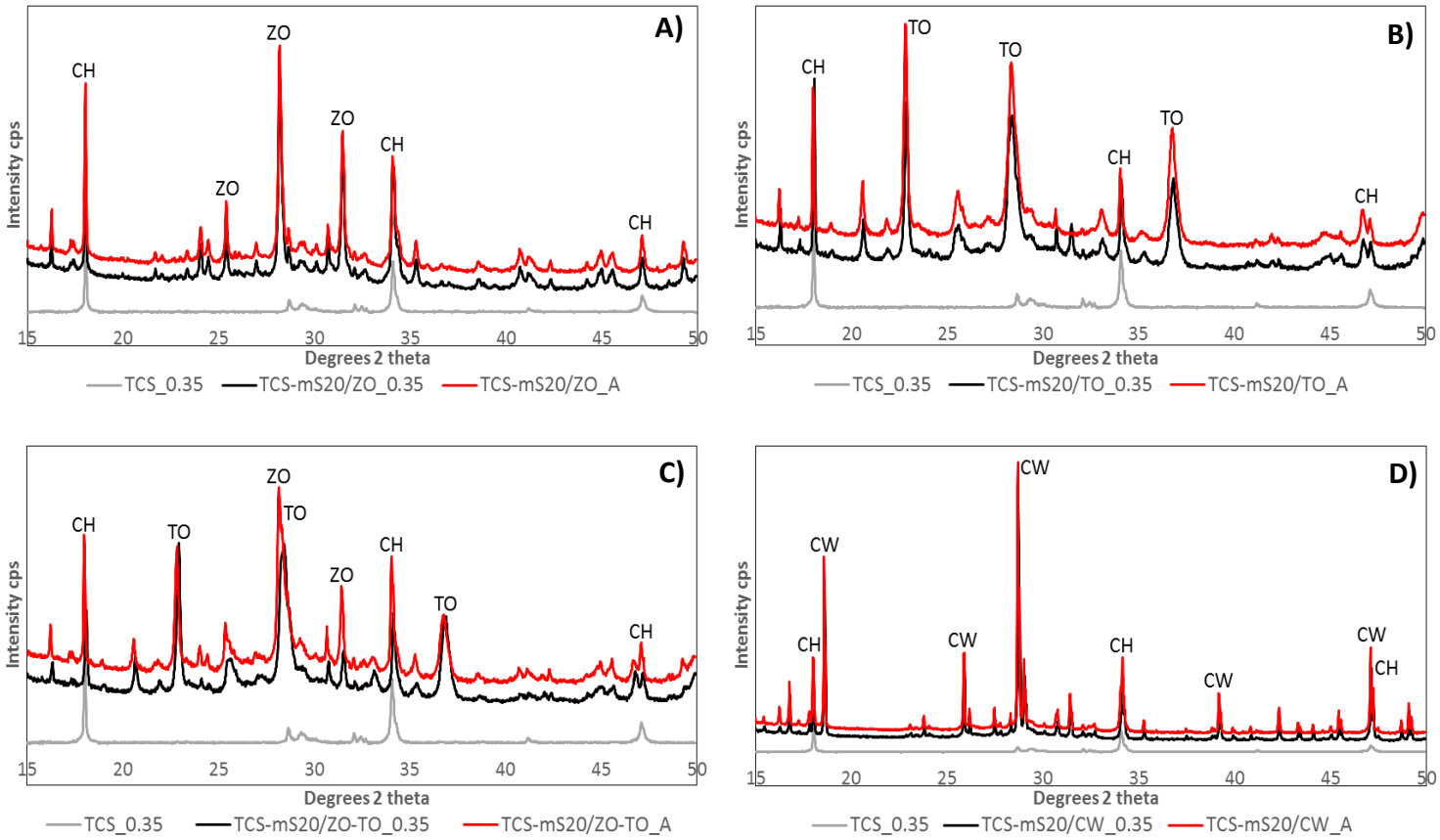


Figure 3.5 X-ray diffraction plots of tricalcium silicate cement and test prototype materials with micro-silica replacement (20%) and different radiopacifiers mixed in a 0.35 w/p ratio (_0.35) or an adjusted ratio (_A) following immersion in HBSS for 1 week.

3.1.4 Calcium release assessment

The calcium ion leaching data is presented in Figure 3.6. All the materials tested leached relatively high levels of calcium ions in solution after 7 days of immersion in HBSS. The reduction in the water added to the calcium tungstate radiopacified material resulted in a reduction in calcium ion leaching. Increased calcium ion leaching was shown for the incorporation of calcium phosphate with both zirconium oxide and the combination of zirconium oxide and tantalum oxide. The micro-silica replacement did not affect the calcium ion release significantly except for the 20% replacement with the tantalum oxide radiopacifier where a relatively higher calcium ion leaching pattern was observed in the adjusted w/p ratio.

Compared with the cement containing no radiopacifier, the zirconium oxide radiopacified TCS and the materials with inclusion of micro-silica and tantalum oxide mixed with the 0.35 ratio, released less calcium ($p<0.05$). Only the material with 20% micro-silica incorporation and tantalum oxide presented significantly higher calcium release, following the adjustment of the w/p proportion.

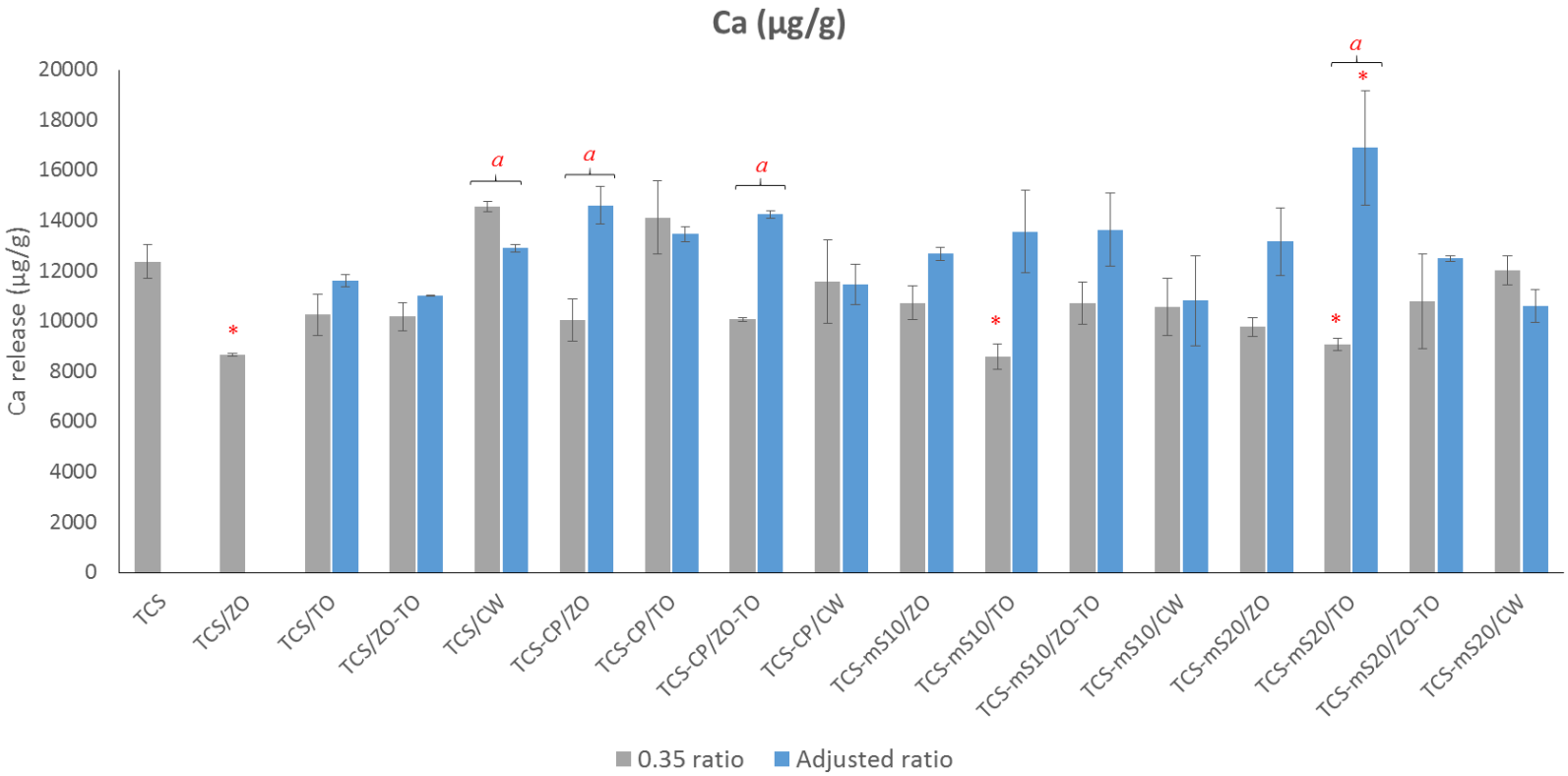


Figure 3.6 Mean and standard deviation of calcium release of prototype materials mixed in a 0.35 w/p ratio or an adjusted ratio after immersion in HBSS for 1 week. Asterisk indicates significant difference with the TCS_0.35 ($p<0.05$); the latin letter '*a*' represents statistical difference between materials with the same components and different w/p ratios ($p<0.05$).

3.1.5 SEM-EDS characterisation

Scanning electron micrographs in backscatter mode and EDS analysis of specific microstructures are presented in Figures 3.7-3.10.

The prototype cements were all based on tricalcium silicate with different radiopacifiers replacing them by 30%. Calcium phosphate monobasic and micro-silica were also used as replacement materials. The cement particles in the pure TCS prototype cement exhibited a halo of hydration product surrounding unhydrated cement particles of a similar size in a dense matrix. Some of the cement particles appeared fully hydrated and the purity of tricalcium silicate was verified by the EDS analysis (Figure 3.7).

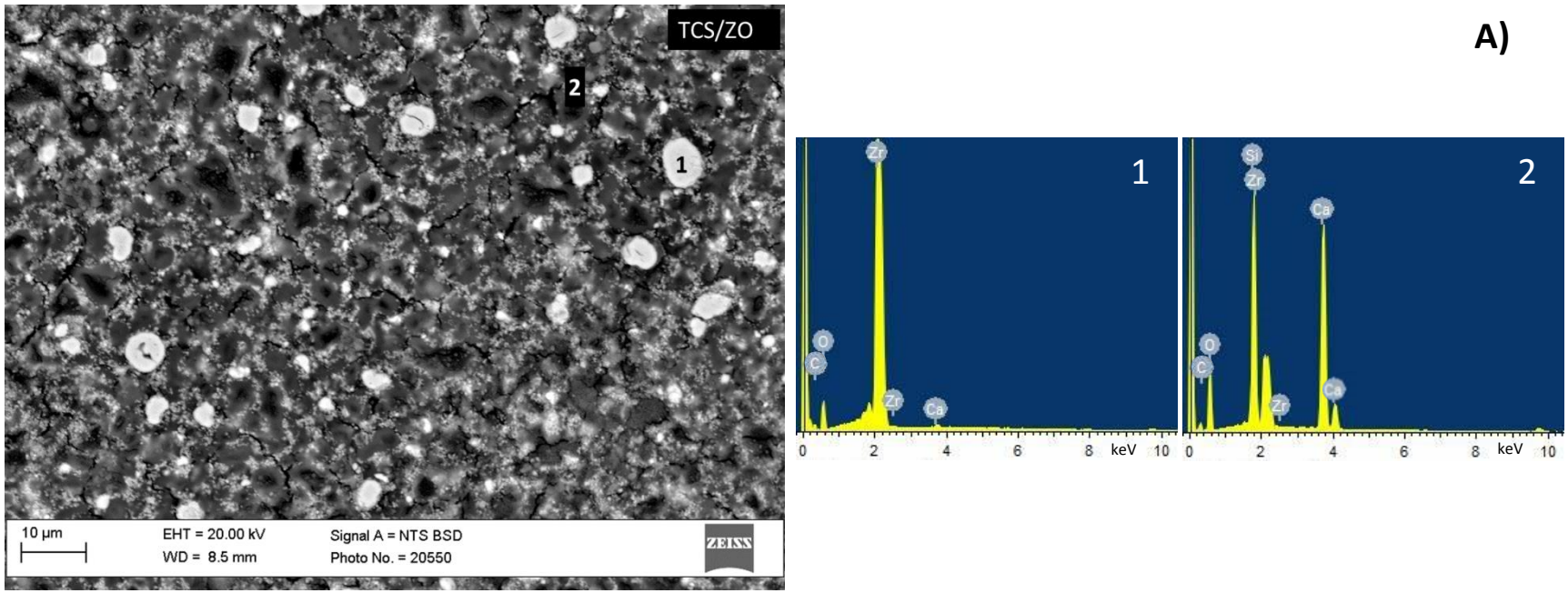


Figure 3.7 Back-scatter scanning electron micrographs of polished sections of tricalcium silicate cement with different radiopacifiers (2500× magnification) showing microstructural components and energy-dispersive spectroscopic scans of selected spectrums (A-D).

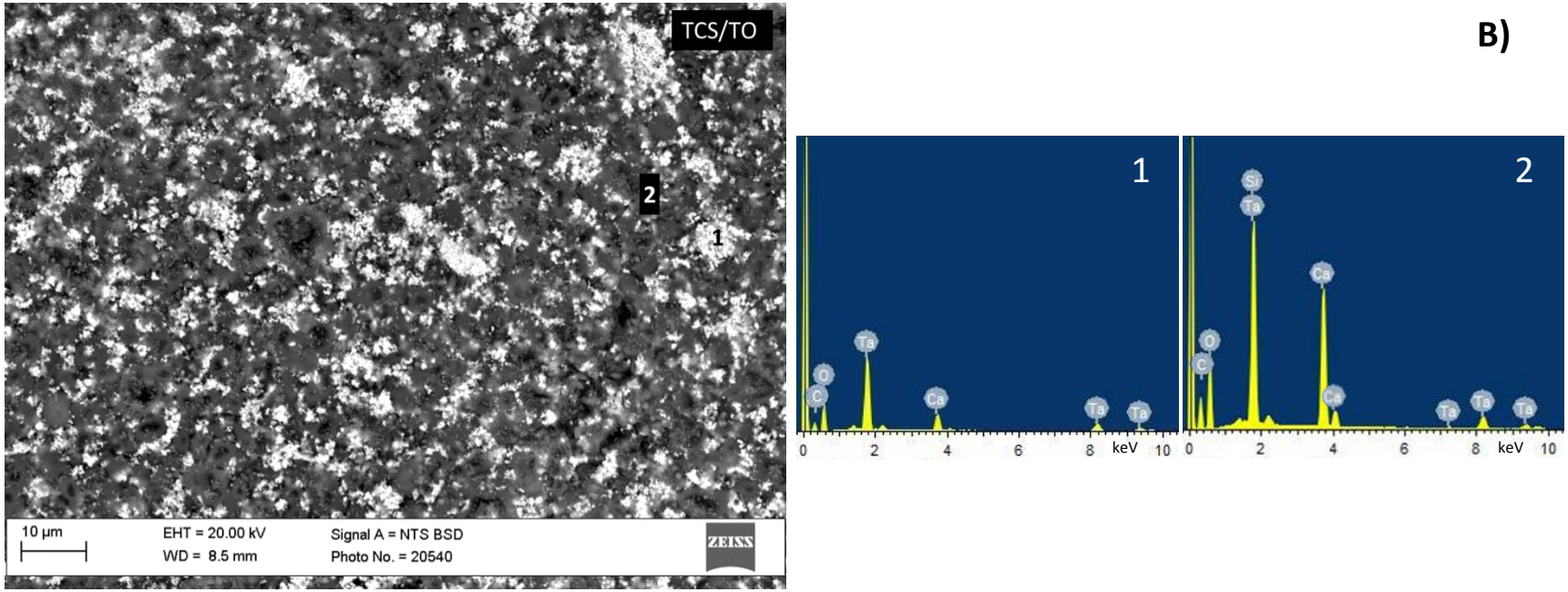


Figure 3.7 (Continued)

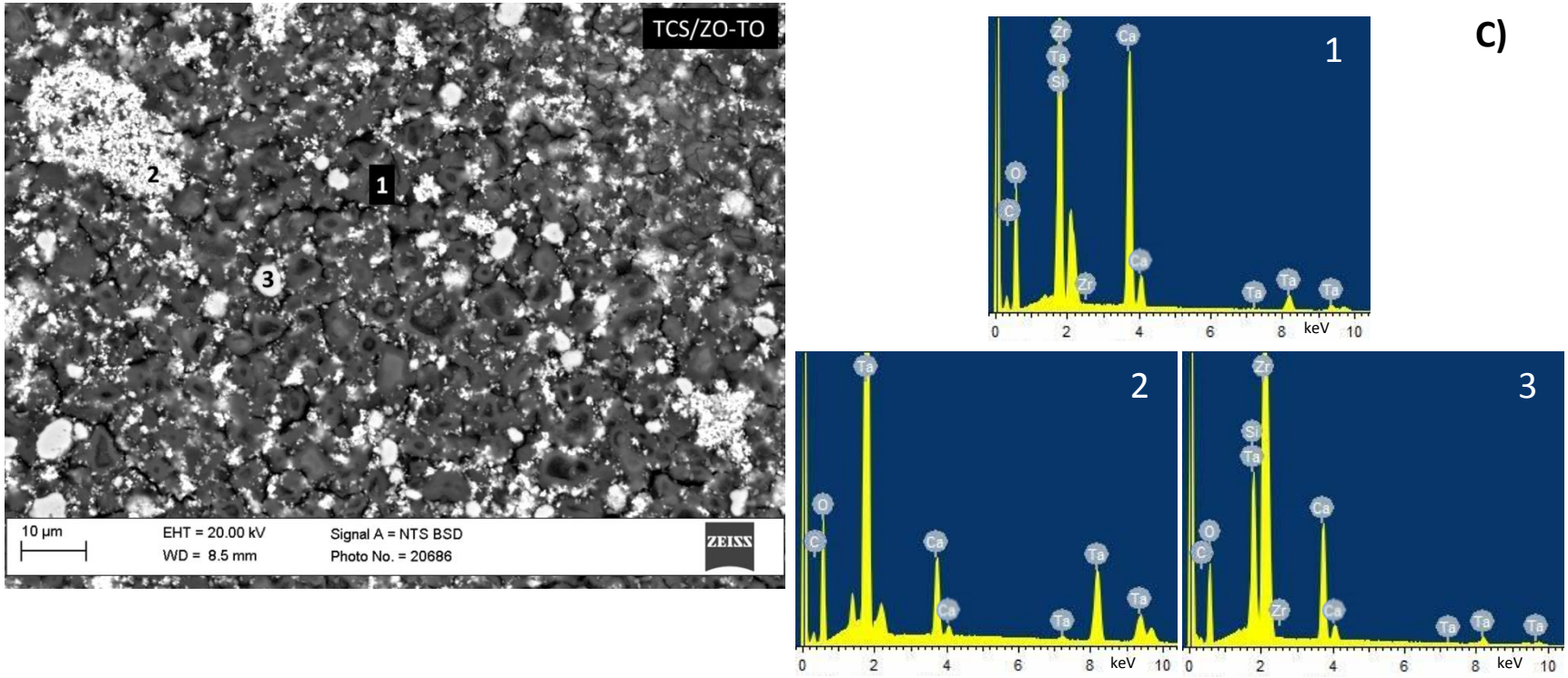


Figure 3.7 (Continued)

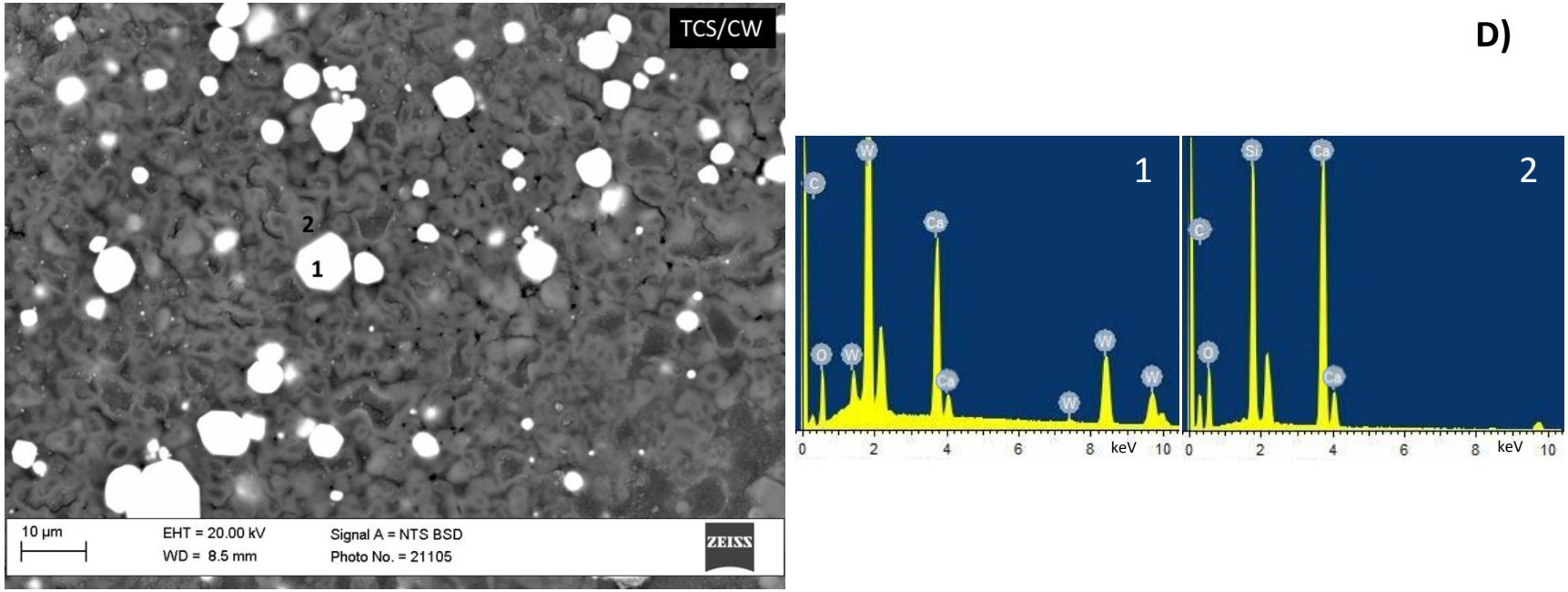


Figure 3.7 (Continued)

The incorporation of the calcium phosphate monobasic changed the hydration kinetics of the tricalcium silicate cement. The calcium phosphate cement particles of the TCS-CP-based materials consisting of calcium, phosphorus and silicon were evident in the micrographs by large halo rims surrounding the unhydrated particles (Figure 3.8).

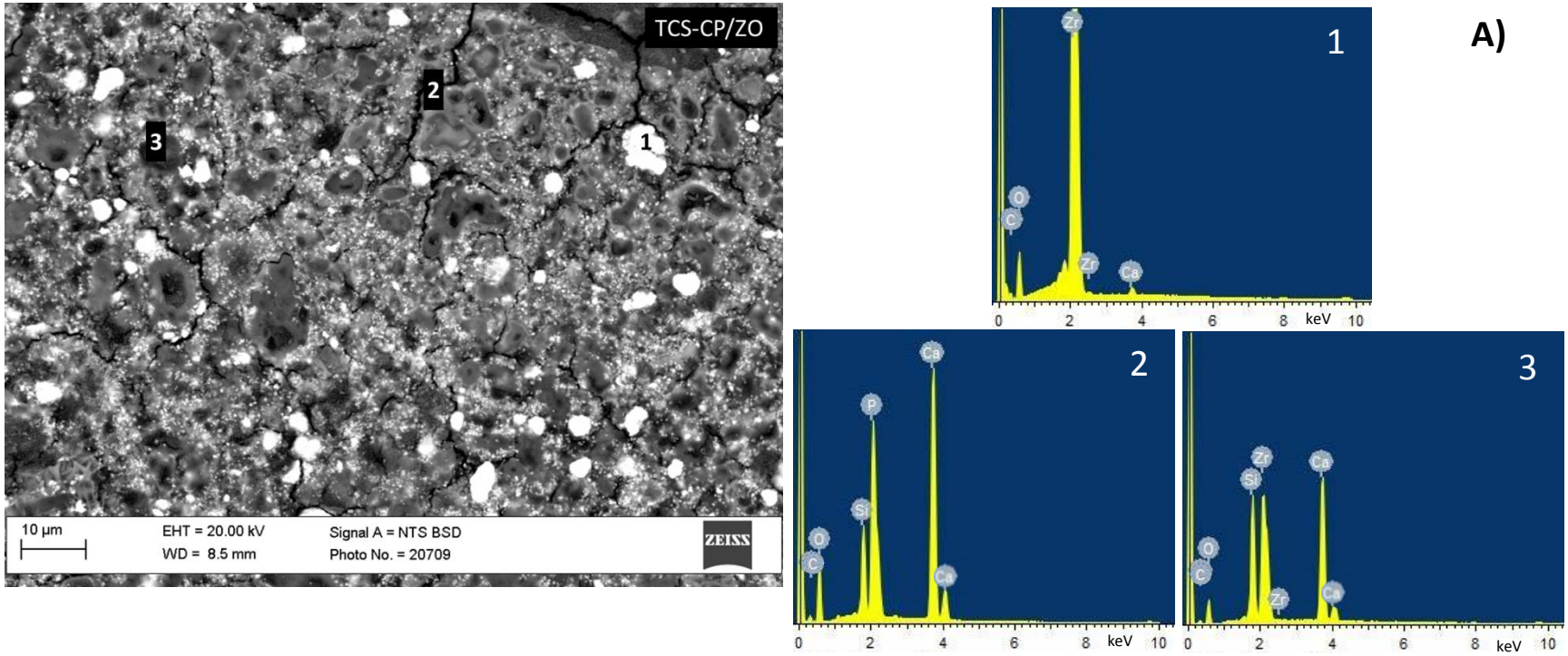


Figure 3.8 Back-scatter scanning electron micrographs of polished sections of tricalcium silicate cement replaced with calcium phosphate (15%) and radiopacifiers (2500× magnification) showing microstructural components and energy-dispersive spectroscopic scans of selected areas (A-D).

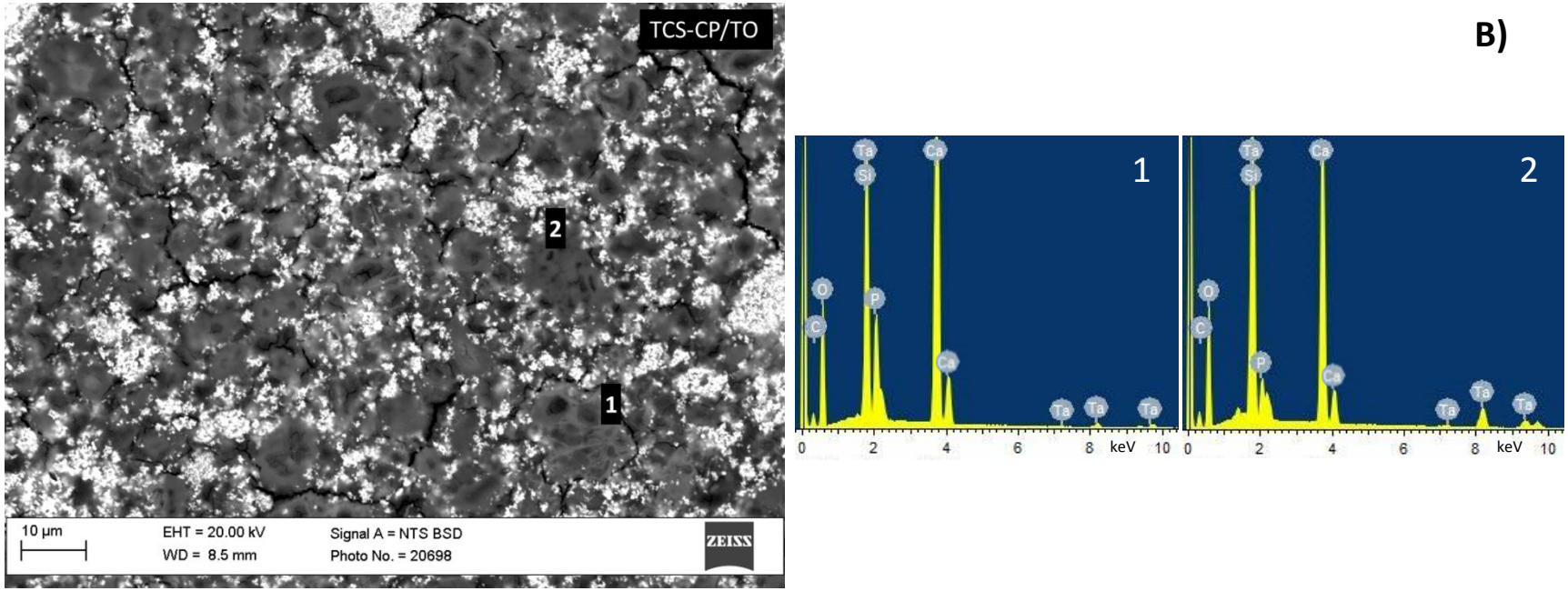


Figure 3.8 (Continued)

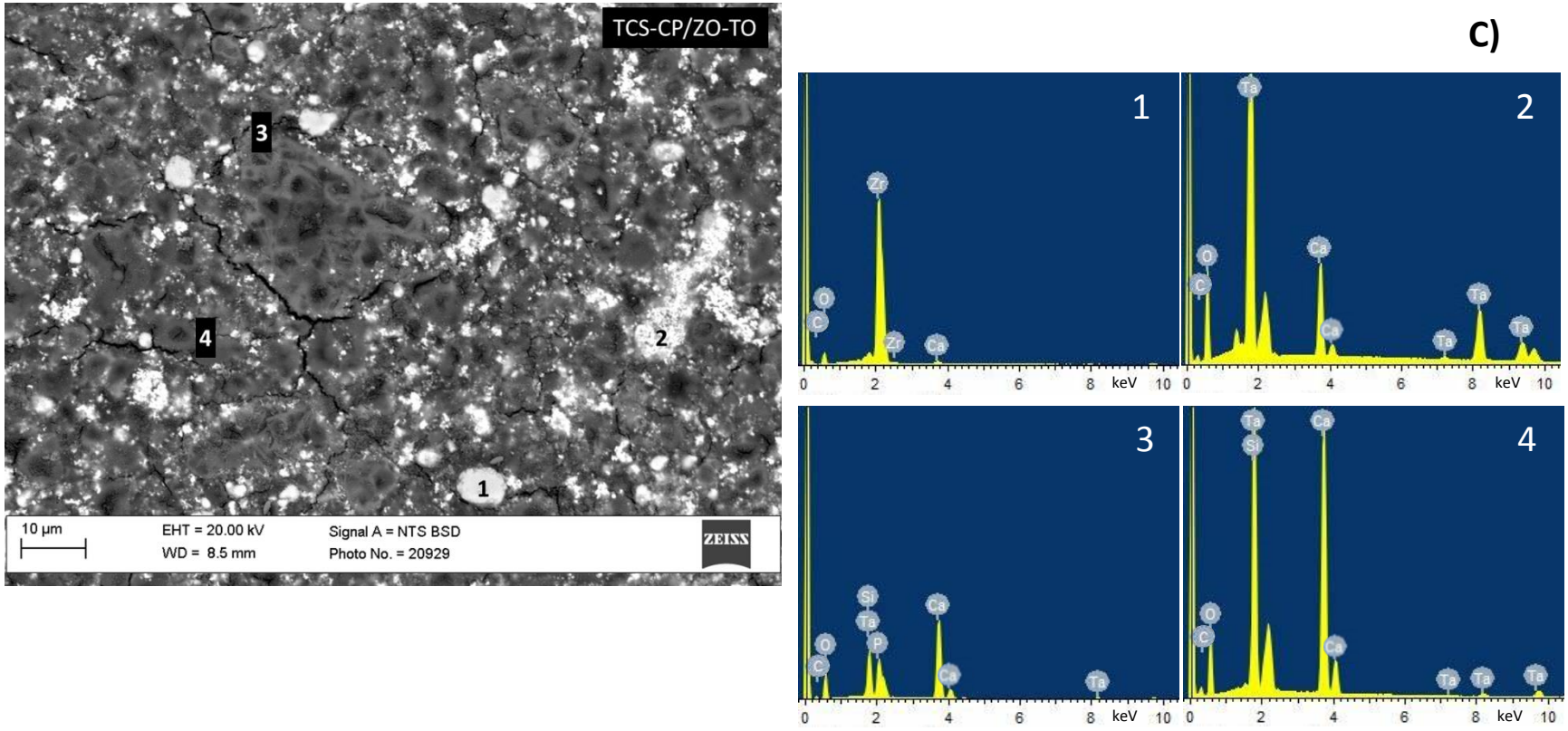


Figure 3.8 (Continued)

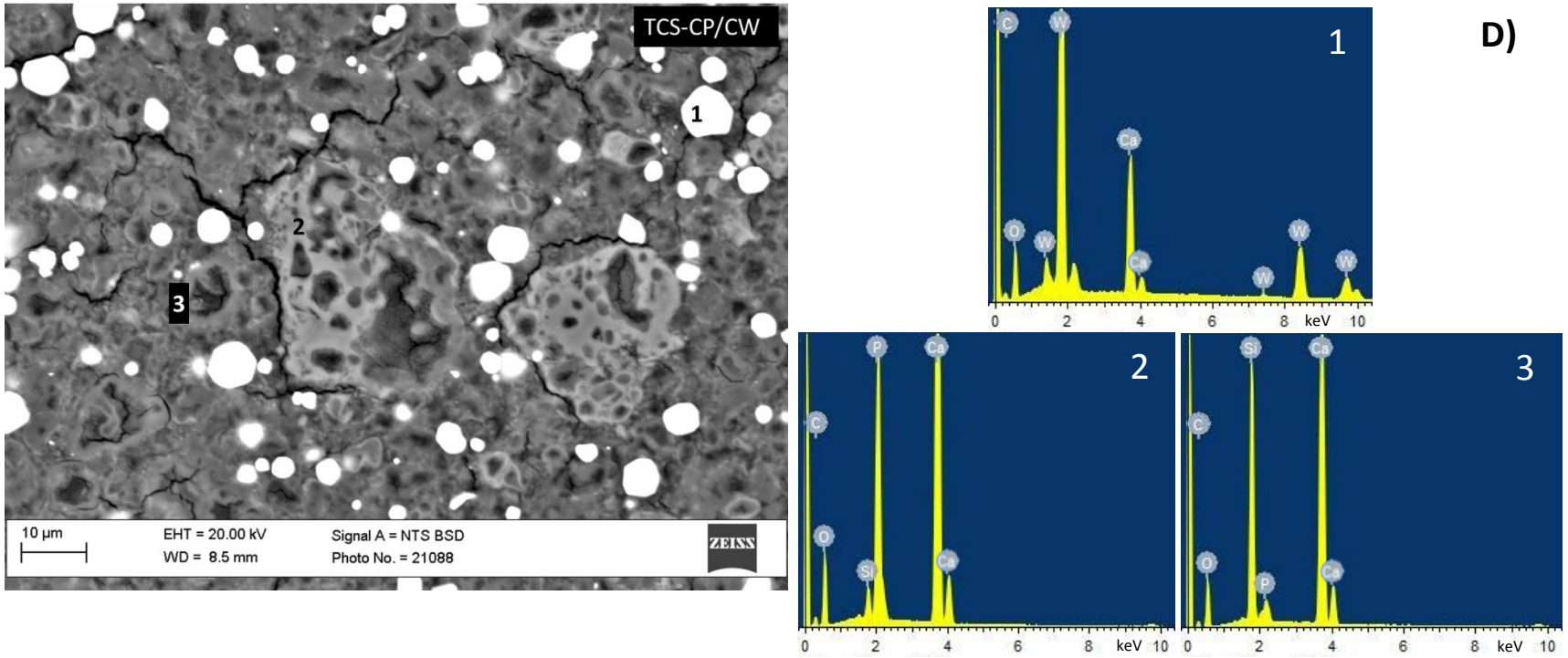


Figure 3.8 (Continued)

The addition of micro-silica also modified the hydration kinetics. In the 10% replacement, a dense matrix of hydrated cement particles was evident (Figure 3.9). In the 20% replacement specimen, fewer unhydrated cement particles were apparent in the micrograph. The matrix was densely filled with micro-silica particles (Figure 3.10).

Different radiopacifier particles and a combination of them (ZO and TO) were included in each cement. All of them appeared bright in the micrographs and they were well distributed throughout the bulk of each material. Zirconium particles were round in morphology and exhibited a range of sizes (Figures 3.7-3.10 A). The radiopacifier particles composed of tantalum oxide presented a relative smaller size while they were occasionally organized in high proximity to each other (Figure 3.7-3.10 B). In the materials consisting both of zirconium and tantalum oxide, similar findings were evident (Figures 3.7-10 C). Finally, the radiopacifier particles in the calcium tungstate containing prototypes, were spherical in morphology and relatively large in size (Figures 3.7-10 D). The elemental analysis undertaken on each radiopacifier particle, verified its constituents.

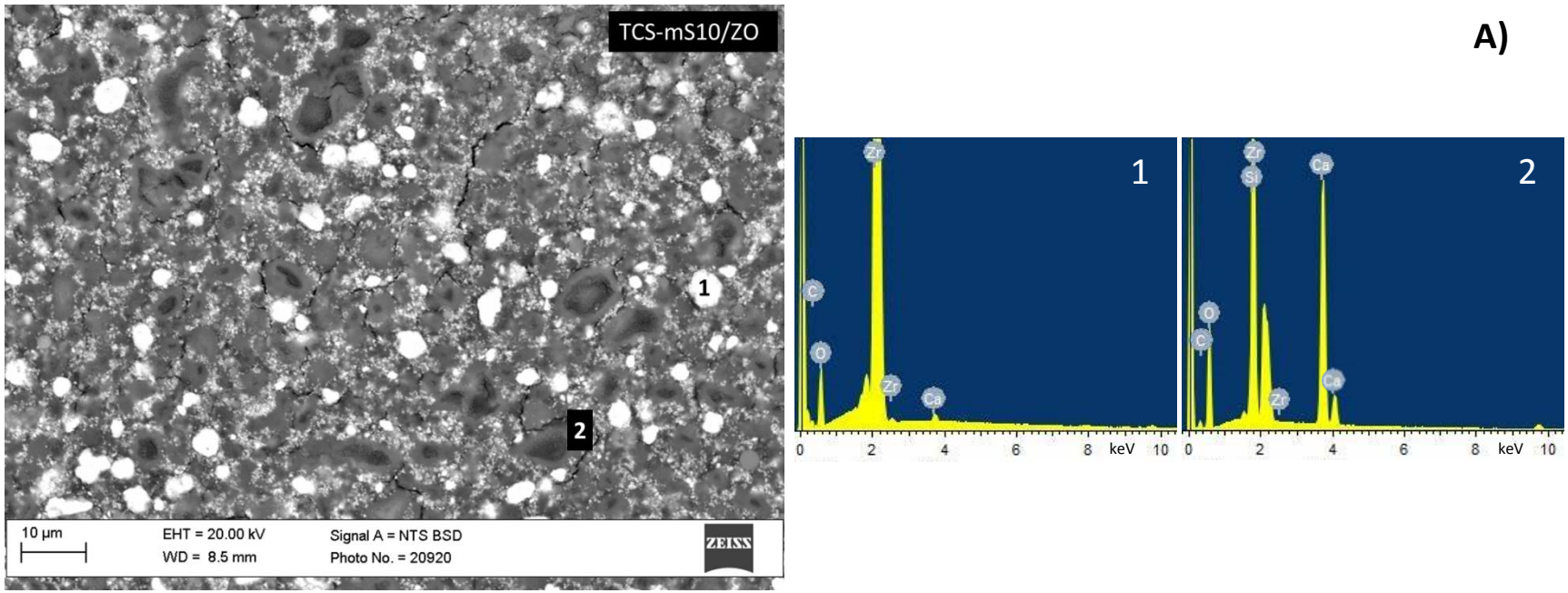
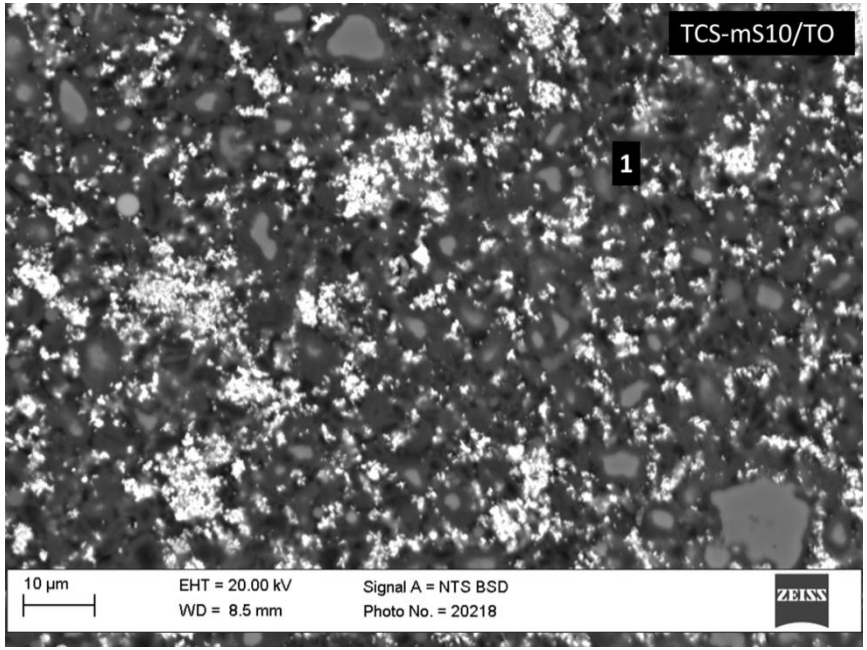


Figure 3.9 Back-scatter scanning electron micrographs of polished sections of tricalcium silicate cement replaced with micro-silica (10%) and radiopacifiers (2500× magnification) showing microstructural features and indicative energy-dispersive spectroscopic scans of selected spectrums (A-D).



B)

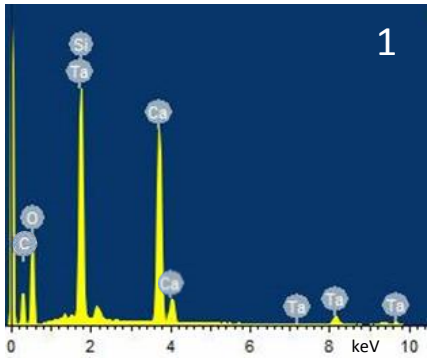


Figure 3.9 (Continued)

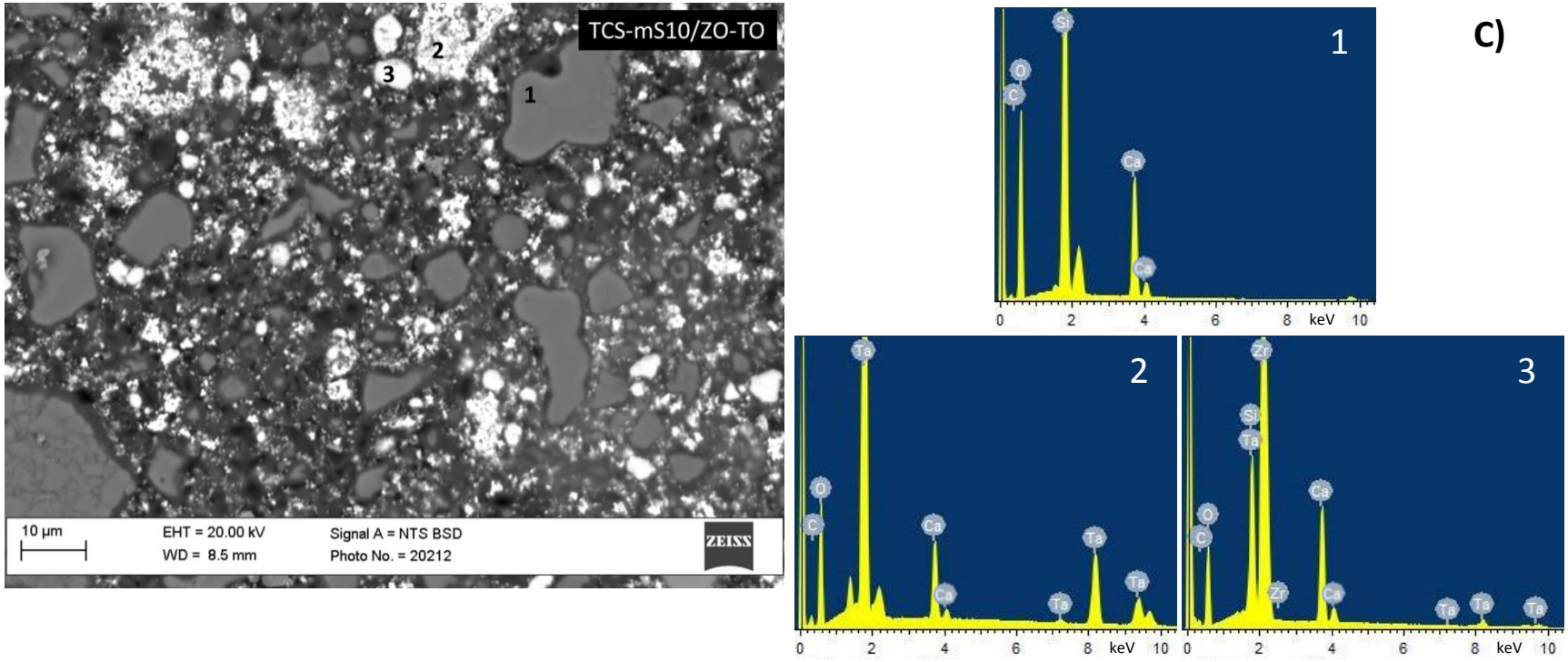


Figure 3.9 (Continued)

D)

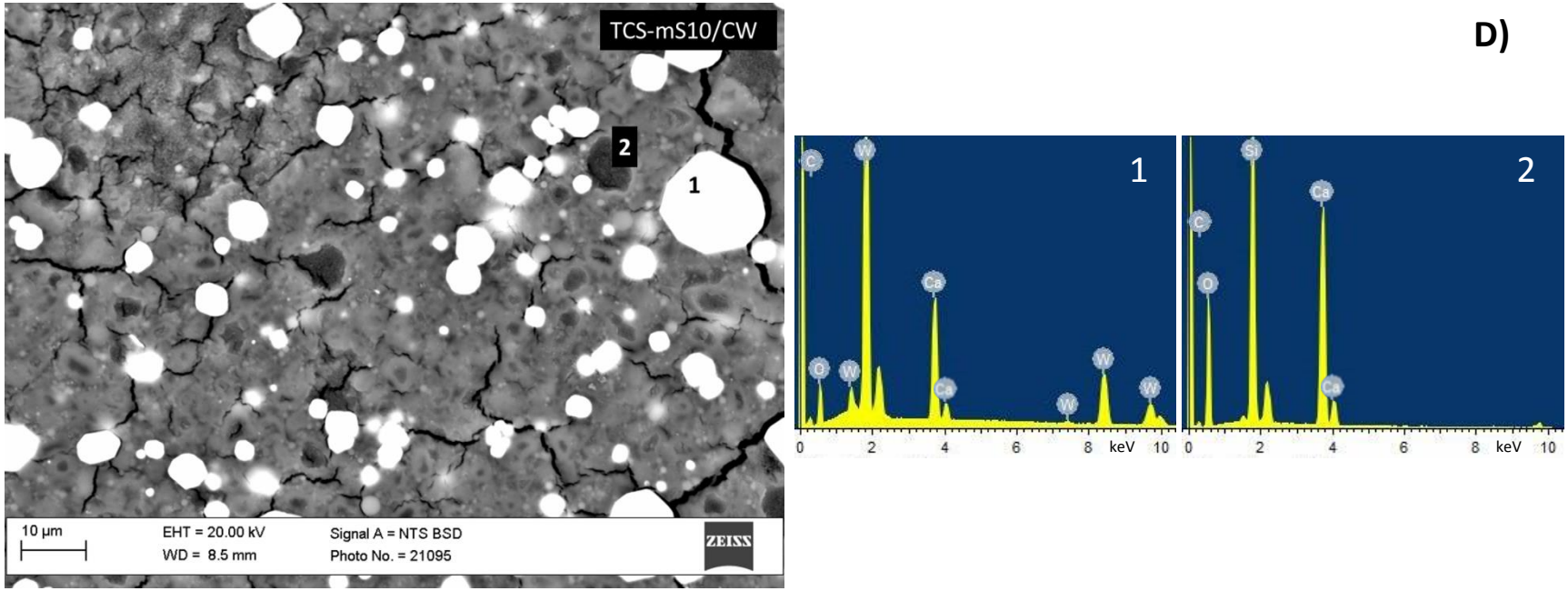


Figure 3.9 (Continued)

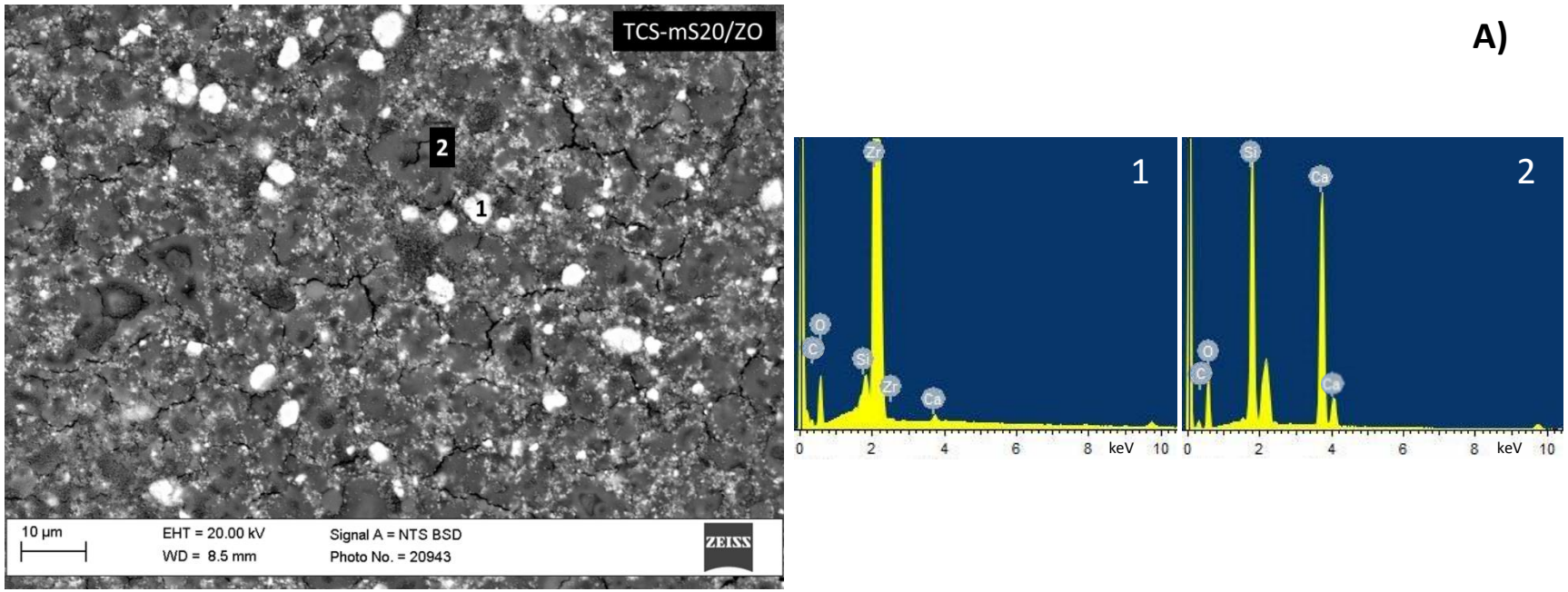


Figure 3.10 Back-scatter images of polished sections of tricalcium silicate cement replaced with micro-silica (20%) and different radiopacifiers (2500× magnification) and energy-dispersive spectroscopic scans showing elemental composition (A-D).

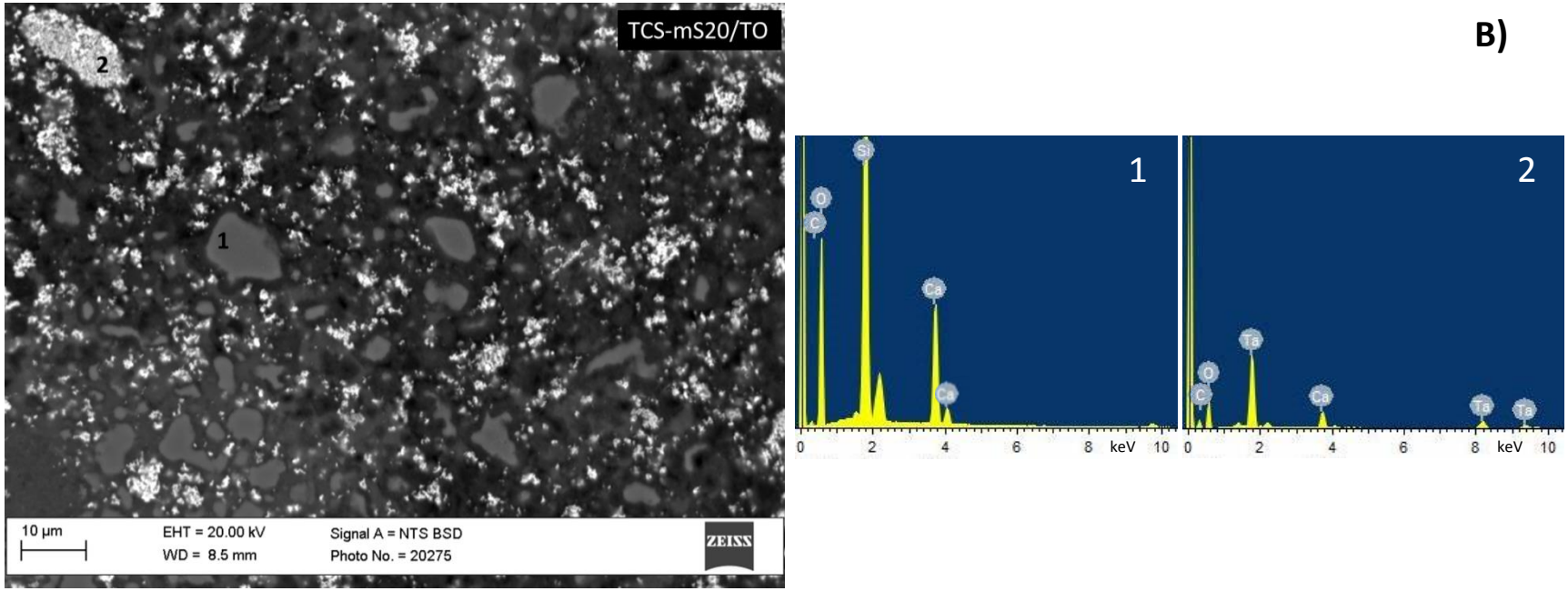


Figure 3.10 (Continued)

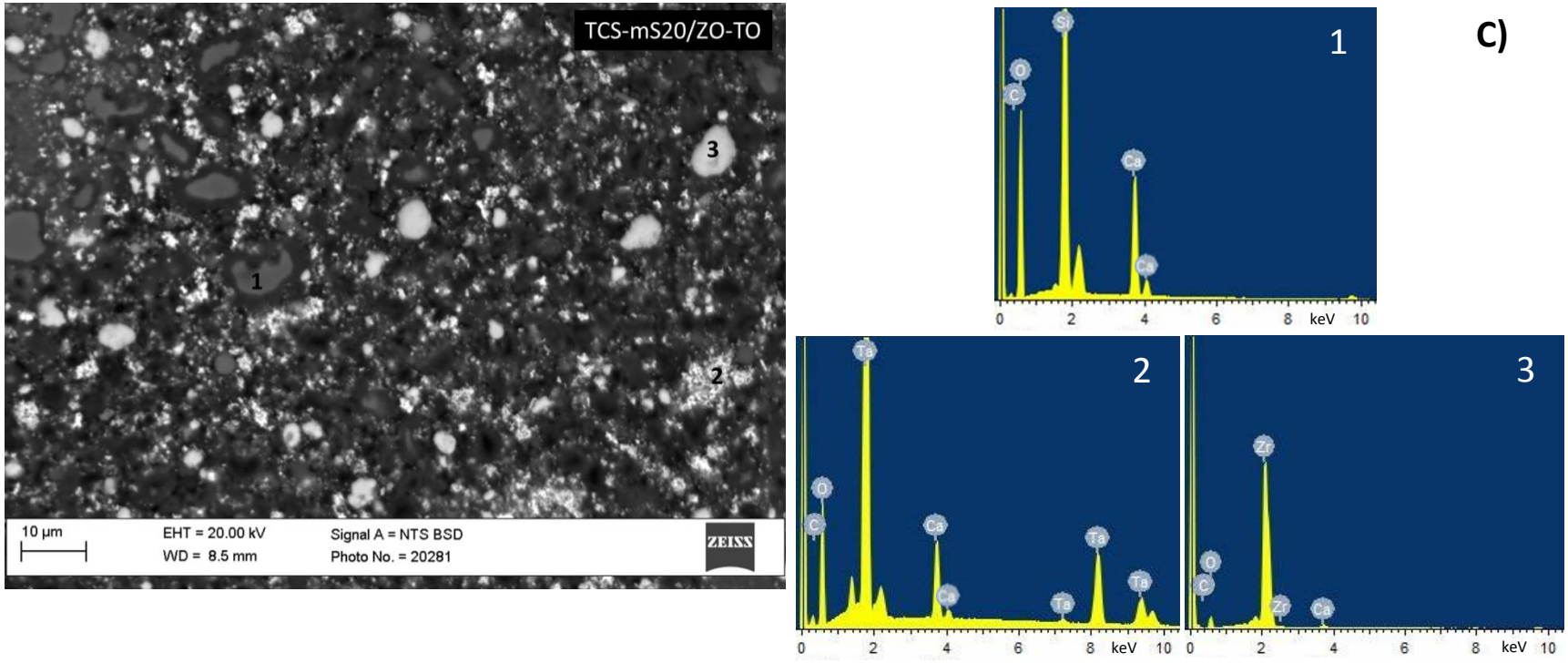


Figure 3.10 (Continued)

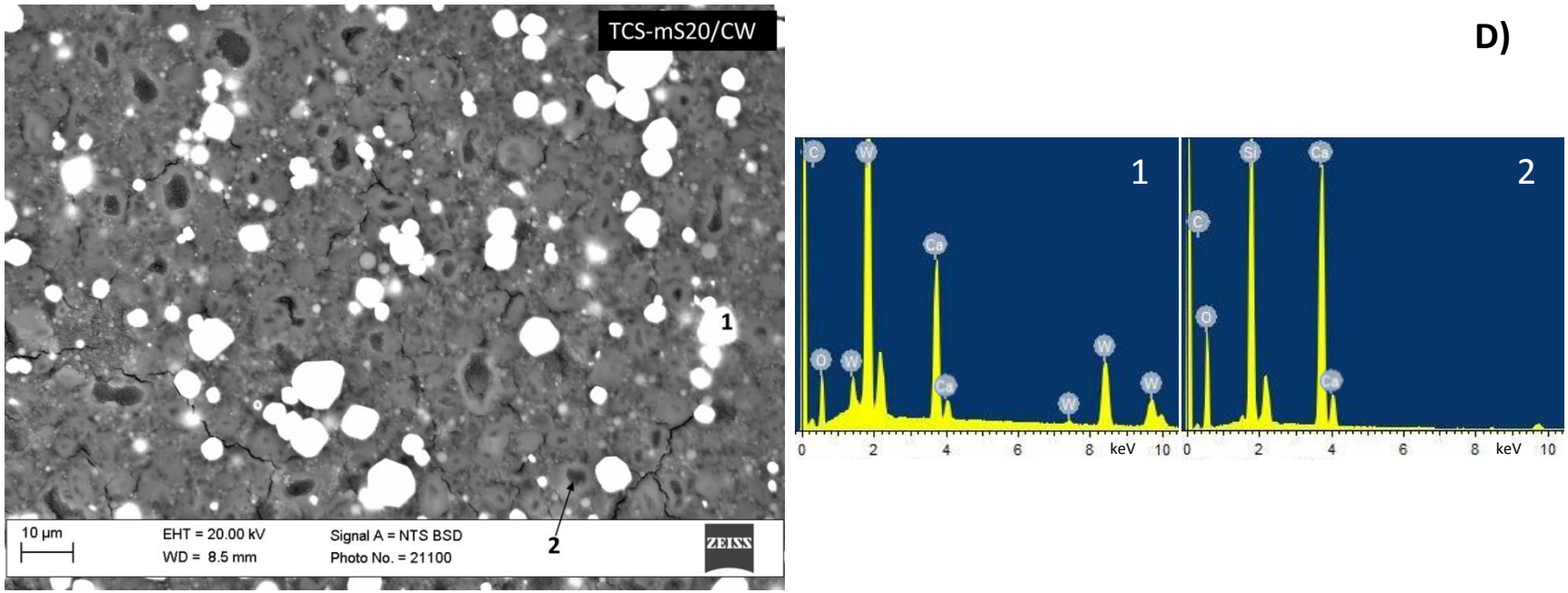


Figure 3.10 (Continued)

3.1.6. Evaluation of radio-opacity

All materials exhibited acceptable radio-opacity (≥3 mm aluminium) after the adjustment of the w/p ratio as specified by ISO 6876:2012 [3] (Table 3.4). The micro-silica addition with calcium tungstate and the 10% micro-silica with zirconium oxide, generated higher values of radio-opacity, with significant differences compared with the majority of the other materials, especially those containing tantalum oxide ($p<0.05$). No statistically significant differences were identified between the other materials tested ($p>0.05$).

Table 3.4 Mean and standard deviation of radio-opacity values of materials (mm aluminium) after the adjustment of the w/p ratio. Latin letters *a*, *b* and *c* indicate statistical significant differences from TCS-mS10/CW, TCS-mS10/ZO and TCS-mS20/CW respectively ($p<0.05$).

TCS	*30% radiopacifier*			
	ZO	**TO**	**ZO-TO**	**CW**
No addition	3.6 ± 0.2	3.1 ± 0.1^{abc}	3.5 ± 0.2^{b}	3.1 ± 0.1^{abc}
15% CP	3.1 ± 0.1^{abc}	3.1 ± 0.02^{abc}	3.3 ± 0.2^{abc}	3.5 ± 0.1^{b}
10% mS	4 ± 0.1	3.3 ± 0.1^{abc}	3.6 ± 0.04	4.2 ± 0.4
20% mS	3.1 ± 0.2^{abc}	3.2 ± 0.3^{abc}	3.3 ± 0.05^{abc}	3.9 ± 0.3

3.2 Chemical, biological and antimicrobial characteristics of pulp capping cements

3.2.1 Condition of TCS-based materials prior to immersion

Mean initial setting time and standard deviation of the TCS-based materials is shown in Table 3.5. The setting time of Bio-C Pulpo and Biodentine was in accordance with the data sheets. TotalFill RRM set approximately one day after placement in the rubber rings and incubation in humidity. In the prototype materials, the calcium phosphate and the 20% micro-silica incorporations had a more prolonged setting period slightly below and above 50 min respectively (Table 3.5).

Table 3.5 Mean setting time and standard deviation of commercial and prototype TCS-based materials expressed in hours or minutes, following which, materials' pellets were immersed in HBSS.

Materials	Setting time
Bio-C Pulpo	7 ± 1.1 min
Biodentine	12 ± 0.9 min
TotaFill RRM	24.7 ± 0.6 h
TCS/ZO	36 ± 2 min
TCS-CP/ZO	48.7 ± 1.2 min
TCS-mS10/ZO	39.3 ± 3.1 min
TCS-mS20/ZO	53 ± 2 min

3.2.2 Degree of conversion of the resin-based materials

Results from FT-IR analysis for the two resin-based materials are shown in Figure 3.11. Both Activa and Theracal failed to reach the manufacturers' claimed DC values during the recommended photo-curing time. More specifically, Theracal's DC began to plateau beyond 90 s timeline, while Activa reached a plateau at a much earlier time-point (~60 s) with lower final DC (marginally above 50%) following the 120 s illumination period.

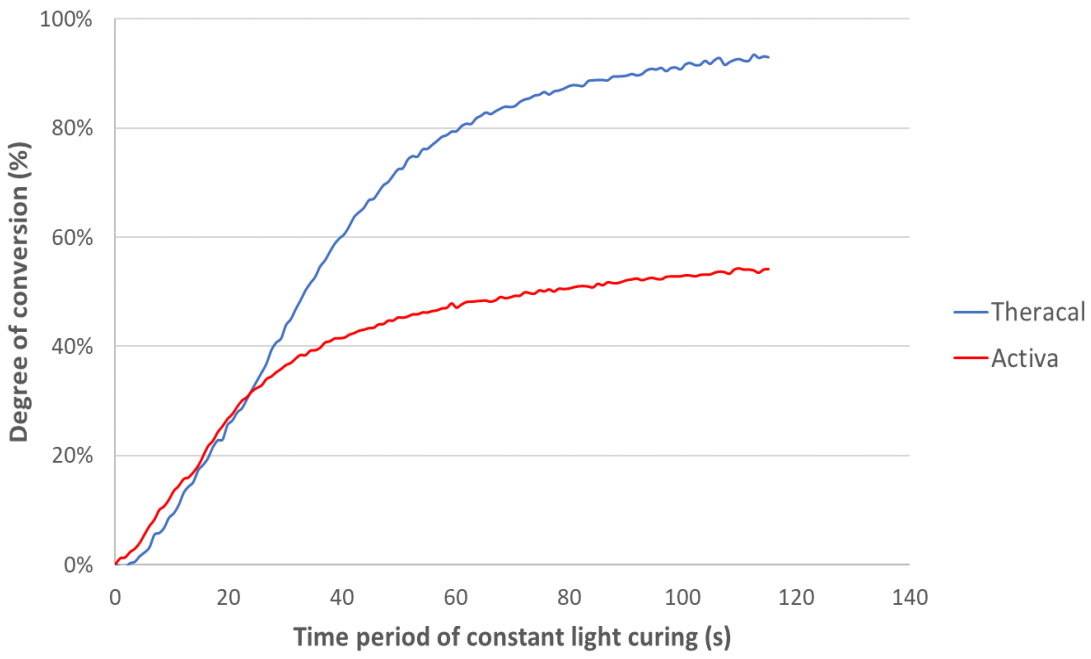


Figure 3.11 Mean degree of conversion for Theracal and Activa during exposure to light curing for a period of 120 s.

3.2.3 Characterisation of 28-day set materials

3.2.3.1 SEM and EDS

The scanning electron micrographs in backscatter mode as well as the EDS analyses of specific microstructural components are presented in Figures 3.12-3.17.

Bio-C Pulpo and Biodentine had a similar composition. Both materials were composed of particles rich in calcium, silicon and zirconium oxide radiopacifier. The cement particles in Bio-C Pulpo were spread in a hydrated matrix; white radiopacifier particles of significantly larger size were found occasionally in its bulk, in proximity to other smaller radiopacifier particles (Figure 3.12). The elemental analysis revealed the presence of aluminium as well as magnesium, suggesting that the cement is a Portland type, thus containing an aluminate phase and other trace oxides such as magnesium oxide. Presence of iron and fluoride were also evident. Zirconium and oxygen were the main elements identified in the radiopacifier particles.

The Biodentine was mainly characterised by reaction products, while few unhydrated cement particles and white radiopacifier particles were also detected. Cracks had also occurred throughout the bulk of the material. In comparison with the Bio-C Pulpo, the hydration was more advanced as the cement particles could not be viewed within the hydrated matrix. The zirconium oxide particles were also smaller in the Biodentine compared to the Bio-C Pulpo. The EDS analysis confirmed the composition of silica particles from fine tricalcium silicate and the presence of zirconium as a radiopacifier in the brighter particles (Figure 3.13).

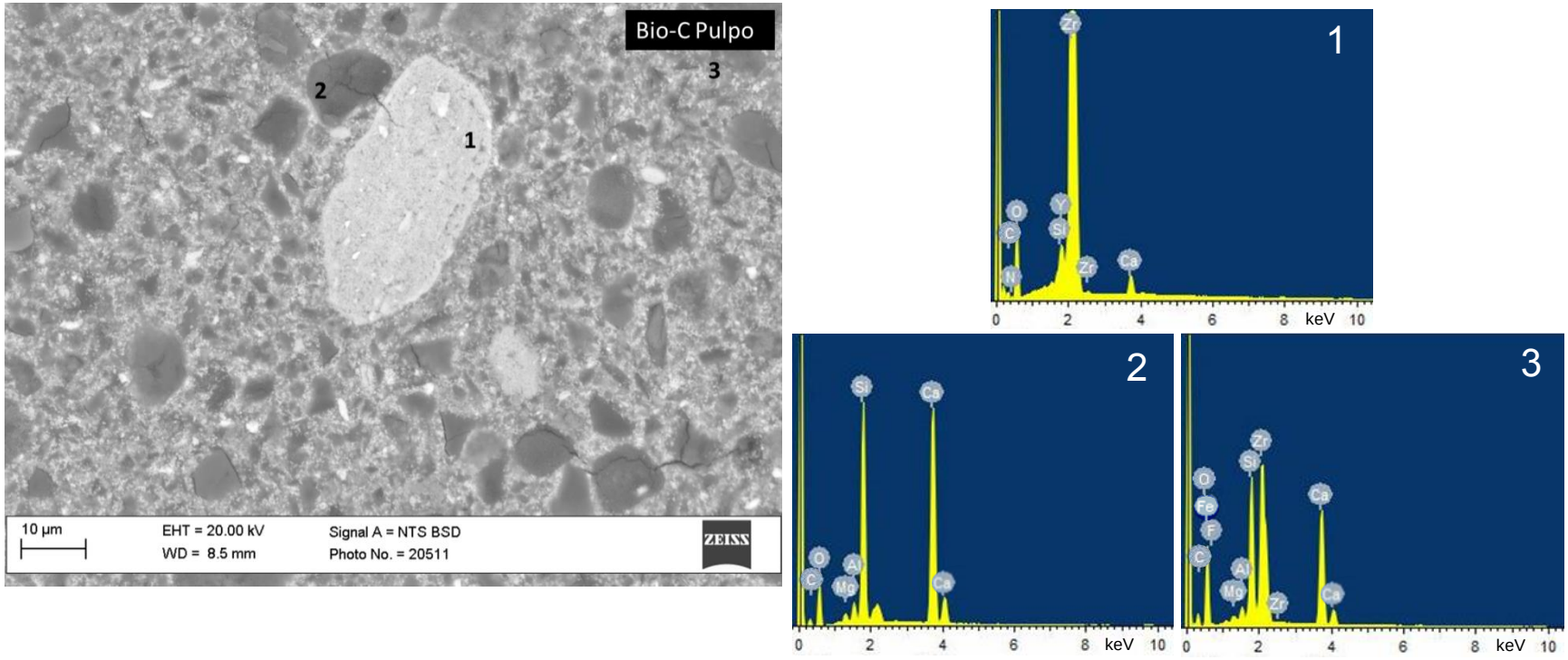


Figure 3.12 Back-scatter scanning electron micrograph of Bio-C Pulpo (2500× magnification) showing microstructural components and energy-dispersive spectroscopic scans of selected spectrums.

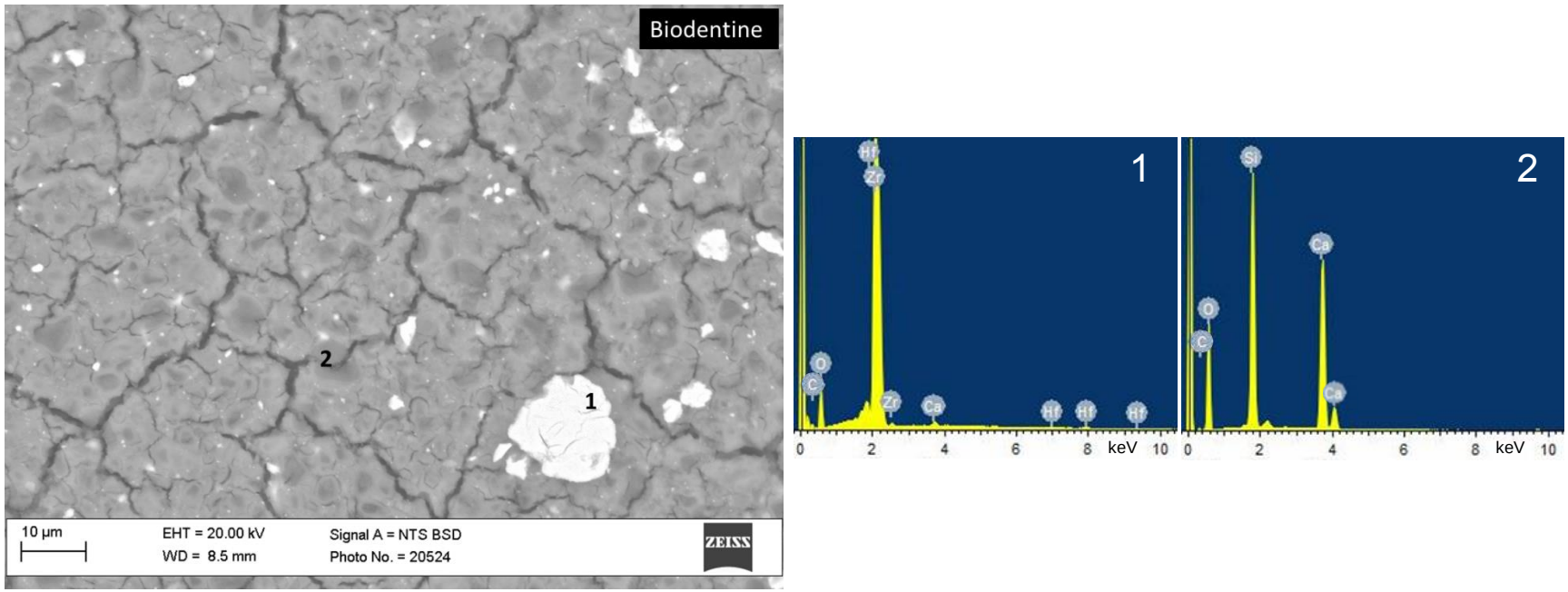


Figure 3.13 Back-scatter image of Biodentine (2500× magnification) showing microstructural features and energy-dispersive spectroscopic scans of selected particles.

The TotalFill RRM was characterised by a dense matrix of cement and radiopacifier particles with large variation in size, which were distributed in a uniform pattern throughout the bulk of the material (Figure 3.14). Two main types of cement particles were identified and their elemental composition was assessed by EDS; the majority of them exhibited peaks only for calcium and silicon, while the rarely distributed ones presented higher peaks for calcium and phosphorous and small peaks for silicon verifying the presence of calcium phosphate in the material (Figure 3.14_5). Finally, white tantalum particles (Figure 3.14_1) and less frequent small elongated zirconium particles (Figure 3.14_3) served as radiopacifiers in the material. The size of the tantalum oxide particles varied, as they were detected both independently spread throughout the matrix and as agglomerates forming large particles; the zirconium oxide particles were elongated, significantly lower in size and fewer in number.

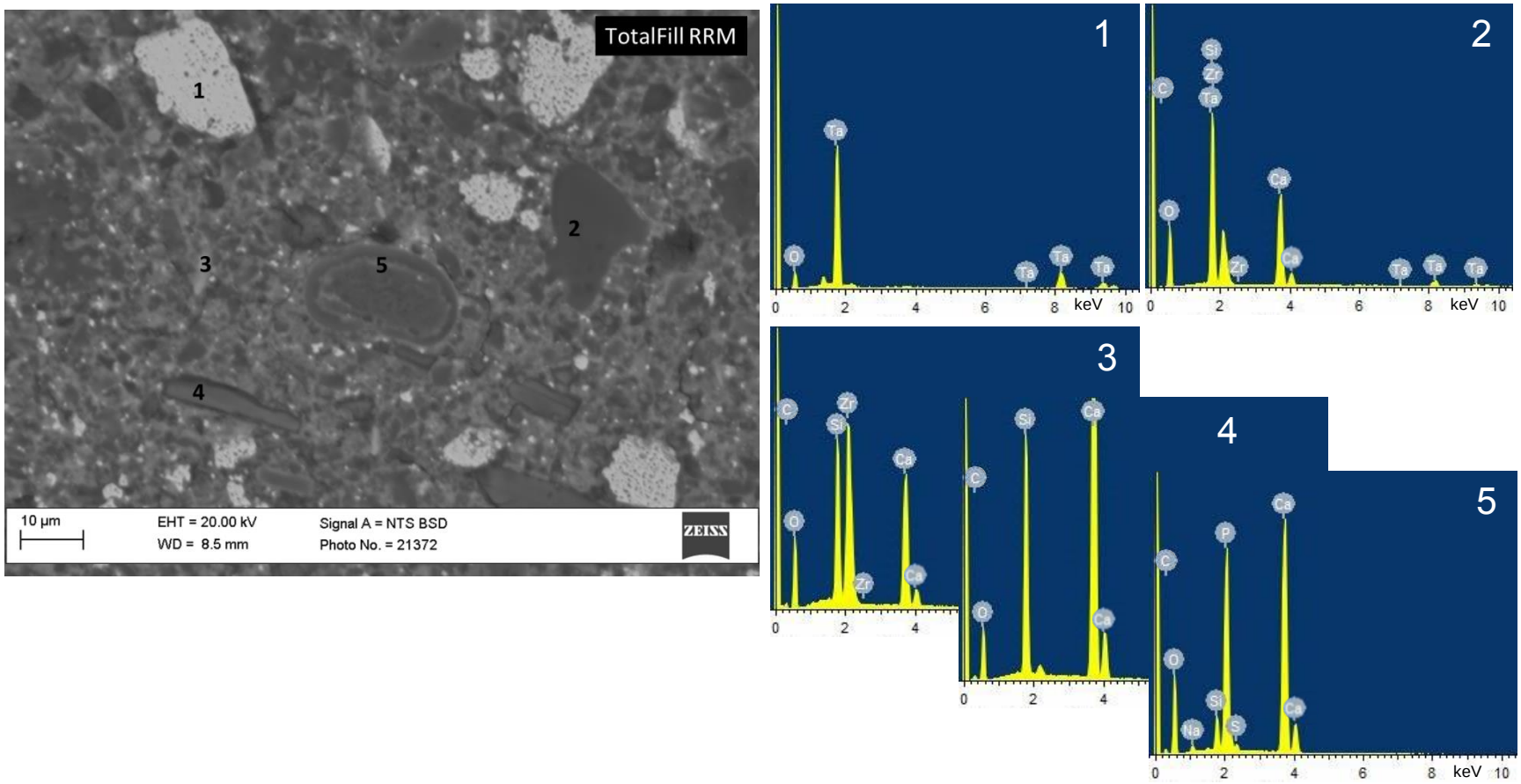


Figure 3.14 Back-scatter scanning electron micrograph of TotalFill RRM (2500× magnification) showing microstructural components and energy-dispersive spectroscopic scans of selected spectrums.

Both Activa and Theracal were resin-based. In the Theracal, cement particles in various sizes were evident throughout the resin matrix (Figure 3.15). Many of them, exceeded by far the size of particles identified in the other materials, while the extent of their hydration was significantly lower. The elemental analysis identified silicon, strontium, calcium, aluminium and magnesium, suggesting a potential glass phase (Figure 3.15_4). Conversely, the radiopacifier particles in the matrix had a smaller size (Figure 3.15_1). Presence of aluminium in the EDS of cement particles confirmed that they are Portland cement-based. Zirconium and barium elements were identified in the radiopacifier particles.

Similarly, the matrix of Activa was heavily filled with particles of small size (Figure 3.16). Silicon was identified as the main constituent of the particles along with aluminium; calcium and minor peaks for sodium and fluorine were also detected. Barium was found in the radiopacifier particles (Figure 3.16_1).

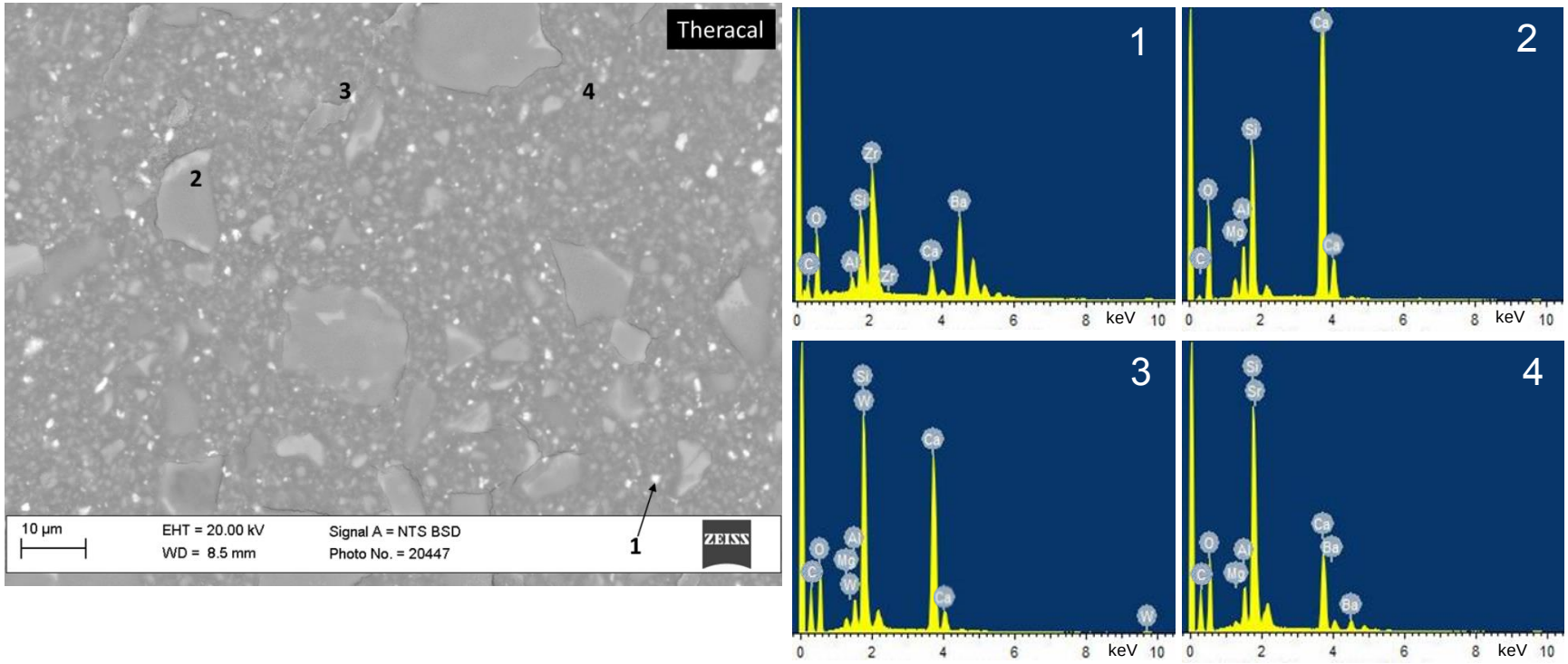


Figure 3.15 Back-scatter image of Theracal (2500× magnification) showing microstructural features and energy-dispersive spectroscopic scans showing elemental composition.

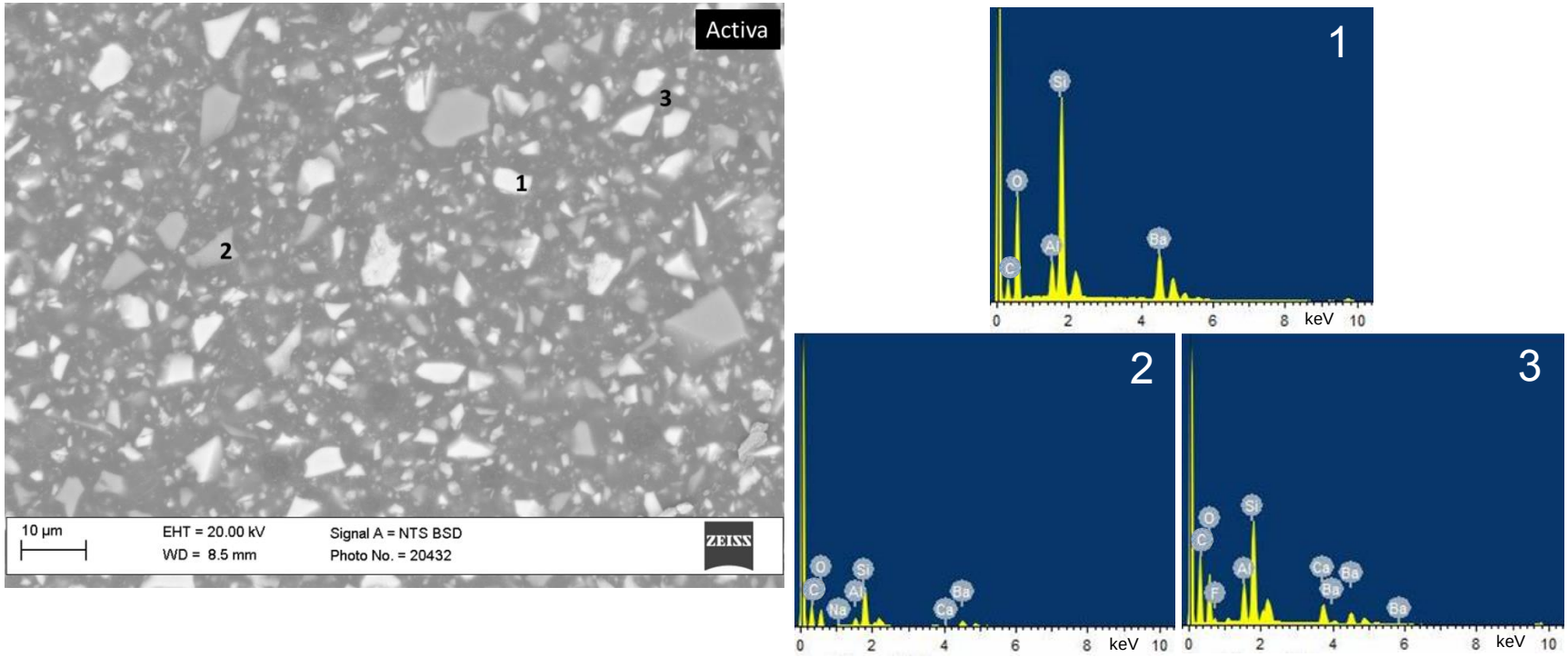


Figure 3.16 Back-scatter scanning electron micrograph and energy-dispersive spectroscopic scans of Activa (2500× magnification) showing microstructural components and elemental composition of selected spectrums respectively.

The prototype cements were all based on tricalcium silicate with zirconium oxide radiopacifier. Calcium phosphate monobasic and micro-silica were used as replacement materials. The cement particles in the TCS/ZO prototype cement showed a halo of hydration product. Extensive hydration had occurred (Figure 3.17). As shown in Section 3.1.5, replacement of TCS with calcium phosphate monobasic or micro-silica, altered the hydration kinetics. In the TCS-CP/ZO, a second type of cement particles consisting of calcium, phosphorus and silicon is evident in the micrograph as lathlike particles (Figure 3.17 B_2). The cement containing 10% micro-silica exhibited a bigger extent of hydration of cements particles (Figure 3.17 C), while in the 20% replacement the cement particles were not visible (Figure 3.17 D). Finally, round radiopacifier particles of various sizes were spread in the bulk of the prototype materials.

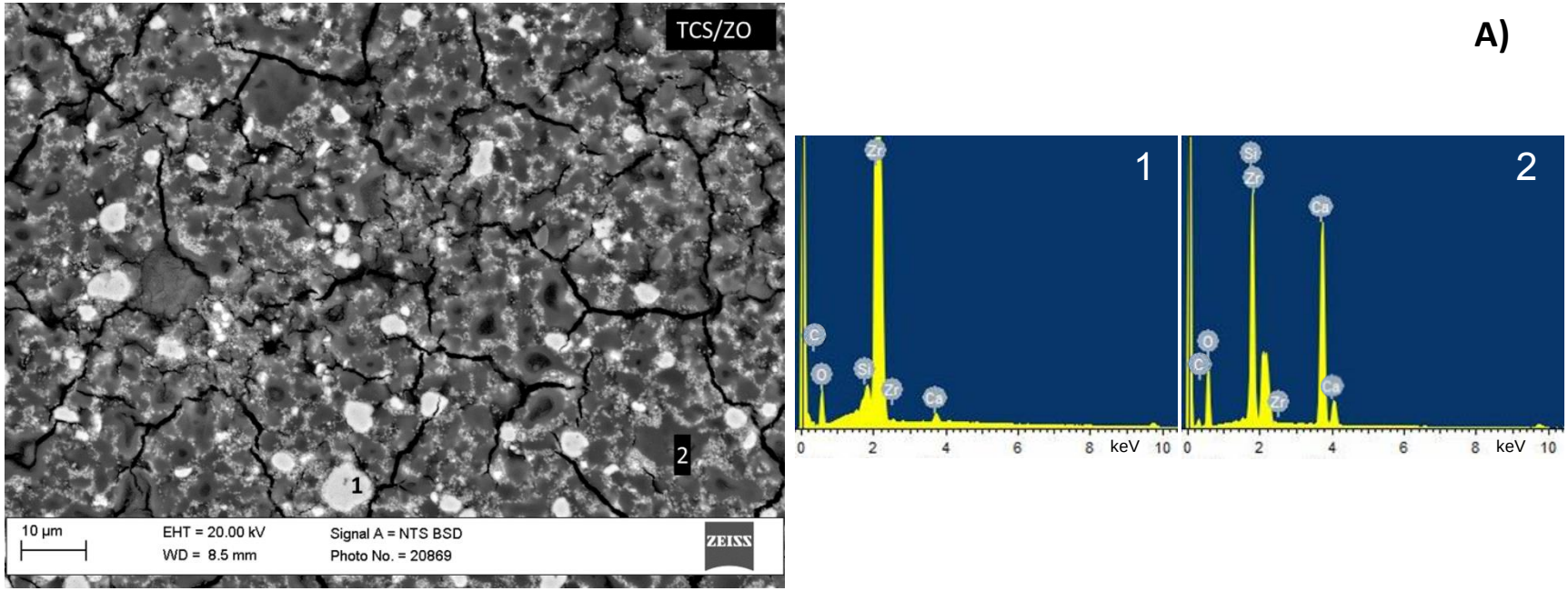


Figure 3.17 Back-scatter scanning electron micrographs of polished sections of prototype radiopacified TCS-based materials without any other incorporation (A) or with inclusion of calcium phosphate (B) or micro-silica (C, D) (2500× magnification) showing microstructural features and energy-dispersive spectroscopic scans of selected spectrums.

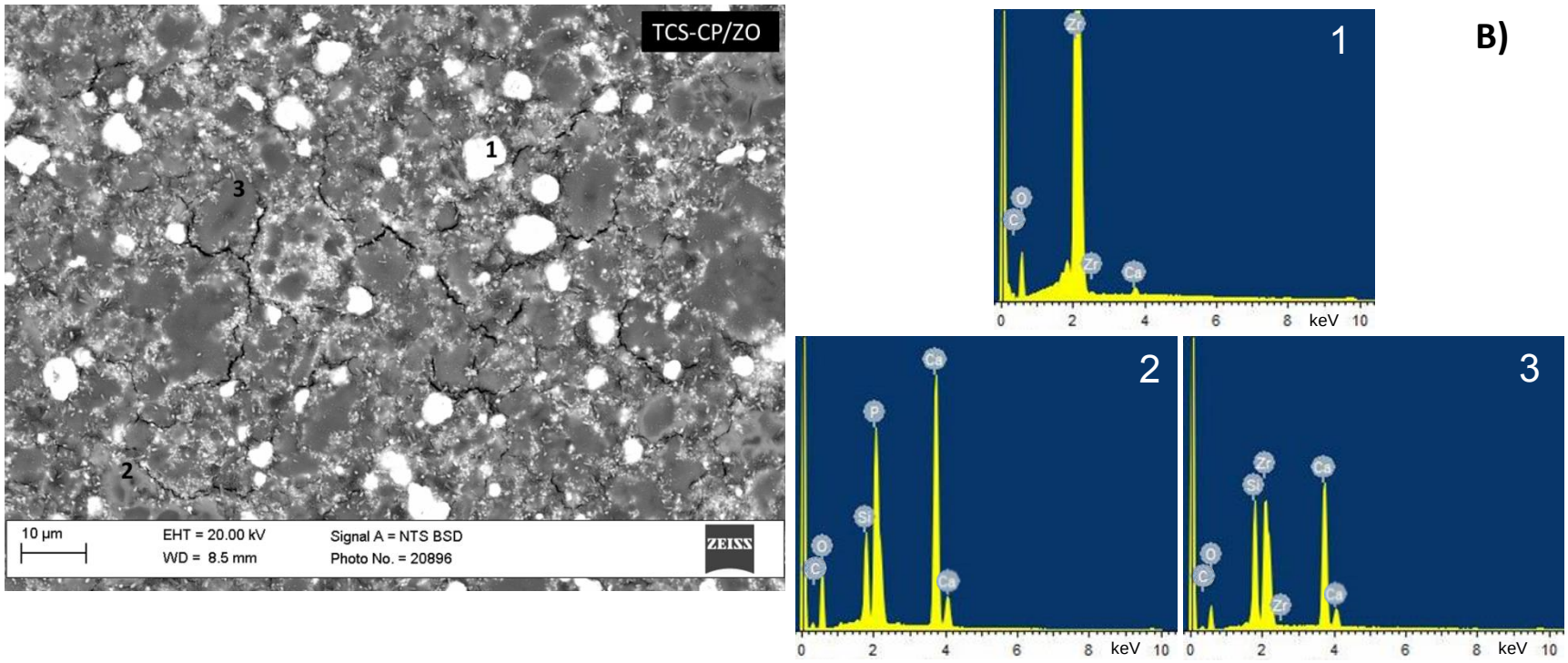


Figure 3.17 (Continued)

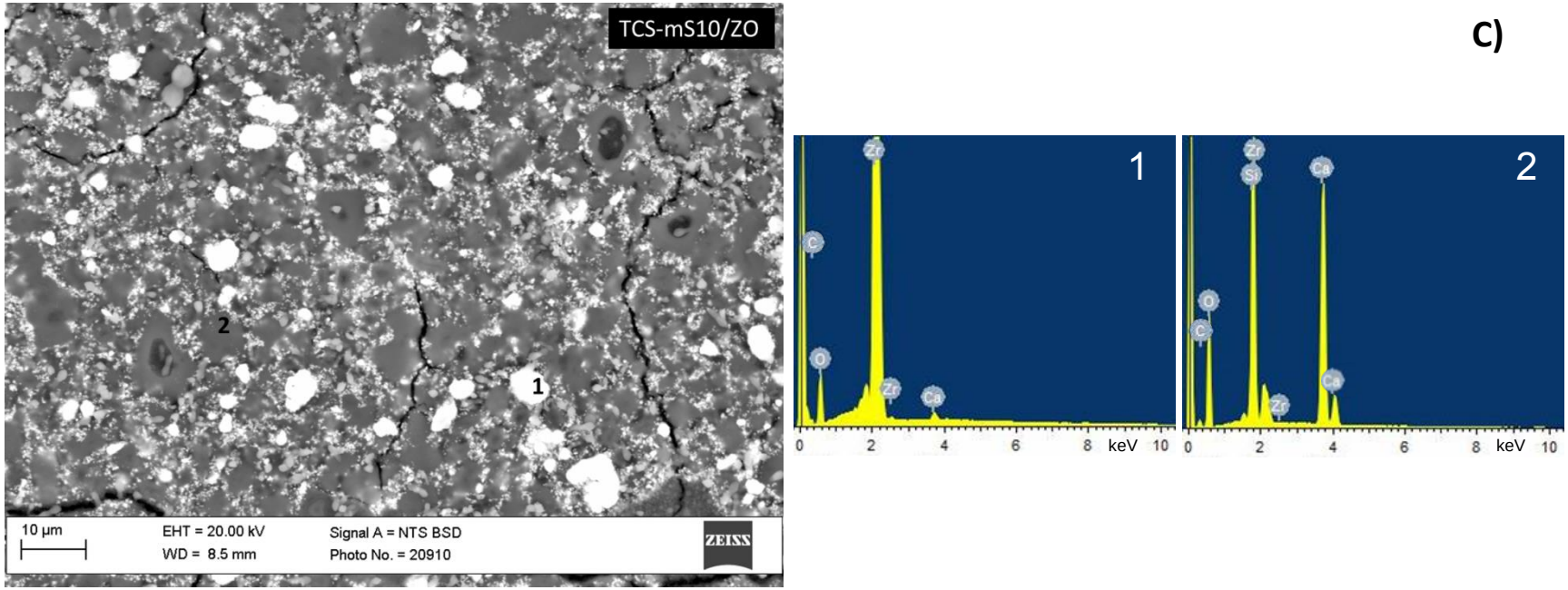


Figure 3.17 (Continued)

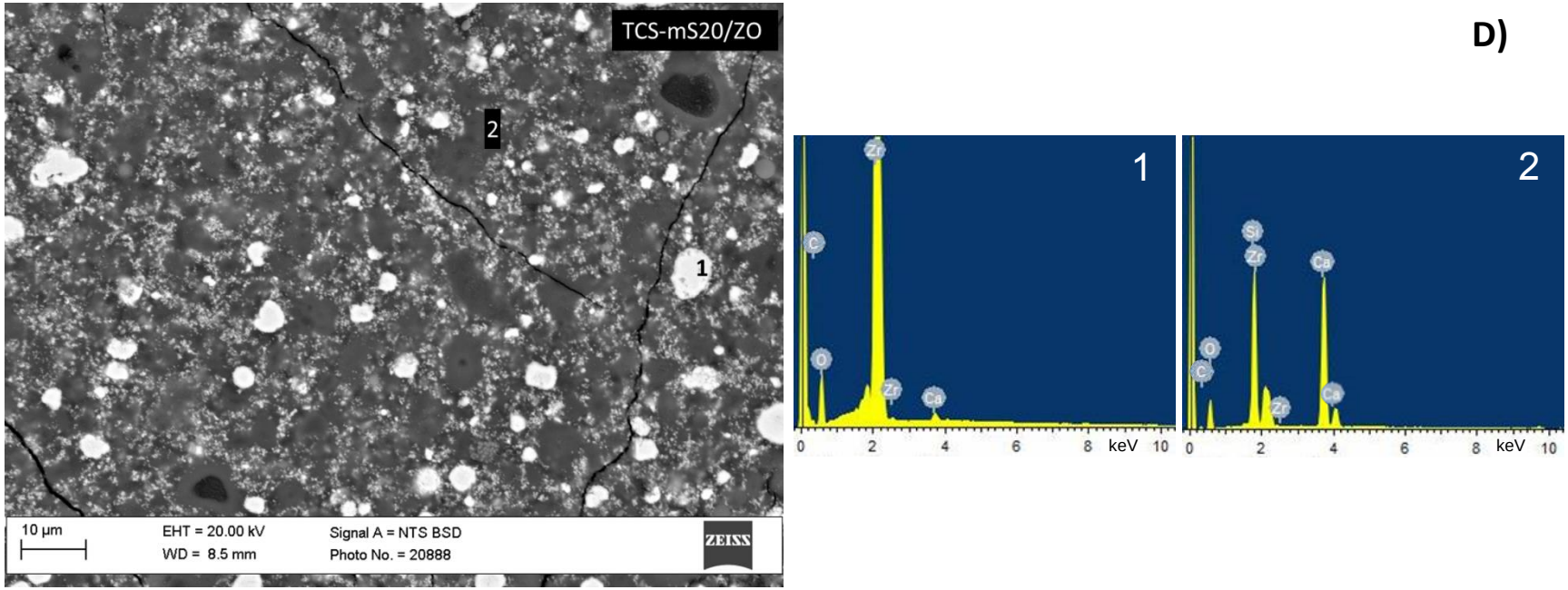


Figure 3.17 (Continued)

3.2.3.2 XRD analysis

The diffractograms obtained for each material are shown in Figures 3.18 and 3.19. The Bio-C Pulpo exhibited peaks for zirconium oxide (ICDD: 00-050-1089), silicon oxide (ICDD: 04-002-8291) and calcium hydride (ICDD:00-001-0881). No peaks for tricalcium silicate were detected, which indicated its complete reaction (Figure 3.18 A).

Conversely, tricalcium silicate peaks (ICDD: 00-055-0739) were evident in the Biodentine. Peaks for calcium carbonate (ICDD: 04-012-8783) of relatively high intensity were detected, as well as minor peaks for zirconium oxide (ICDD: 01-073-8590, Baddeleyite) and calcium hydroxide (ICDD: 00-002-0969, Portlandite) (Figure 3.18 B).

The diffractogram for TotalFill RRM exhibited a similar peak for Portlandite (ICDD: 00-004-0733) and peaks of higher intensity for zirconium oxide (ICDD: 00-065-0729); peaks for tantalum oxide (ICDD: 00-019-1299) were also evident. Additionally, peaks for calcium phosphate (ICDD: 00-002-0647) and calcium phosphate silicate (00-050-0905) were identified (Figure 3.18 C).

The Theracal had peaks containing barium strontium zirconium oxide (ICDD: 04-018-8311); other peaks consisted of barium zirconium oxide (ICDD: 00-003-0641) or zirconium oxide (ICDD: 04-011-8815). A minor peak for tricalcium silicate (ICDD: 00-033-0303) was also identified (Figure 3.18 D).

The Activa exhibited relatively few minor peaks for calcium fluoride (ICDD: 04-002-2191) and sodium fluoride (ICDD: 01-073-1922, Villiaumite) but it was mainly amorphous (Figure 3.18 E).

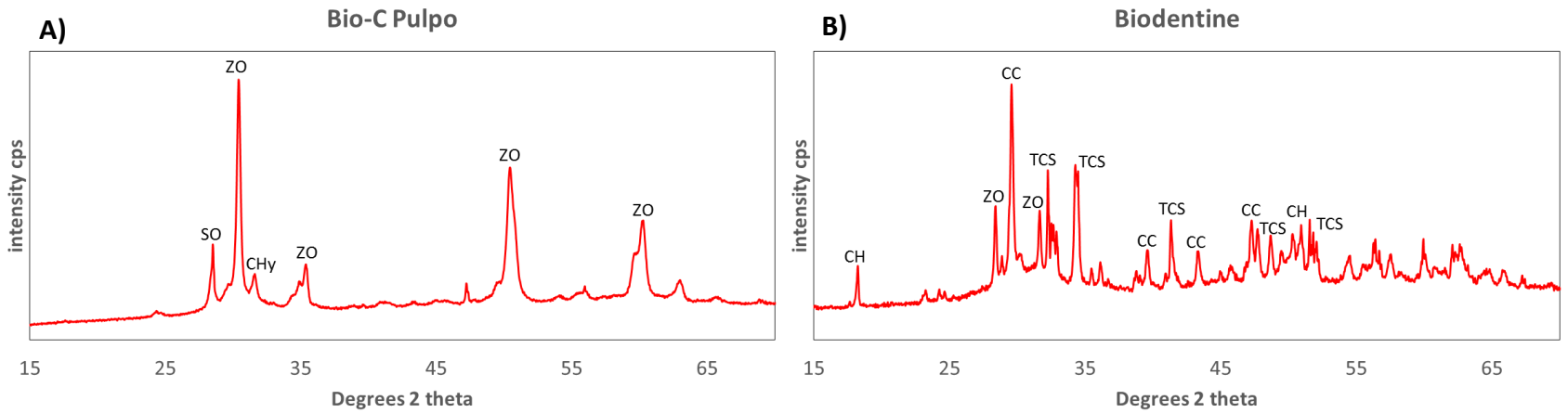


Figure 3.18 X-ray diffraction plots of the test commercial materials after the 28-day immersion period in HBSS, showing the crystalline phases formed (A-E). BStZO: Barium strontium zirconate oxide, BZO: Barium zirconate, CH: Calcium hydroxide, CHy: Calcium hydride, CC: Calcium carbonate, CF: Calcium fluoride, CPS: Calcium phosphate silicate, NF: Sodium fluoride, SO: Silicon oxide.

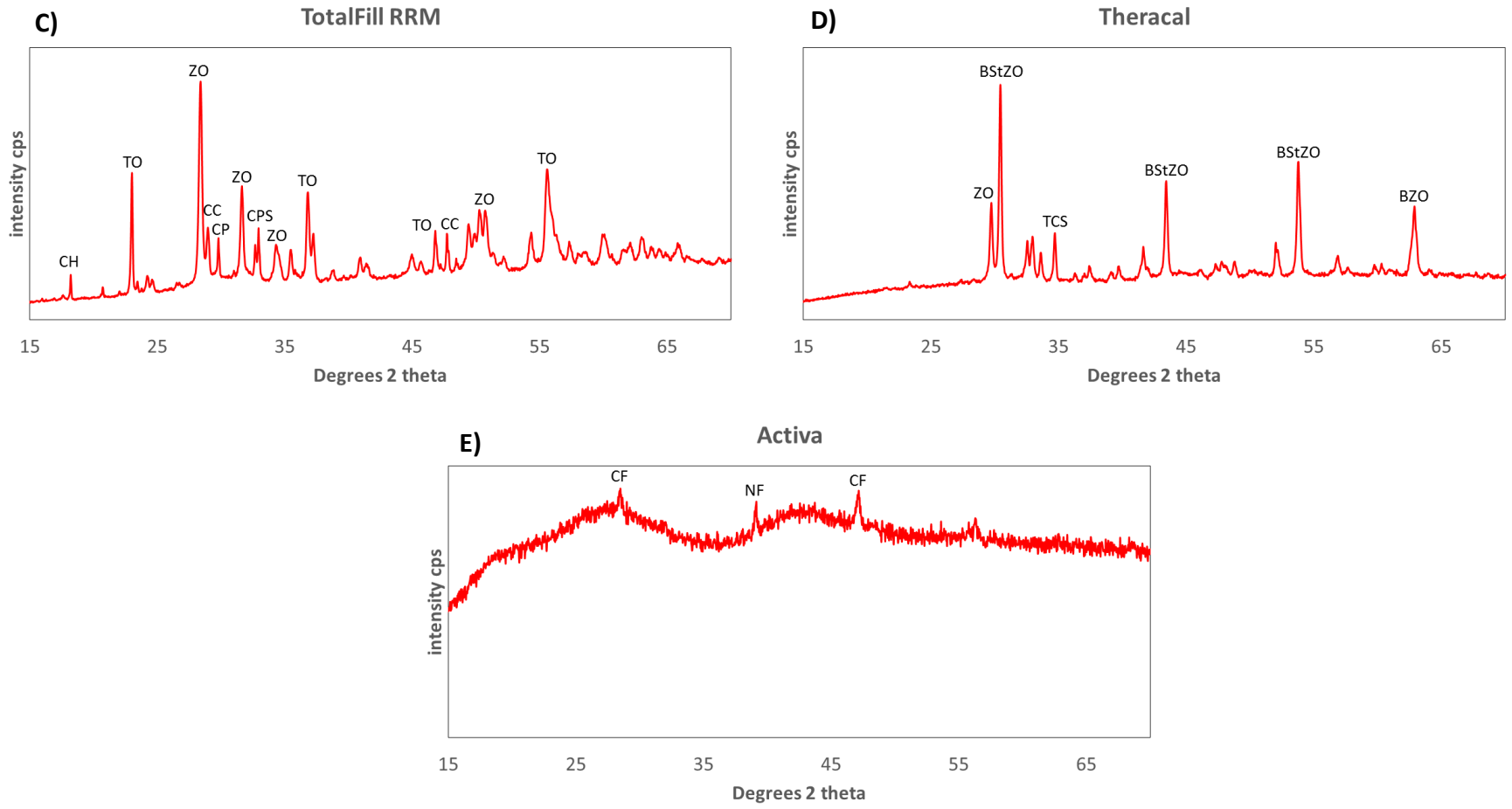


Figure 3.18 (Continued)

The prototype materials exhibited a similar pattern in peaks for calcium hydroxide (ICDD: 01-078-0315, Portlandite), zirconium oxide (ICDD: 00-065-0728) and tricalcium silicate (ICDD: 00-003-1105) as shown in Figure 3.19. Incorporation of calcium phosphate or micro-silica were not evident in the diffractograms; however, changes in the cementitious phase of the materials appeared to alter the intensity of the existing peaks. More specifically, the micro-silica containing cements presented peaks of lower intensity for calcium hydroxide which was positively correlated with the percentage of micro-silica replacement. A decrease in the intensity of the Portlandite peak was also evident for the calcium phosphate-containing prototype. Notably, lower intensity peaks for tricalcium silicate were detected in the materials that a compound incorporation had been performed in its cementitious phase. The peaks for zirconium oxide were similar for all the prototype materials tested (Figure 3.19).

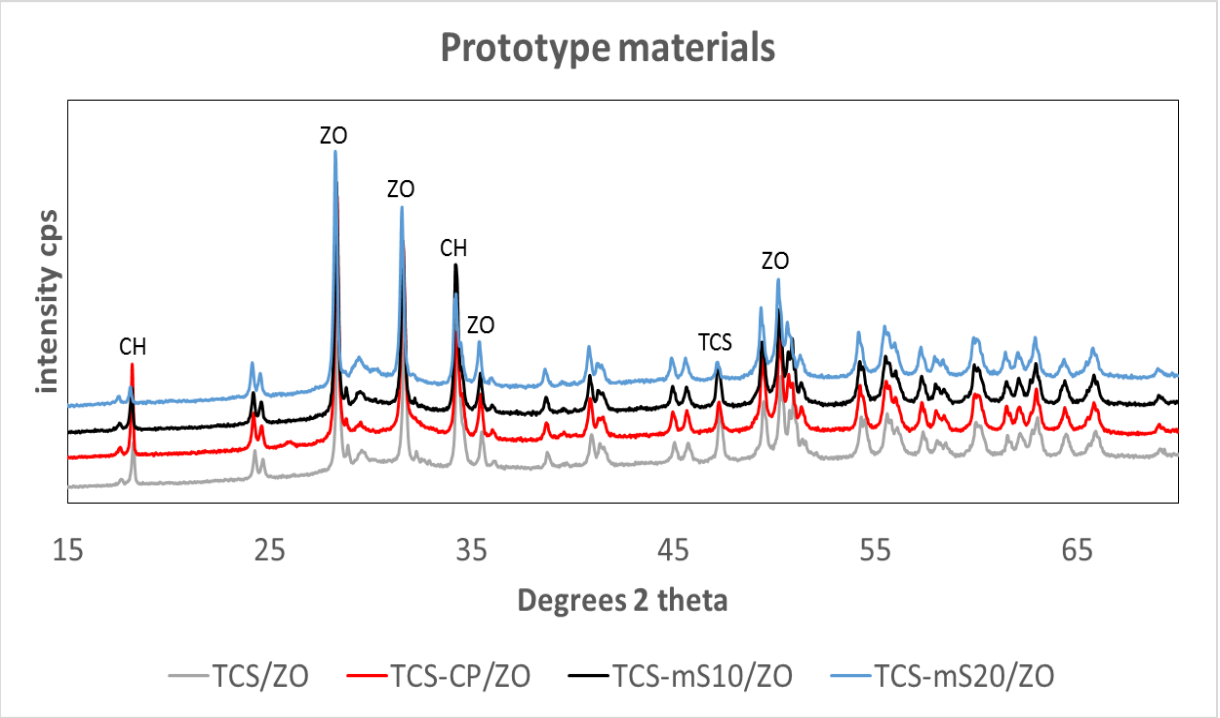


Figure 3.19 X-ray diffraction plots of the test prototype materials after the 28-day immersion period in HBSS, showing the crystalline phases formed.

3.2.4 Leachate analysis

3.2.4.1 pH assessment

All materials alkalinised significantly the pH of the solution ($p<0.05$), except for the resin-based Activa ($p>0.05$); notably, the 14-day leachate of Activa exhibited a significant decrease in its pH ($p<0.05$) (Table 3.6). The alkalinisation of the soaking solution in all other materials occurred from the first 24 h with no significant alterations in most of them. In the Biodentine and the pure tricalcium silicate with zirconium oxide, a significant increase was reported between the first day and after 14 days ($p<0.05$), while the pH of TotalFill RRM rose significantly after 7 days ($p<0.05$) and plateaued after that period. This indicates the gradual release of calcium hydroxide by these materials and especially Biodentine. All the TCS-based materials-both commercial and prototypes reported higher pH values than the resin-containing Theracal ($p<0.05$).

Table 3.6 Mean pH and standard deviation of leachates of commercial and prototype materials during the 28-day period. The pH of the extract vehicle (HBSS) was 8.8 ± 0.1.

Materials	1 day	7 days	14 days	21 days	28 days
Bio-C Pulpo	13.01 ± 0.08	13.23 ± 0.05	13.4 ± 0.07	13.3 ± 0.08	13.36 ± 0.04
Biodentine	12.79 ± 0.12	13.23 ± 0.05	13.48 ± 0.06	13.38 ± 0.07	13.48 ± 0.05
TotaFill RRM	13.1 ± 0.03	13.87 ± 0.03	14 ± 0	13.83 ± 0.76	13.93 ± 0.08
Theracal	11.92 ± 0.29	11.5 ± 0.04	11.72 ± 0.26	11.58 ± 0.21	11.75 ± 0.24
Activa	8.86 ± 0.43	8.52 ± 0.48	8.3 ± 0.37	8.44 ± 0.38	8.43 ± 0.15
TCS/ZO	13.14 ± 0.07	13.62 ± 0.03	13.77 ± 0.03	13.76 ± 0.03	13.77 ± 0.05
TCS-CP/ZO	13.31 ± 0.08	13.71 ± 0.02	13.8 ± 0.05	13.82 ± 0.06	13.86 ± 0.03
TCS-mS10/ZO	13.26 ± 0.09	13.57 ± 0.04	13.7 ± 0.05	13.77 ± 0.08	13.67 ± 0.01
TCS-mS20/ZO	13.26 ± 0.03	13.5 ± 0.05	13.65 ± 0.05	13.69 ± 0.01	13.65 ± 0.03

3.2.4.2 Calcium release

The materials released varying amounts of calcium into the HBSS solution. Mean calcium release with standard deviation for each material is presented in Figure 3.20. The 28-day eluates of Bio-C Pulpo and TotalFill RRM reported the highest calcium release, while the former reached a plateau in its release after 24 h. Calcium amounts in the 28-day leachate of Biodentine were similar to Bio-C Pulpo ($p>0.05$), although significantly lower compared with the TotalFill RRM ($P<0.05$). Activa was the only material that released minimal amounts of Ca^{2+} into the solution. The Theracal released lower amounts of Ca^{2+} from the other commercial tricalcium silicate-based materials for both aging periods studied ($p<0.05$), however no differences were identified with the prototype materials for the same time periods ($p>0.05$). Calcium release of the micro-silica containing prototype materials was not significantly altered after the 24-h aging period ($p>0.05$).

Overall, the materials containing additions or incorporation of silicon and the resin-based Theracal released calcium in a faster pattern, without reporting significant changes in the calcium amount after 28 days.

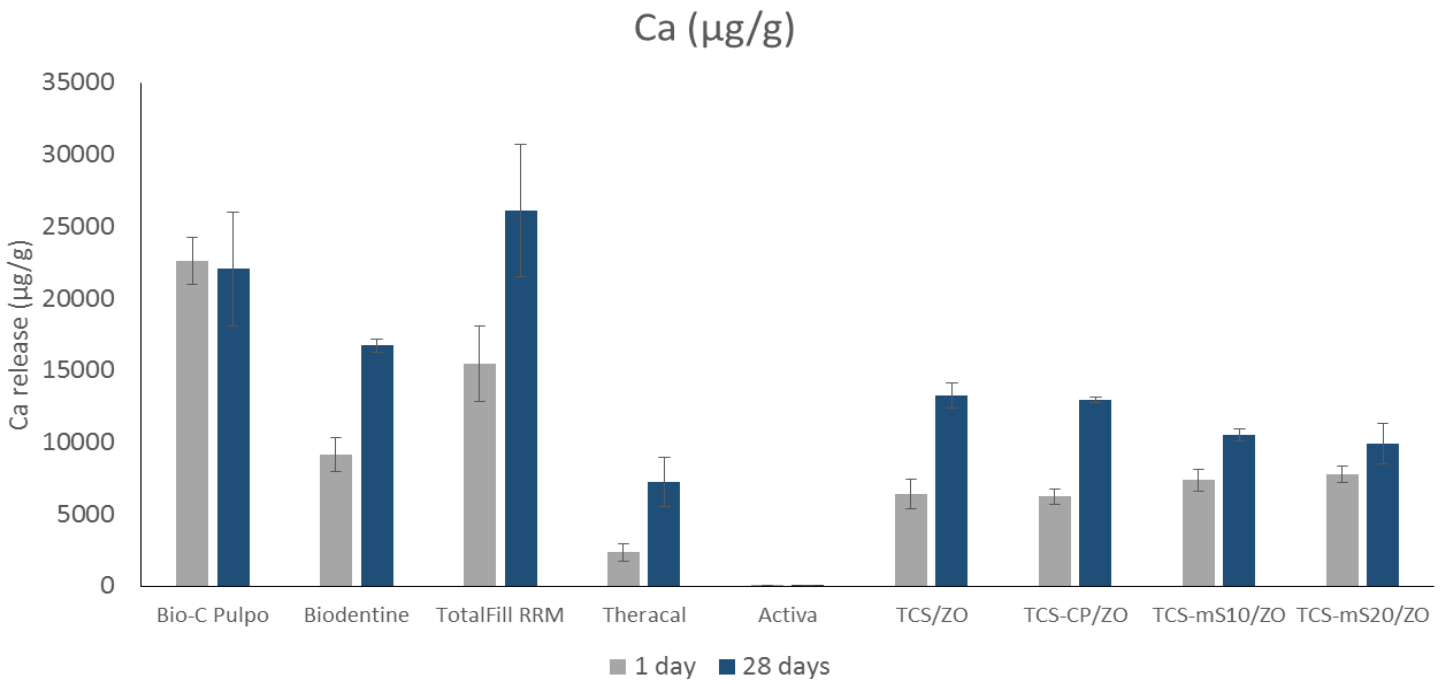


Figure 3.20 Mean calcium release and standard deviation of eluates of commercial and prototype materials aged for 1 or 28 days in HBSS.

3.2.5 Biocompatibility assays

3.2.5.1 Cell mitochondrial activity

3.2.5.1.1 Effect of 1-day leachates

The 1-day leachates and their dilutions exhibited varying effects on the metabolic activity of 3T3 cells and HDPCs. Figure 3.21 presents the cell metabolic activity of cells after exposure to different dilutions of 1-day leachates as a percentage of the blank extract vehicle (HBSS). According to ISO 10993-5;2009, relative MTT values below 70% of the blank group suggest a potential cytotoxic effect [280].

Undiluted leachates of Bio-C Pulpo, TotalFill RRM, Activa and all the prototype TCS-based cements reduced the metabolic activity of both cell types to different extents ($p<0.05$); pure eluates of Biodentine and Theracal were regarded as biocompatible for both cell types, while Biodentine reported higher MTT values in HDPCs than Theracal ($p<0.05$). The pure leachate of TotalFill RRM was more cytotoxic in HDPCs ($p<0.05$). The radiopacified tricalcium silicate (TCS/ZO) presented a moderate decrease in MTT activity in HDPCs, with a mean value marginally above 70%, which is the cytotoxicity threshold according to ISO 10993-5;2009 specifications [280]; on the contrary, it had a significantly higher cytotoxic effect in 3T3 cell cultures ($p<0.05$). In a similar pattern, all the undiluted eluates of the other prototype materials affected significantly less the metabolic activity of HDPCs comparing to 3T3 cells ($p<0.05$).

All the diluted leachates of the test samples were biocompatible for all materials; in particular, some dilutions of the test leachates enhanced the MTT activity

in a different pattern between the two cell types ($p<0.05$), except for the radiopacified tricalcium silicate ($p>0.05$).

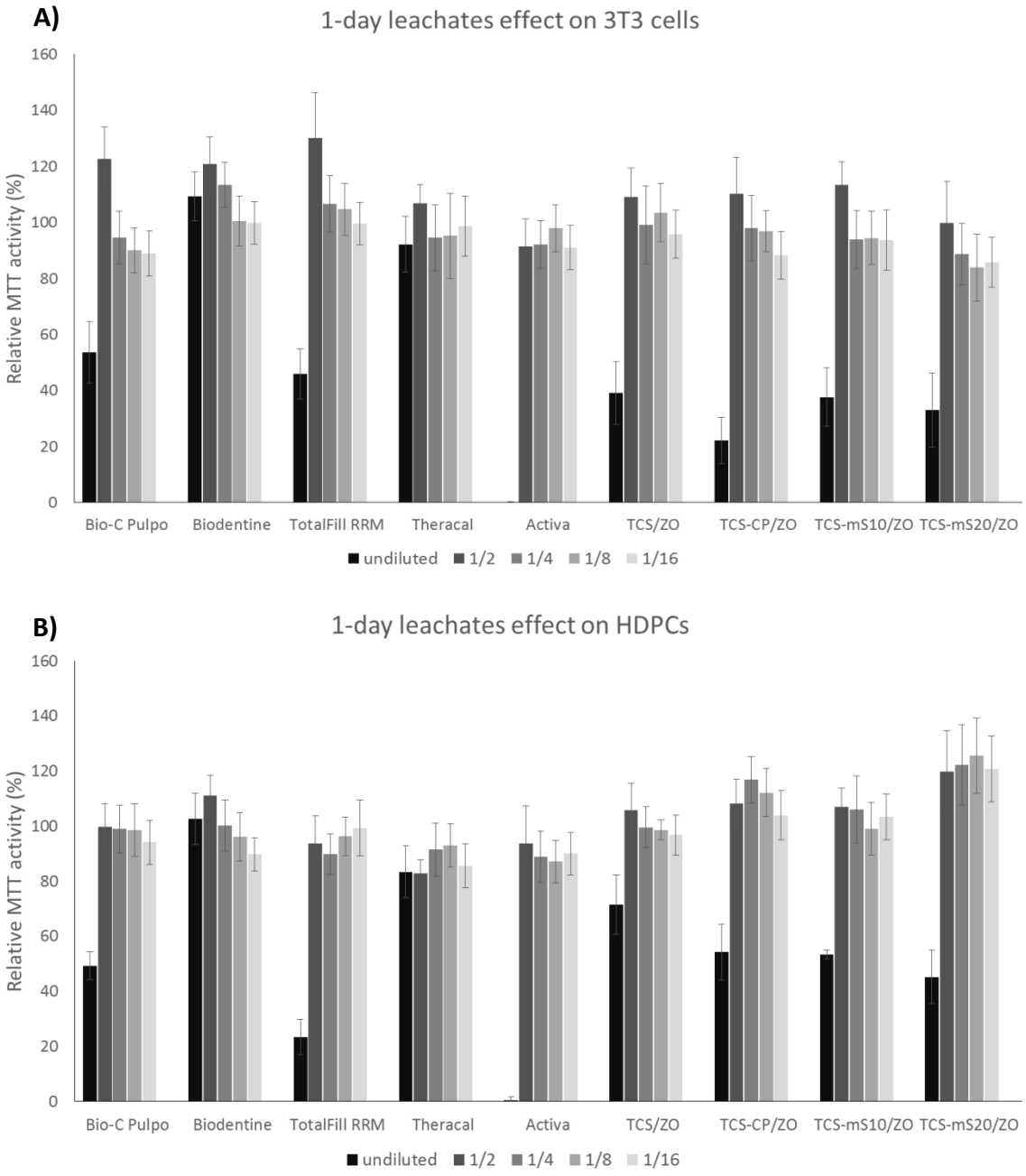


Figure 3.21 Mean relative MTT activity and standard deviation in 3T3 cell cultures (A) and HDPC cultures (B) following a 24-h exposure to 1-day leachates of commercial and prototype materials and their dilutions.

3.2.5.1.2 Effect of 28-day leachates

Figure 3.22 represents results from the metabolic activity of cells after exposure to the 28-day leachates. Results were normalised in respect to performance of blank HBSS and presented as relative MTT activity. The non-diluted eluates of the tricalcium silicate-based Bio-C Pulpo, Biodentine, TotalFilll RMM and the prototype materials were regarded as being cytotoxic. However, Bio-C Pulpo, Biodentine and TCS-mS20/ZO had a lower cytotoxic effect than the aforementioned cytotoxic eluates in both cell types ($p<0.05$). The pure leachate of TCS-mS20/ZO reduced more the metabolic activity in 3T3 cells than in HDPCs ($p<0.05$).

The non-diluted resin-based Theracal and Activa were regarded as being biocompatible and reported significantly higher viability values compared with all the other pure leachates for both cell types ($p<0.05$). Notably, both eluates reported higher values for 3T3 cells comparing to HDPCs ($p<0.05$).

Diluted leachates of all materials were cytocompatible; however, values of relative MTT activity in the solutions varied between materials and cell type in many cases. Several of the diluted leachates of the prototype materials (1/2 for TCS/ZO and TCS-CP/ZO, 1/4 for TCS-CP/ZO and TCS-mS10/ZO, 1/8 and 1/16 for all) reported higher values for HDPC cultures ($p<0.05$). Values for the first three dilutions of eluates of TotalFill RRM were also higher for HDPCs ($p<0.05$). No pattern was evident in the other commercial materials, with few significant differences being observed between the same eluates and the two different cell types.

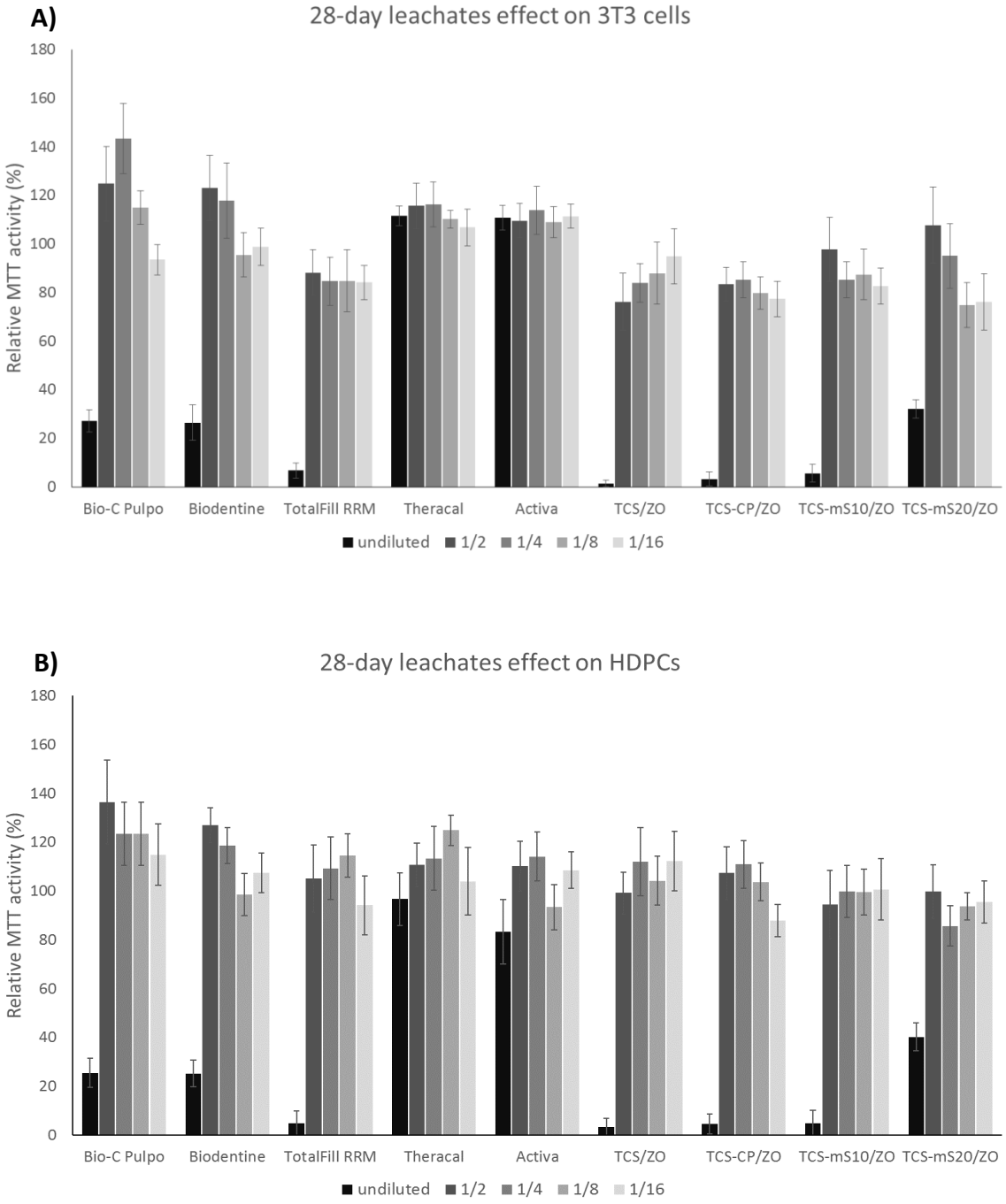


Figure 3.22 Mean relative MTT activity and standard deviation in 3T3 cell cultures (A) and HDPC cultures (B) following a 24-h exposure to 28-day eluates of commercial and prototype materials and their dilutions.

3.2.5.1.3 Transwell system

Results for the metabolic activity of cells exposed in a transwell model in a 96-cell culture plate are presented in Figure 3.23. The TotalFill RRM was the only material that did not affect the mitochondrial activity of both cell types and reported significantly higher values compared with all the other materials ($p<0.05$). The prototype material containing 20% micro-silica did not affect the activity of HDPCs (normalised activity>70%) [280], but it exerted a more moderate cytotoxic effect against the 3T3 cell cultures.

Bio-C Pulpo, Biodentine, Theracal, Activa and the remaining prototype materials were cytotoxic. No significant differences between them were detected and their effect on MTT values of 3T3 cells were evident ($p>0.05$). However, the Theracal, the radiopacified tricalcium silicate without any incorporation (TCS/ZO) and the prototype with calcium phosphate replacement (TCS-CP/ZO) exhibited improved biocompatibility compared with the other cytotoxic materials in HDPC cultures ($p<0.05$), but they remained overall cytotoxic (relative MTT activity <70%) [280]. Theracal was the only material that reported a significant difference in its effect between the two cell types, presenting lower MTT values in 3T3 cells compared with HDPCs ($p<0.05$).

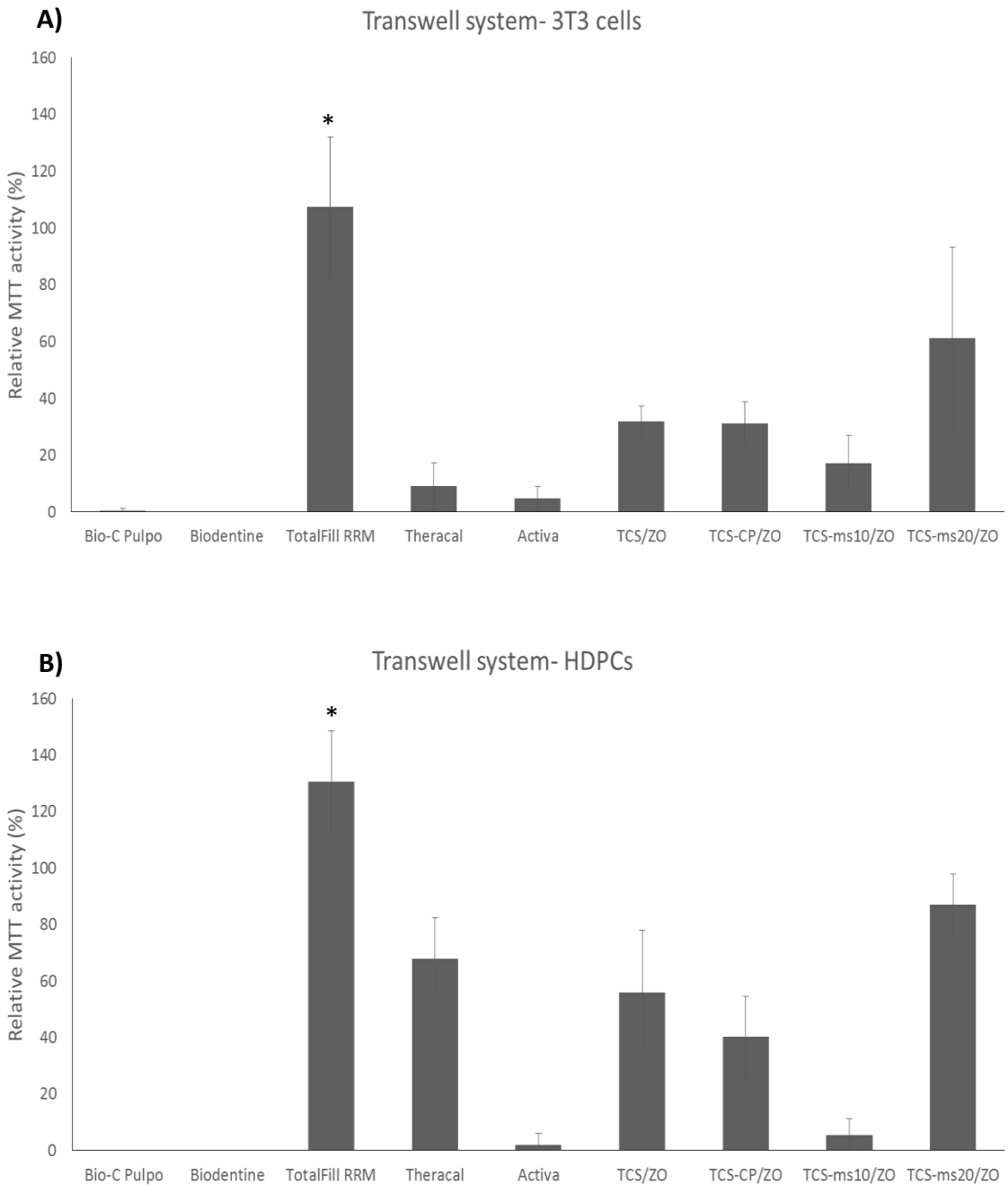


Figure 3.23 Mean relative MTT activity and standard deviation of 3T3 cell cultures (A) and HDPCs (B) following a 24-h exposure to a transwell system. Asterisk indicates significant difference in relative MTT activity from all other materials ($p<0.05$).

3.2.5.1.4 Effect of leachates on cell confluency and corresponding cell numbers

Values obtained for cell confluency following the indirect testing period provided false positive results in several materials due to sedimentation occurring upon the bottom of the well (Figure 3.24 A). The carry-over constituents could not be removed even after washing out the wells with PBS before assessment (Figure 3.24 B), and therefore this technique was considered non reproducible for testing leachates of TCS-based materials. Sedimentation was verified also in the inverted optical microscope (Figure 3.25).

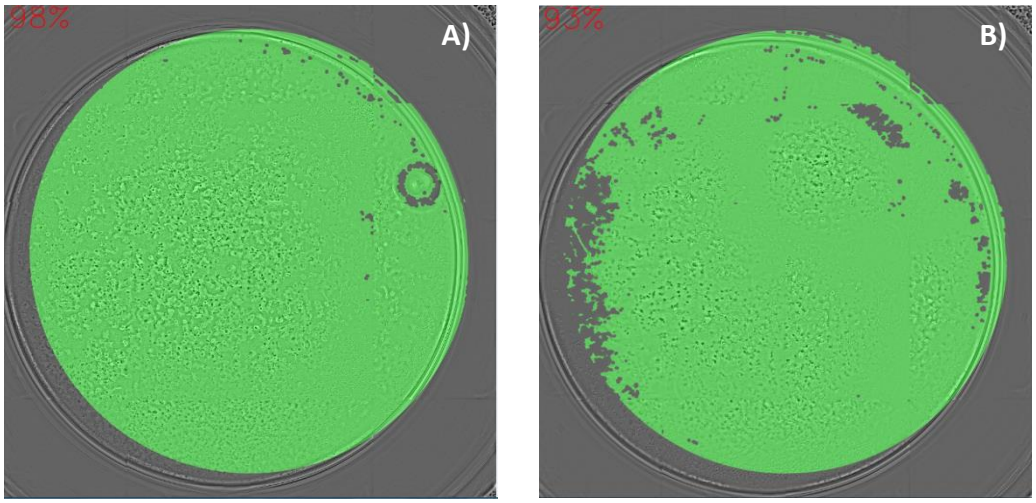


Figure 3.24 Images obtained from the multi-mode micro-plate reader following exposure of 3T3 cell cultures with the 28-day leachate of Bio-C Pulpo, before (A) and after washing out with PBS (B). Cell confluency cannot be assessed due to heavy sedimentation. Sedimentation occurring from undiluted leachates of TCS-based materials was similar with that presented for Bio-C Pulpo.

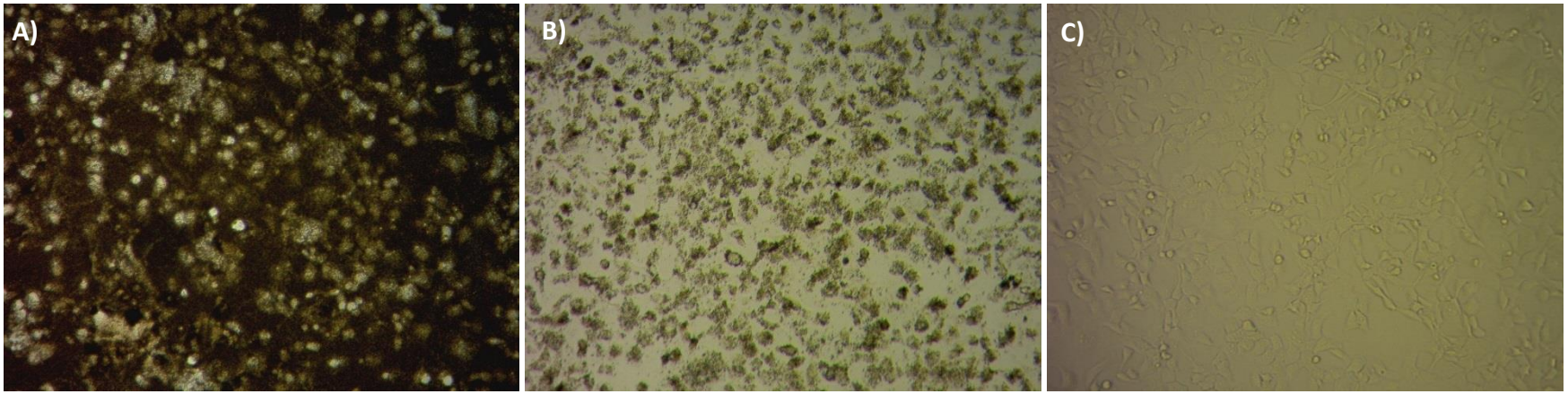


Figure 3.25 Sedimentation from carry-over compounds occurring from pure (A) and 1/2 dilution (B) of the 28-day eluate of Bio-C Pulpo on 3T3 cell cultures (10× magnification). 3T3 cell cultures after a 24-h exposure to HBSS (C). Images obtained from the inverted optical microscope (10× magnification).

3.2.6 Antibacterial assays- Inhibition of bacterial growth

3.2.6.1 Efficacy of 1-day eluates

Results from the MIC assays of 1-day eluates for *S. mutans* and *L. casei* are presented in Figure 3.26. The undiluted leachates of Bio-C Pulpo, TotalFill RRM and the prototype material containing calcium phosphate inhibited the growth of both bacterial species ($p<0.05$). The pure leachate of Bio-C Pulpo was significantly more antibacterial than all the other leachates against *S. mutans* ($p<0.05$) and remained antibacterial even after 2 two-fold dilutions of the pure eluate. However, its antibacterial activity was reduced following the first serial dilution ($p<0.05$). Against *L. casei*, the inhibitory effect of Bio-C Pulpo was also significantly greater compared with all other test leachates ($p<0.05$) except for the non-diluted eluate of TotalFill RRM ($p>0.05$).

The pure leachates of Biodentine, Theracal and TCS-mS10/ZO were antibacterial only against *S. mutans* ($p<0.05$). Activa, the radiopacified tricalcium silicate and the prototype material with 20% micro-silica incorporation did not present any antibacterial effect ($p>0.05$). Regarding the other diluted leachates, most of them had no effect on the bacterial growth while others exhibited an increase in the ODs. Specifically, diluted leachates of Bio-C Pulpo (1/16 for *S. mutans*, 1/8 for *L. casei*), Biodentine (1/2 for *S. mutans,* 1/8 and 1/16 for *L. casei*) and the radiopacified tricalcium silicate with 20% micro-silica (all dilutions for *L. casei*) enhanced bacterial proliferation ($p<0.05$).

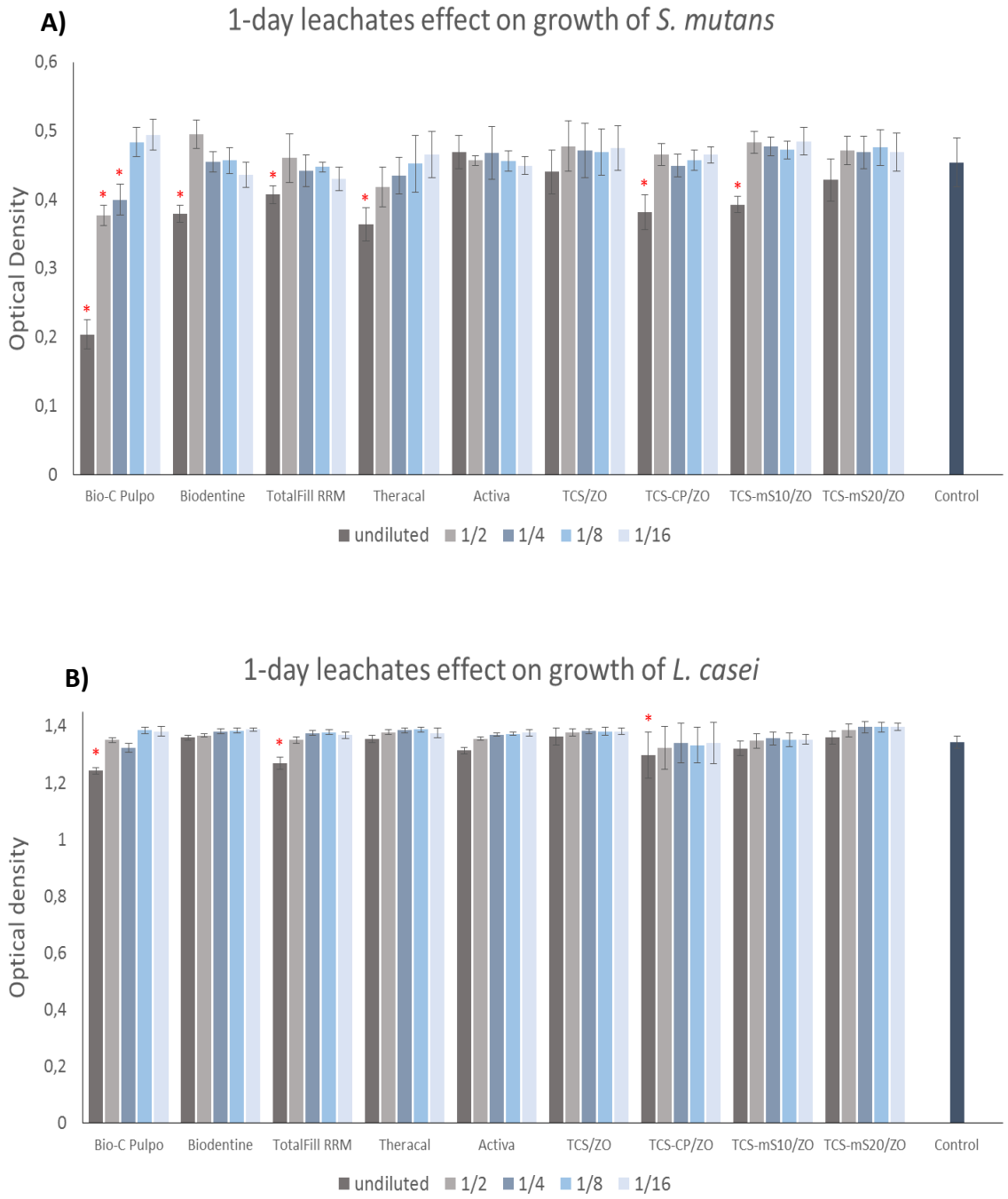


Figure 3.26 Mean optical density (OD) and standard deviation *S. mutans* (A) and *L. casei* (B) suspensions following overnight exposure to 1-day eluates of different concentrations of commercial and prototype materials. Asterisks indicate significant reduction of OD compared with the positive control ($p<0.05$).

3.2.6.2 Efficacy of 28-day eluates

Figure 3.27 shows results from the 28-day leachates on growth of the two bacterial species. Bio-C Pulpo exhibited the greatest inhibitory effect on the bacterial growth in both species, since its leachate remained antibacterial even after the third dilution ($p<0.05$). Pure concentrations of Biodentine, TotalFill RRM, Theracal and pure leachates of the radiopacified tricalcium silicate with or without incorporation of calcium phosphate or 10% micro-silica inhibited the growth of both bacterial species ($p<0.05$); the undiluted leachate of the prototype cement with 20% micro-silica incorporation reported a reduction only in the growth of *S. mutans* ($p<0.05$). Theracal remained antibacterial against *S. mutans* even after two dilutions of the neat leachate ($p<0.05$). The undiluted leachate of Activa inhibited the growth of *L. casei* ($p<0.05$), but it had no effect against *S. mutans* ($p>0.05$).

None of the other diluted eluates exhibited a significant effect on the proliferation of the bacterial species ($p>0.05$), while Biodentine's fourth two-fold dilution appeared to enhance the growth of *S. mutans* ($p<0.05$).

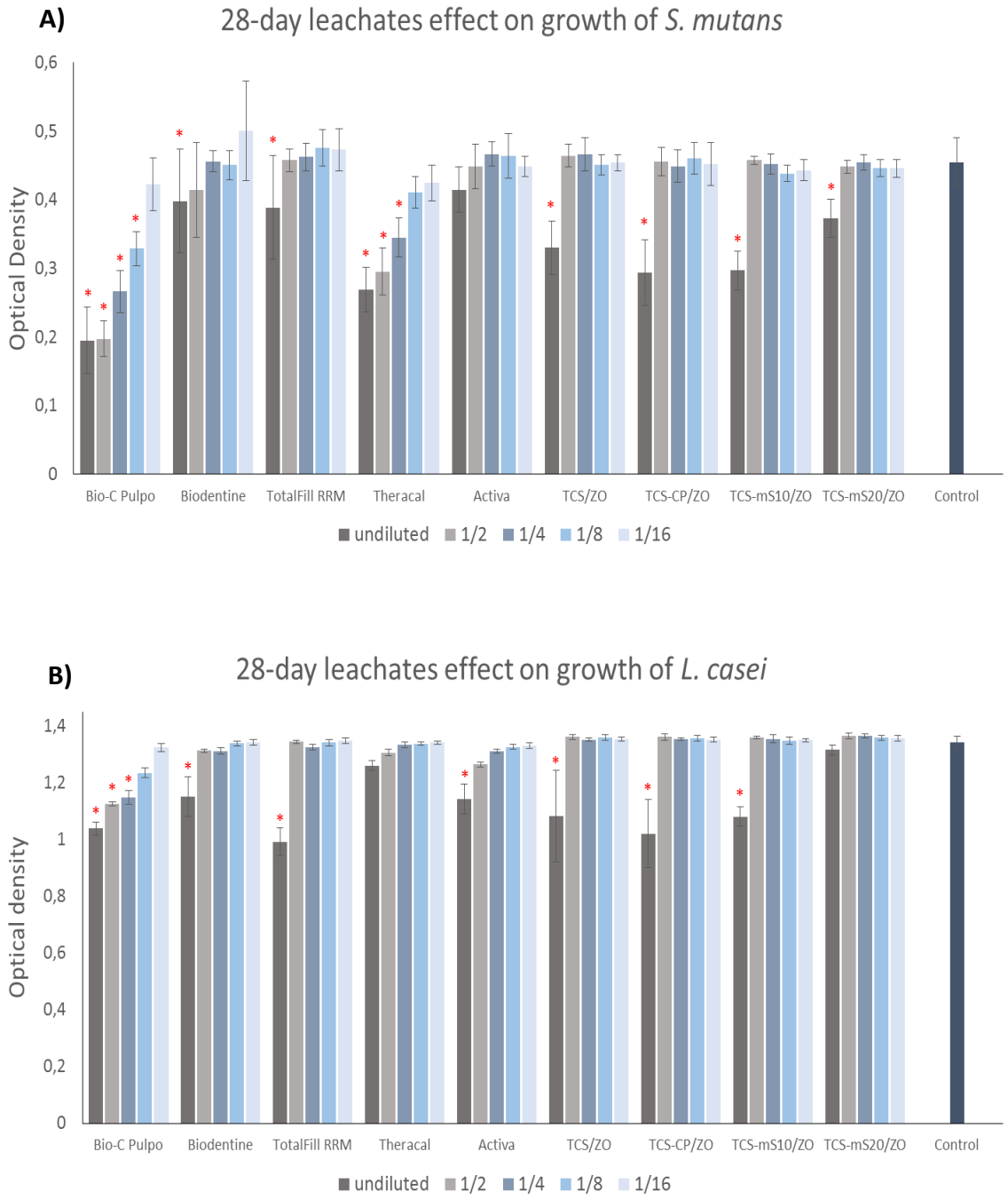


Figure 3.27 Mean optical density (OD) and standard deviation of inoculums of *S. mutans* (A) and *L. casei* (B) following overnight exposure to 28-day leachates of different concentrations of commercial and prototype materials. Asterisks indicate significant reduction of OD compared with the positive control ($p<0.05$).

3.2.7 Correlation tests

For cytotoxicity, a medium correlation coefficient ($0.3<r<0.49$) was reported in both cell types which was non-significant ($p>0.05$). In terms of antimicrobial effectiveness, a significant correlation was observed for both bacterial species studied ($p<0.05$).

CHAPTER 4

DISCUSSION

Sections 4.5.1 and 4.6.1 have been publised in Dental Materials as part of the discussion of the article (Koutroulis A, Batchelor H, Kuehne SA, Cooper PR, Camilleri J).

The author has performed the writing of these sections.

4.1 Selection of materials

The pulp-capping materials studied here were mainly based on a tricalcium silicate cement phase and radiopacifier. The commercial TCS-based materials tested contain different additives in their formulation which have been verified in the current study. The prototype cements tested were developed following incorporation of calcium phosphate monobasic, micro-silica and a series of radiopacifiers that are present in commercial TCS-based materials to identify the effect of these compounds in the properties of the classic formulation of a radiopacified cement.

More specifically, CP monobasic has been identified in all TotalFill products (endodontic sealer and root repair materials) [210, 287], while several new generation TCS-based materials contain additions of silicon oxide, such as Bio-C Pulpo, MTA Angelus and BioAggregate (Verio Dental Co, Vancouver, Canada) [128, 130, 171]. From the radiopacifiers available, zirconium oxide (ZO) was selected for use as it was one of the initial compounds to substitute for bismuth oxide in the original formulation of MTA [190, 288]. Notably, ZO is inert and biocompatible, without compromising the cement's properties [147] and was further used in the experiments here as it is present in the formulation of several commercial materials that were tested, namely Bio-C Pulpo [171], Biodentine [130], and TotalFill RRM [174]. Tantalum oxide is another compound that has adequately replaced bismuth oxide in the commercial hydraulic cement formulations either identified as the only radiopacifier present, such as in Bioaggregate [128] and Neo MTA Plus (Avalon Biomed Inc, Houston, USA) [128] or in a combination with ZO which is found in the TotalFill RRM products [174]. Lately,

calcium tungstate has been identified in the formulation of a resin-modified MTA-based endodontic sealer, MTA FIllapex (Angelus, Londrina, Brazil) [150]. Therefore, these radiopacifiers were also included in the experimental materials studied.

The prototype materials developed did not contain any setting accelerators or other additives for enhancement of physical properties in contrast to the commercial ones. Thus, a direct correlation between inclusion of specific additives and change in properties could be explored.

From the commercial materials tested, Biodentine is the material that introduced the second generation of TCS-based materials on the market following MTA, suggesting the use of pure calcium silicate cement over PC, substitution of bismuth oxide with inert zirconium oxide and incorporation of a setting accelerator (calcium chloride) and a water soluble polymer in the liquid form in an effort to enhance the materials' mechanical properties and improve their handling characteristics [190]. The TCS/ZO, which provides a simpler laboratory composition without inclusion of calcium carbonate in the powder and the additives in the liquid phase, namely the water-soluble polymer and calcium chloride was included in the study for comparison to Biodentine as it has previously been assessed [130, 180]. Overall, despite the use of the prototype materials to identify the role of particular compounds in modifications of chemical properties of the hydraulic cements, it is worth reporting that inclusion of the water-soluble polymers can independently affect chemical properties of the materials due to the reduction in the water demand [130] as was indicated in the results Section 3.1 and this will be discussed further below.

A significant amount of research has been undertaken in Biodentine, resulting in it being the second most studied hydraulic cement after MTA in terms of biological properties [229]. The material has been well characterised [7, 179, 180, 289] and is considered to have a good potential as a pulp capping agent due to its biocompatibility and stimulation of different types of pulp cell differentiation among other desirable physical properties [6, 186-190]. Therefore, it was included in the study as a control material.

Bio-C Pulpo is a recently developed material that has not been characterised so far. Only one study has been published assessing its biological effect [200]. The material contains silicon oxide in a non-specified concentration [171]. Therefore, two prototype materials with different concentrations of micro-silica incorporation (10% and 20%) were developed for comparison purposes. Typically, additions of silicon are used in concrete in order to enhance the strength of the mixture [168]. However, its presence can compromise the amount of the calcium hydroxide by-product. Following the initial reaction of calcium silicate with water which results in the formation of calcium silicate hydrate and calcium hydroxide, silicon oxide can react with the available calcium hydroxide favouring the production of more calcium silicate hydrate [172].

TotalFill RRM is a premixed TCS-based material, which is also marketed under the commercial names Endosequence RRM and iRoot BP. One main characteristic is the inclusion of a second cementitious phase (CP monobasic) for enhancement of physical, chemical and biological properties. However, results from studies in biphasic cements are controversial [223]. Notably, the amount of CP in TotalFilll RRM is

unknown. Taking into consideration a previous study that did not identify any phosphate in the elemental analysis by EDS of the unset putty formulation, it was postulated that the CP concentration is low [210]. Therefore, a 15% CP incorporation of the cementitious phase in the laboratory developed material was used for comparison purposes with the TotalFill RRM. In addition, preliminary studies in Section 2.2 assessing the w/p ratio in hydraulic cements, indicated a high water demand for incorporation of CP at high concentrations (data not shown) that could lead to flocculation of the mixture. Findings in the literature are also controversial whether the CP in TotalFill is amorphous or not. Xuereb *et al.* identified a CP phase in the diffractogram of a 14-day set TotalFill sealer [287], while no similar phase was identified in the surface of the unset putty formulation in an investigation carried out by Moinzadeh and coworkers [210].

Theracal is a single-paste light-curable material consisting of a cementitious phase (PC), a resin matrix and a glass phase [179]. The rationale behind the addition of the resin phase was to present an optimised material with the desirable chemical properties of PC in combination with the mechanical characteristics, handling and adhesive properties as well as command cure of a resin [167]. However, the use of Theracal in proximity with the pulp has been questioned, mainly due to the potential cytotoxicity induced by resin monomers [6], while a recent clinical study reported very low values of reparative dentine-like barrier formation (11%) following direct application in pulp tissues of caries-free human molar teeth [258]. The hydration of PC has also been shown to remain incomplete due to insufficient amount of water supply in the paste [180].

Finally, Activa is a resin-modified glass ionomer that serves as a base/liner. The manufacturer states that the material contains bioactive compounds that can release significant amounts of calcium and respond to environmental changes by diffusion of ions and adjustment of the pH [268]. The chemical composition of Activa has not been well characterised so far. Overall, this base/liner shall meet similar standards of biocompatibility and antibacterial effectiveness with the other pulp capping materials [23], even though it is only indicated for indirect pulp-capping [3, 268], as their leachable components can interact with the vital pulp tissue throughout diffusion inside the dentinal tubules. Furthermore, assessing properties of the two resin-containing materials, Theracal and Activa in the same project assisted in the evaluation of modifications of the performance of a resin-modified glass ionomer by inclusion of a cementitious phase.

4.2 Calcium hydroxide release and its correlation to biological activity

The role of calcium ions in proximity with the pulp tissue was highlighted in Chapter 1. In brief, leaching of calcium is considered an important parameter for endodontic materials as it has been correlated with the beneficial biological properties of the hydraulic cements. Calcium has been regarded to stimulate the differentiation potential of dental pulp stem cells to odontoblast-like cells in the site of the pulp-material interface [6, 35] and contribute to the induction of a mineralised matrix [36]. Overall, it enables dentine bridge formation [25, 35].

Even though the mechanism with which calcium acts to promote a biological response from the pulp system has been reported, no evidence regarding the desirable calcium leaching pattern is available [7]. Therefore, materials with different calcium leaching profile are usually tested using different biocompatibility and cell differentiation assays in order to assess a dose-response relationship between calcium ion release and desirable biologic stimuli. However, several limitations exist in the current approach, as the calcium ion values assessed *in vitro* do not reflect the exact concentrations that can be diffused throughout the dentinal tissues in the clinical scenario in cases of indirect pulp capping [39].

At the same time, abundance of hydroxyl ions can trigger pulp tissue healing potentially by inducing an initial necrotic layer in the field of placement [6]. It was thus interesting to investigate a potential correlation of the chemical leaching profile with the biological performance in early and late stages of hydration and identify whether a depletion of calcium and hydroxyl ions would occur in the tested time period as a result of further reaction of silicon oxide with calcium hydroxide by-product. Additionally, the effect of inclusion of other compounds, namely calcium phosphate or resins was assessed.

4.3 Antimicrobial potential of pulp capping materials

During removal of the carious lesion and preparation of the operating field for placement of the repair material in proximity with the pulp tissue, bacteria such as

Streptococci and *Lactobacilli* can remain due to incomplete excavation of the caries affected dentine [290, 291]. Additionally, it has been well described that the application of a composite restoration above TCS-based materials such as Biodentine (sandwich technique) cannot provide a hermetic seal in the material interface [292]. Notably, deterioration of chemical properties and significant microleakage have been reported, due to chemical modifications induced in the hydraulic cements during the procedures of acid etching prior to placement of a composite material [289]. Conversely, the light-curable Theracal has reported significantly better values of bonding to the overlying composite restorations [292].

Overall, pulp capping materials should present antimicrobial properties to effectively kill bacteria and prevent recontamination as a result of microleakage. Notably the high pH in the field of application has been regarded to provide a bactericidal effect in TCS-based materials [6]. However, despite the recognised distinct mechanisms of the antimicrobial effectiveness of pH, Estrela *et al.* suggested that damages to bacterial enzymatic activation which are introduced by the alkaline pH can be reversible should the pH returns to normal values over time [33]. Therefore, induction of a long-lasting alkaline environment is a desirable property in pulp capping cements.

4.4 Determination of the effect of the w/p ratio

During the initial stages of devising the experimental plan for the current project, it was necessary to determine the amount of water needed to hydrate tricalcium silicate-based materials with incorporation of different chemical

compounds. So far, properties of experimental materials in the literature are assessed by either using a standard 0.30-0.35 liquid: powder ratio [159, 166, 222], or using pilot values to provide a visually acceptable mixture [160, 273, 293, 294], while the w/p ratio might also not be reported by the researchers [223]. Despite this approach, there is strong evidence that alterations in the water amount can affect the physicochemical properties of cements [295, 296]. Standardisation of the w/p ratio and further assessment of the effect of this adjustment on chemical characteristics of the cement was therefore of high importance.

Following this adjustment, both commercial and experimental materials were thoroughly characterised and a potential interrelationship between the leaching profile and biological and antimicrobial properties was investigated.

4.5 Methodological design

The w/p ratios were adjusted using data obtained from analysis on a shear rheometer. Comparative flowability values prior to and following the adjustment of the water demand were measured according to ISO 6876:2012 indications [277]. The chemical characterisation of the materials was performed with SEM, EDS and XRD analyses. Calcium leaching was assessed with ICP-OES. Materials that were developed with the new w/p ratio were evaluated for radio-opacity values with ISO 6876:2012 specifications [277]. For the second part of the study, condition of TCS-based materials prior to immersion in soaking solution was determined with an initial setting time according to ASTM C266-18 instructions [279]; FT-IR spectroscopy was performed in order to assess the extent of polymerisation of the resin containing materials. The pH

of leachates was monitored weekly. For biological and antimicrobial experiments indirect leachate assays were performed with MTT and MIC respectively to correlate their performance with their chemical profile.

4.5.1 Viscosity measurements

The use of a rheometer has been proposed by several previous studies for the assessment of setting and handling properties [297] of endodontic sealers [298, 299], impression materials [300] and composites [301-304]. For endodontic sealers [305, 306], the materials are loaded between two parallel plates and different shear strain tests are applied. The change in viscosity values as a result of the deformation of the material can thus be monitored. In the present study, a rheometer was used to determine viscosity values for increasing values of shear rate for each prototype material with different additives included. Portland cement was mixed with a 0.35 w/p ratio as a control, since this mixture has the most acceptable handling properties for clinical use and the process of its hydration reaction has been extensively studied [307].

Parallel plate geometry was used as it is preferred for slurries and pastes compared to the cone and plate model as solid materials can accumulate under the "point" of the cone plate which can affect interpretation of the results. Additionally, parallel plates provide flexibility in selecting a gap to account for the particulate matter in the sample. Within this configuration the shear rate varies due to the gap size selected and according to the radius of the plate [308, 309]. A cross-hatched plate design was used to minimise slip. A standard amount of water was added to each test

material immediately after mixing to render them adequately flowable and ensure reproducibility of the experiments. This was considered important since the maximum loading force applied by the upper plate (50 N) was not adequate enough to achieve an even contact surface throughout the mixture due to the cement's stiffness. Addition of a water-soluble plasticiser to the mixture was also considered as an alternative option, as it has been suggested to improve flowability of hydraulic cements [158, 310]. However, it was observed that the effect of a standard plasticiser addition had varying effects in flowability of mixtures, while no adequate sample loading could be achieved in all samples. The volume of water addition was determined through pilot experiments evaluating reproducibility in execution and absence of flocculation in the final mixture in each material. Overall, the use of a smaller diameter cross-hatched upper plate (20-25 mm) might have allowed the undertaking of experiments without any water addition as higher pressure values would have been applied to the mixture during the loading procedure.

The temperature was established at 20^{o}C, as higher temperatures could potentially result in rapid dehydration of the test samples, which would lead to variability in the data. As the primary purpose of the rheology experiments was to compare test formulations to a reference material through matching rheological performance, the temperature was set to ensure reproducible data. Such temperatures have been reported in previous published work [311]. Additionally, although a 2-min flow-sweep test was initially designed, it was noted that viscosity values for shear rate values above 20 s^{-1} could result in separation of the material with

the upper plate and they were thus not significant for evaluation. Therefore, results were assessed for the first 23 s of the experiment.

ISO 6876:2012 flowability tests were also performed to compare results from the rheological assessment with a standard procedure for evaluation of flowability [277]. ISO flowability tests provide information on the compressed diameter of the material after a specific amount of load is applied. In contrast, the protocol design of the rheological adjustment is dynamic; apparent viscosity values are calculated as progressively higher shear strain is applied to the material. Moreover, the ISO flowability tests are designed for endodontic sealers; such materials are expected to present relatively higher flowability compared with hydraulic cements [277]. To overcome these limitations, a new method using micro-computed tomography has also been proposed recently for reproducible flowability assays of root-repair materials [271]. This model is applied in 3 dimensions and could thus be potentially more relevant to the clinical scenario.

4.5.2 Chemical characterisation of materials and leachates

The techniques used to characterise the materials' microstructure and composition provided precise information regarding the arrangement of hydrated products and non-hydrated particles and radiopacifiers in the bulk of each set material (SEM), their elemental composition (EDS) and the mineral phases identified in them (XRD). These methods have been extensively used in the literature for assessment of chemical composition of dental materials [312].

Specimens were observed under the back-scatter detector of the SEM, as this signal obtained enables the monitoring of the hydration process of the hydraulic cements, by identifying the different structures in the bulk of the material [312]. The image contrast which is generated is a result of reflected electrons (back-scattered) from the different atoms in the surface of the material [313]. Notably, the presence of non-hydrated cement particles surrounded by hydration products (reaction rims) as well as radiopacifiers and potentially other compounds serving as fillers was identified. The EDS analysis provided information on the elemental composition of selected particles or designated areas.

The prototype materials with the adjusted w/p ratio were viewed under the SEM at an early stage of the hydration procedure to monitor the hydration process. Characterisation of set materials after 28 days of immersion in HBSS for commercial and prototype materials was performed as it has been proposed that TCS-based cements reach their almost complete hydration state after this period [57].

X-ray diffraction was used for crystalline phase analysis and differences in the intensity of the formed peaks were evaluated. The importance of the presence of mineral structures in the hydraulic cements has been highlighted as they are decisive parameters for the long-term performance of the material in the application site [57]. The extent of hydration can also be monitored semi-quantitatively, by evaluating the presence of peaks for mineralised hydration products such as calcium hydroxide or the absence of initial constituents of the unhydrated materials, namely TCS [312].

FT-IR spectroscopy examined the degree of polymerisation in the resin-containing materials after the indicated time of polymerisation. Leaching out of

potentially cytotoxic monomers can occur if these remain unpolymerised [314], while mechanical properties of the material can also be compromised [315]. Reduction in the intensity of the double bond in $–CH=CH_2$ was followed throughout time, corresponding to a percentage conversion of monomers into a polymer. This technique has been vastly applied for measuring the DC [316]. However, a potential limitation exists due to the background noise in the absorption at the specific wavelength that could potentially lead to variations in results or underestimated values of DC in the presence of unreacted C=C fillers [314, 316].

Analysis of the calcium release in the soaking solution provided a holistic approach in the characterisation assays, quantifying the calcium concentration deriving from the by-products of hydration. ICP-OES was used for testing as it is one of the most reliable available methods [317], and is significantly more accurate in comparison with other techniques commonly applied, namely the use of a calcium probe [223]. Specifically, assessment of the elemental concentration in a solution with ICP-OES is carried out by heating at high temperatures part of the samples, leading to excitement of elements in the solution. As a result, elements irradiate in specific wavelengths. As the intensities of emissions are unique for each element, these can be identified as well as quantified with high precision [92, 318].

For the investigation of the effect of the water adjustment on the prototype materials, comparative evaluation of crystal phases and calcium leaching of materials with a similar composition and different w/p ratios was carried out after a 7-day incubation period. The pure TCS mixed in a 0.35 w/p ratio served as a reference material in these comparisons. The rationale was to identify any differences in the

amount of calcium hydroxide by-product formed and its release into the local environment. Consequently, the materials that were studied in the 28-day immersion period were assessed for the presence of crystalline phases at the same stage with the other characterisation assays (28 days) to provide a clear insight into their chemical composition and characteristics.

Additionally, the pH of the soaking solution of materials was monitored after one day and then weekly for the 28-day period. It is worth reporting however, that the material pellets were not re-immersed in fresh medium following each measurement. A saturation effect could exist, therefore not allowing accurate interpretation of the alkalinisation potential of an aged material in the extract solution. Thus, conditions under which the pH of materials were tested cannot provide directly translatable results to the clinical environment where tissue fluids can be drained by the lymph system of the dental pulp in an effort to preserve a fluid equilibrium [319].

4.5.3 Radio-opacity of prototype materials following the water adjustment

Evidence derived from the literature indicates that an increase in the w/p ratio is negatively correlated with the radio-opacity of hydraulic cements [296]. Therefore, it was considered important also from a clinical viewpoint to assess whether materials remain adequately radiopaque. Radio-opacity of the materials after modifying the water amount was evaluated using ISO 6876:2012 instructions [277] and interpretation of results was performed using an appropriate software correlating pixel

values of the digital radiograph to the radio-opacity measurement unit (thickness of aluminium).

4.5.4 Condition of materials prior to extraction

For the materials used for assessment of their chemical, biological and antibacterial properties of their leachates in the 1- and 28-day periods, immersion of the TCS-based ones in the solution began as soon as they reached their initial setting time. This was considered more relevant to the clinical scenario for the application of a pulp capping material in proximity with the pulp system, as they begin to interact immediately with the tissue fluids. Thus, initial setting time was preferred rather than the final endpoint. Conversely, in several studies reported in the literature, immersion of materials in the extraction medium takes place following a prolonged incubation period during which materials are allowed to set [201, 204, 246].

A Gillmore-needle model according to ASTM C266-18 specifications was used as these standards define both initial and final setting time periods for hydraulic cements [279]. The ISO 6876:2012 and ISO 9917-1:2007 setting time assays for root canal filling materials and water-based cements respectively are similar approaches to that of the experimental procedure described in the ASTM but they do not separate an initial from a final setting state [277, 279, 320].

The light-curable materials were immersed immediately after the indicated period of photo-polymerisation.

4.5.5 Biological and antimicrobial assays

In the present project, biocompatibility and antibacterial assays were performed on materials' leachates. It has been well described that eluates of endodontic materials interact with the root canal system, the surrounding tissues and bacteria [321]. The indirect method of assessing the eluates was considered more relevant to the scopes of the current project, as the amount of leached calcium hydroxide into the extraction solution could be directly correlated to any potential antimicrobial or cytotoxic behaviour, without interference of the surface characteristics of materials with the results [72, 199, 312, 322]. HBSS was used as the extraction vehicle since its inorganic composition of ions is similar to that of human blood plasma [323] and it has been vastly used in similar biocompatibility assays allowing comparison of the current study with other scientific work [7, 223]. Additionally, the use of water as extraction vehicle is not clinically relevant. The same extract vehicle was used throughout the chemical characterisation and the biological and antimicrobial assays as a variation in leaching ability exists between different media [324].

Testing was performed after 1 and 28 days of leaching, same as the calcium release analysis, to assess how potential changes in elemental composition affect the behaviour of leachates during early and late stages of the hydration process.

4.5.5.1 Biocompatibility experiments

The biocompatibility of materials' eluates was assessed using two cell types and a commonly employed cytotoxicity assay. Both 3T3 cell cultures and HDPCs were tested in order to investigate if there was any difference in the cytotoxic effect between a cell line and primary cell cultures isolated from the pulp of a healthy young patient. The 3T3 cells are relatively easily cultured, relatively resistant and overall similar in behaviour to the L299 cell line which is proposed by ISO 10993-5:2009 for cytotoxicity assays [280]. They were thus used instead of the L299 cell cultures due to their availability. Additionally, execution of experiments with HDPCs provided a more clinically relevant testing condition.

The MTT assay is considered the gold standard for initial screening of material cytotoxicity [312]. The amount of the blue-violet coloured formazan produced from the metabolic conversion of the mitochondrial dehydrogenase which was added to the cell cultures was solubilised and consequently quantified assessing the colour intensity using photo-spectronomy. Therefore, results provided information regarding the extent to which the cell metabolic activity was affected, correlating the optical density measured to cell viability [280]. However, an important limitation of this assay is its sensitivity: reduction of the cell mitochondrial activity which results in less formazan production occurs during the stage of cellular apoptosis [325]; thus, results cannot be directly translated to true cell cytotoxicity but they can indicate cell death. The test therefore might underestimate the cytotoxic potential of the tested agents.

Two different types of indirect contact test were assessed. The first type of MTT assay for 1-day and 28-day leachates was performed according to ISO 10993-

5:2009 instructions [280], to obtain results more easily comparable with the literature. The undiluted eluates that were tested were also assessed for pH and calcium release (see above). Results could therefore be interpreted in respect to their chemical profile. The use of the transwell system provided an indication of the cytotoxicity of materials in a more clinically relevant scenario, since the outflux of by-products commenced as soon as the materials were compacted upon the bottom of the well and were immediately immersed in an equal amount of HBSS and cell growth medium (DMEMsup-10FBS). However, direct comparison of results from the conventional MTT assay with those from the transwell method could not be carried out due to alterations in the experimental parameters, namely the differences in the surface area to volume ratio in each procedure and alterations in the extract vehicle. Leaching for the materials tested in the former was carried out solely in HBSS while an equal volume of HBSS and DMEMsup-10FBS served as extract vehicle in the latter. Notably, leaching in DMEM has been reported to be higher than in HBSS [324]. Therefore, differences in results between the two tests could be attributed to the different conditions mentioned.

Consequently, a new method to assess the effect of leachates on cell proliferation was also used. An equation specific to each cell type translating cell confluency values to cell numbers was created, but this failed to provide reproducible results for all the eluates studied. Sedimentation of constituents that overlapped the cell-layer led to false positive results of confluency following assessment with a micro-plate reader. This effect was mainly evident in the TCS-based materials. Constituents passed through the 0.22 μm filter pore and could not be effectively removed from the

wells following a washing step with PBS. Centrifugation of the leachates prior to the filtering process up to 10,000 rpm also did not have any effect on limiting the carry-over. Therefore, it was concluded that the current method cannot be used for TCS-based materials.

4.5.5.2 Antimicrobial assays

Studies reported in the literature have mainly assessed antibacterial activity using the ADT [198, 217, 266, 326] or different methods of direct contact tests of materials with planktonic bacteria or biofilms [68, 327]. ADT is a relatively low cost and simple to perform technique [328]. However, this test exhibits several limitations, the most important of which is that results are dependent on the material's diffusion ability through the agar. Therefore its use is no longer recommended [329].

In the current study, an indirect test was selected for the evaluation of the antimicrobial potential of the eluates. The MIC assay is a relatively inexpensive and reproducible technique that can recognise the lowest concentration of an antibacterial agent able to inhibit the growth of a microorganism [330]. Dilutions of the test leachates were also assessed to identify the antimicrobial strength of the test eluates in a similar rationale with the cytotoxicity assays. Interestingly, only two studies have evaluated the antimicrobial effect of leachates thus far [7, 67]. Despite the advantages of MIC, this assay can only provide an initial indication of the antimicrobial effect of an agent against planktonically grown bacteria. In clinical cases, endodontic pathology is biofilm induced [331, 332] and therefore materials should be tested also against multispecies biofilm communities.

For the experiments reported here, two caries-associated bacteria were selected. *S. mutans* is an early coloniser in the dental plaque biofilm and contributes significantly in biofilm maturation communicating with neighbouring bacteria by secretion of signal peptides, a process known as quorum sensing [285]. Additionally, the role of *Lactobacillus* species in the progression of dentinal caries has been well described [286, 333]. Notably, *Lactobacilli* have been isolated in higher concentrations in active dentinal lesions [334]. Overall, elimination of these microorganisms through diffusion of leachable elements in the dentinal tubules could have a detrimental effect both on maturation and metabolic activity of the biofilm.

Bacteria were grown overnight prior to exposure to the test leachates. Differences in optical density in growth between the two bacterial species can be attributed to different nutrient capacities between the two growth media used.

4.6 Interpretation of results

4.6.1 Adjustment of the w/p ratio on hydraulic cements and the effect on chemical properties

4.6.1.1 Rheological assessment and comparative evaluation of cements' properties

Data obtained from the rheological experiments showed an increase in the water demand when calcium phosphate, micro-silica or tantalum oxide was incorporated into the material. Replacement of zirconium oxide did not affect the

water amount and resulted in similar viscosity values compared with the unmodified cement, while calcium tungstate decreased the water demand. Apart from zirconium oxide, a standard percentage change of the w/p ratio was determined when a 30% replacement of the radiopacifiers was incorporated compared with the unradiopacified cement (Table 3.2). Thus, the hypothesis that different compounds will not alter the water demand of the cement was rejected. Results from the compression glass plate analyses demonstrated a significant change in flowability values of most prototype materials after the adjustment of the w/p ratio, especially for the calcium phosphate and micro-silica containing cements ($p<0.05$) (Table 3.3). However, comparisons with the unradiopacified cement were only in partial agreement with results obtained using the rheometer. Therefore, the pattern in the adjustment of the w/p ratio could not be supported in all cases. This can partially be attributed to the lower sensitivity of the compression glass plate test [298].

In terms of mineral structures identified in the cements, all materials formed calcium hydroxide while the minor peaks for tricalcium silicate indicated that a significant part of the hydration reaction had occurred by 7 days. The adjustment of the w/p ratio resulted in differences in the calcium hydroxide peaks mainly for the tantalum containing materials, where an increase of the water amount included in the mixture was correlated with higher intensity peaks. No differences in the diffraction patterns were detected in the 10% micro-silica replacement. Micro-silica affects material hydration and calcium ion release in the long term as described above [172]. The current testing was performed after 7 days so the effect of the micro-silica on the calcium ion release may have been masked by the short-term evaluation period. In a

similar pattern, increase of the w/p ratio resulted in higher calcium release. This is in accordance with a previous study which reported a positive correlation between the water amount in the mixture and calcium release in MTA [296].

Overall, the hydration mechanism was altered after the replacement of calcium phosphate and micro-silica as is evidenced by the micrographs (Figures 3.8, 3.9, 3.10). The different radiopacifiers were organised throughout the bulk of the material or spread as independent particles, especially following calcium tungstate inclusion. Finally, all the materials were adequately radiopaque after modification of the water amount according to ISO 6876:2012 specifications [277], subsequently they all have the potential of clinical application.

4.6.1.2 Outcome from the w/p adjustment

Results from the physicochemical analyses indicated that the null hypothesis regarding the modification of material's properties following the change in the hydraulic cement's water demand can be partially rejected. The effect of different amounts of water on the physical properties of Portland cement has been described previously [276]. Therefore, this should always be taken into consideration when evaluation of physicochemical properties of TCS-based cements is carried out after addition of different compounds and radiopacifiers. As a consequence, a potential alteration in characteristics could be incorrectly attributed to the additives. It is also evident that the powder to liquid proportions suggested by the manufacturer, should be strictly followed.

In conclusion, the rheological assessment provides a reproducible method for the calculation of the water amount in hydraulic cements by establishing similar viscosity values using a reference material. The water demand for materials which include radiopacifiers and additives varies depending on the type of radiopacifier and additive. However, the amount of water demand for each cement is affected by the size of the cement particles [85]; therefore it cannot be determined *a priori*.

4.6.2 Assessment of properties of commercial and prototype pulp capping cements

Results from the characterisation assays indicated that the tricalcium silicate-based materials, although reporting a similar basic chemical composition with few alterations in incorporation of different compounds, exhibit significant differences in their hydration profile, mineral structure as well as their calcium leaching profile. The reactivity of leachates in terms of cytotoxicity and antimicrobial effectiveness was enhanced in the TCS-based materials between the two periods studied.

4.6.2.1 Correlation of calcium release with the reactivity of eluates

Calcium ion release affected positively the antibacterial effectiveness of eluates, while it had a medium non-significant correlation with cytotoxicity. Therefore, an escalating reactivity was reported in leachates of TCS-based materials between the early and late stages of hydration.

The relationship of the hydroxyl ions with biological properties and antimicrobial performance is also well-known [6]. However, as the pH of most TCS-based materials appeared saturated, a correlation analysis with the pH would not provide clear conclusions. Overall, calcium ions leached from hydraulic cements derive mainly from dissolution of the calcium hydroxide by-product [5]. Therefore, an increase in calcium release is also indicative of higher hydroxyl ion diffusion as well.

4.6.2.2 Analysis of chemical, biological and antimicrobial properties of materials

4.6.2.2.1 Bio-C Pulpo

Even though Bio-C Pulpo had the typical microstructural image of a Portland cement-based material, it did not exhibit any crystalline calcium hydroxide peak in its diffractogram, but only a low intensity peak for calcium hydride, possibly as a result of the dehydration of calcium hydroxide. Silicon oxide, which is added to the material, was evident also in the XRD analysis.

As further reaction of the silicon oxide with the calcium hydroxide by-product has been suggested to occur in another commercial material [128], further investigation at the early stages of hydration of Bio-C Pulpo would assist in determining whether initial Portlandite peaks are formed and are consequently depleted. However, the calcium ion release was the highest even at early ages, which consequently plateaued, possibly due to reaction with the silicon oxide. Inclusion of calcium chloride as a setting accelerator in the Bio-C Pulpo has been reported to enhance calcium leaching as well [154].

The high values of calcium release and increased alkalinisation from the first 24 h observed in Bio-C Pulpo resulted in a reactive eluate, which induced initial moderate cytotoxicity and exhibited antibacterial activity, particularly strong against *S. mutans* (1/2 and 1/4 dilutions were also antibacterial). In the transwell system, Bio-C Pulpo exposure resulted in cell death, providing an indication of the aggressive leaching taking place during the first few minutes of setting reaction. Despite the minor differences in calcium release and pH values of the 1- and 28-day eluates, the later stage increased cytotoxicity. A strong antibacterial potential was also observed at the 28-day leachate against both bacterial species as several of its dilutions remained effective (1/2, 1/4, 1/8 dilutions against *S. mutans*, 1/2, 1/4 dilutions against *L. casei*). The high reactivity of the 28-day leachate could be attributed to saturation of hydroxyl ions. As Bio-C Pulpo was recently developed, the only study that has been published so far assessing its biological properties has been performed in a rat model and therefore results cannot be easily compared with the findings from the current *in vitro* investigation [200]. In addition, this is the first study to assess its antibacterial properties.

4.6.2.2.2 Biodentine and the radiopacified TCS

Biodentine presented peaks for calcium hydroxide and TCS in its diffractogram. These data indicate that the material's cementitious phase had not reacted completely, although findings from the SEM analysis showed that extended hydration had occurred with a dense matrix of by-products surrounding few unhydrated cement particles. The material has previously been thoroughly characterised and results of the

current study were in alignment with reports from the literature [130, 335]. The micrograph of the radiopacified TCS (TCS/ZO) revealed the presence of more unhydrated cement particles. The difference in the extent of hydration between these two chemically relevant materials has been mainly attributed to the presence of calcium chloride in the Biodentine [180]. Similarly, the optimisation in Biodentine resulted in a moderately improved calcium leaching profile compared with the TCS/ZO, as it has been reported previously [180]. It is also worth noting that the amount of zirconium oxide in the prototype material (30% inclusion) is significantly higher than that included in Biodentine (5%) [130]. The amount of active compounds that are incorporated in Biodentine's formulation are therefore higher, as TCS comprises 80% of the mineral phases in the powder of the material, while calcium carbonate is also evident (15%). The latter is regarded to accelerate the hydration reaction as well [170].

Biodentine's 1-day leachates appeared biocompatible and moderately antibacterial against *S. mutans,* with no effect on growth of *L. casei*. Absence of antimicrobial effectiveness of Biodentine's 1-day leachate has previously been reported in the literature against a multispecies bacterial suspension [7]. In a similar pattern for the radiopacified TCS, even though a cytotoxic effect was induced in 3T3 cell cultures, its effect in HDPC cultures was moderate, with viability values above the cytotoxicity limit that has been suggested by ISO 10993-5:2009 (70%) [280]. The statement by ISO, however, which appears to determine the cytotoxic potential in absolute values, is not supported with any scientific evidence. Additionally, no antimicrobial effectiveness was observed for the 1-day eluate of TCS/ZO. Overall, it can be postulated that these 1-day eluates exhibited acceptable values of biocompatibility

and negligible antibacterial activity due to their similar alkalinisation potential. It has been established that high alkalinisation due to hydroxyl diffusion causes an initial necrotic response from cells in proximity with the material [6]. Thus, after the pH exceeds a critical level, a significant cytotoxic effect could be triggered [216]. Although both Biodentine and TCS/ZO induced a highly alkaline environment, their pH rose significantly after 14 days and thus their cytotoxic potential and bacterial inhibitory effect was increased in the 28-day eluate. Furthermore, the presence of calcium carbonate in the Biodentine might have as well contributed to the moderately lower 1-day pH values [336] comparing to TCS/ZO resulting thus to excellent initial biocompatibility values. The significant overall elevation of the pH in Biodentine, Theracal and the prototype TCS-based materials has been reported in several studies in the literature [7, 181, 223].

Interestingly, Biodentine's fresh leachate behaved more aggressively in the transwell system, possibly due to the accelerated hydration occurring compared with the experimental material, as was also indicated for Bio-C Pulpo (see above). The Biodentine reported initial setting in 12 min while TCS/ZO set initially after 36 min. It has been also shown that the heat flux recorded in Biodentine during the setting reaction is much higher and more aggressive than that of a similar composition prototype material TCS with 20% zirconium oxide [130]. Results from the literature regarding biocompatibility of Biodentine's fresh leachates are also contradictory [7, 219, 337]. These differences can be partially attributed to different cell types being tested and alterations in the experimental design and extract conditions as is discussed in Section 4.5.5.1.

Overall, in the pulp system, induction of an initial necrotic surface in the pulp-material interface can be beneficial, as it can trigger a series of healing events in the underlying vital pulp tissue [27]. It is, however, of importance that the induced stimulus is mild and can shift the balance towards the regeneration process [6]. Thus, a moderate decrease in the MTT activity as shown by several leachates in this study could indicate better biological response in the long-term in *in-vivo* conditions than an inert activity.

4.6.2.2.3 TotalFill RRM

TotalFill RRM is a biphasic cement containing calcium silicate and CP in an unknown concentration. Characterisation of the 28-day aged sample revealed the presence of calcium hydroxide peaks as well as a calcium phosphate phase, which are in accordance with findings from an investigation performed using a 14-day sample of the TotalFill sealer [287]. The calcium carbonate phase which was depicted in the XRD analysis was attributed to potential environmental carbonation of the tested sample following reaction of the calcium hydroxide by-product and the atmospheric carbon dioxide, as observations of the bulk of the material with SEM showed no evidence of associated particles. The material presented an enhanced leaching profile, as high amounts of calcium release and a strong alkalinisation potential were reported from the first day in the soaking solution and were consequently increased. Calcium release was significantly higher than Biodentine, the prototype cements and the resin-modified materials.

The enhanced leaching profile correlated with an escalating cytotoxic potential and stable antibacterial performance. Overall, both 1- and 28-day eluates induced cytotoxicity and bacterial inhibition, with values reporting a more aggressive behaviour in the 28-day eluate. A stronger antibacterial potential was reported in the 1-day immersion period in comparison with Biodentine and TCS/ZO. Notably, as evidenced in the other TCS-based materials as well, reactivity of eluates was positively correlated with the increase in the pH values, albeit the saturation potential could not provide direct evidence in all cases.

Interestingly, TotalFill RRM behaved in a completely different pattern in the transwell assays, as it showed high values of biocompatibility and even enhancement of mitochondrial activity. It is worth reporting that the setting time of TotalFill RRM was found to be 24.7 h, exceeding by far the 2-h period suggested in the data sheet [173]. Consequently, the material remained unset after the end of the experiment. Elution therefore could have occurred in the medium mainly by the dissolution of the calcium phosphate and not due to the by-products of hydration.

Observations in the literature regarding the setting time of TotalFill materials have showed big variation in results, with values ranging from several hours to more than 30 days [338-341]. Assessment of the chemical composition of the tested solutions in the transwell system could elucidate further the differences in the material behaviour between the biological assays.

Despite the fact that higher biocompatibility values have been overall reported for TotalFill RRM in the literature [201, 204, 205, 246], it should be highlighted that in several studies, the material was allowed to set for significantly longer incubation

periods before being immersed in the extract solutions, such as 48 h or even 7 days [201, 204, 246]. Thus, leaching in the extract solution could potentially be altered. On the contrary, in the current study, TotalFill RRM was immersed following the initial setting period (24.7 h) which was calculated according to ASTM C266-18 specifications [279].

4.6.2.2.4 Resin-based cements

The resin modification in Theracal induced significant alterations in the material hydration. Notably, no formation of calcium hydroxide was observed. Additionally, the micrograph of the material included several unhydrated cement particles. These findings have been previously reported [179, 180]. Overall, the material's matrix was complex and apart from the elements deriving from PC and the radiopacifier, the identification of strontium in the EDS along with aluminium and calcium indicated the presence of a glass phase added to the material as it has been previously suggested [179]. The zirconium oxide phase has been speculated to be accidentally included during the procedure of chemical preparation of barium zirconate [179], as it serves as a raw material during sintering performed at 1200^{o}C [342]. Additionally, the alteration in the hydration of the cementitious phase induced by the resin matrix in Theracal has resulted in the induction of a significant compromise in the alkalinisation potential [181] and the calcium release compared with other TCS-based materials [179].

Consequently, leachates of Theracal showed a different performance in the biocompatibility assays compared with the TCS-based materials, without affecting cell viability in both 1- and the 28-day periods. A cytotoxic performance was observed in

the transwell assay for the 3T3 cell cultures while a moderate decrease in the MTT activity of HDPC cultures was also reported. Differences in the extraction conditions might also have affected the pH and the overall leaching pattern of the eluate of Theracal and particularly the release of monomers in this assay, rendering it cytotoxic, while findings in the literature regarding Theracal's effect in cell proliferation are also controversial [7, 217, 219, 337].

Interestingly, an escalating antimicrobial potential was observed only against *S. mutans* even from the 1-day eluate and for two of Theracal's dilutions after 28 days (1/2, 1/4). The enhanced antibacterial performance of Theracal against *S. mutans* provides an indication that high alkalinity is not the only factor responsible for the antimicrobial activity, as has been suggested in another recent study as well [343]. The 1-day leachate of Theracal did not present any inhibitory effect in a previous study against a bacterial cocktail [7]. Differences in susceptibility of bacterial species might be responsible for the variation in the antibacterial performance, as it was also observed in the current assay between the two bacteria tested. Overall, *L. casei* seemed to be more resistant than *S. mutans* against the majority of test leachates, possibly due to higher growth rates reported in the former or differences in the defensive mechanisms of the two species.

Both the two resin-containing materials tested, Theracal and Activa exhibited a poor degree of polymerisation (approximately 30%) after the light-curing period specified by the manufacturers. The acceptable limit of DC for resin-based materials is approximately 50% [344]. Adequate inclusion of light-sensitive initiators in the formulation is important for desirable monomer conversion [345]. In addition, the low

values of DC can be attributed to the materials' shade, not allowing adequate transmission of light throughout their bulk and therefore efficient curing cannot be accomplished [346]. As a result, a significant amount of unreacted monomers is present in the materials following restoration.

The role of the cementitious phase in Theracal's leaching profile despite the compromise in its hydration can be also verified by observations in Activa, which presented the typical microstructure of a glass ionomer [289]. The material was mainly amorphous with only minor intensity peaks of calcium and sodium fluoride. Despite the manufacturer's statement that Activa acts as an ionic deposit [268], no calcium leaching potential or alkalinisation activity was identified, while a moderate decrease in the pH was reported after 14 days. No previous study has characterised the microstructural phases of Activa.

Activa's 1-day leachates in the conventional MTT assay and the fresh eluate in the transwell system were found to be cytotoxic. This could be attributed to leaching of cytotoxic unreacted monomers due to incomplete conversion, as it has been reported previously for materials of similar composition [347, 348], albeit the manufacturer stating that Activa does not contain Bis-GMA or TEGDMA monomers [268], which have been suggested to present a cytotoxic potential [49, 349]. A direct correlation between the amount of fluoride release and cytotoxicity of the glass ionomer based materials has been also suggested [350] and should be further investigated assessing leaching of other elements in the formulation of Activa.

Interestingly, the 28-day eluate was biocompatible, indicating that no long-term degradation of cytotoxic monomers occurs in the material. Activa was mainly

inert in terms of antibacterial effectiveness, except for the 28-day leachate against *L. casei*. The low antibacterial potential is in accordance with results of the negligible leaching profile of Activa.

4.6.2.2.5 Effect of compound incorporation on prototype cements

Overall, values of calcium ion release were significantly lower for the experimental cements due to the absence of setting accelerators and other additives. Inclusion of micro-silica provoked a plateau in calcium release at early stages and depletion of calcium hydroxide, especially in the TCS-mS20/ZO, which resulted in moderate reactivity in terms of both cytotoxicity and inhibition of bacterial growth. Notably, the prototype material with the 20% micro-silica inclusion exhibited higher viability values than the other prototypes in the transwell system and in the 28-day eluate as well as a compromised antibacterial effectiveness. These findings suggest that further analysis of the alkalinisation potential of aged cements should be carried out to identify whether leachates are saturated. The less reactive profile observed in the TCS-mS20/ZO correlated with the lower formation of calcium hydroxide in the 28-day aged material.

No CP phase was identified in the respective biphasic prototype cement (TCS-CP/ZO), indicating its complete reaction, while leaching was not altered, albeit a better initial antibacterial effect was observed. A previous study reported that incorporation of CP monobasic in TCS in 50% replacement affects the pH and calcium release negatively [223]. However, the low amounts used in the current study did not induce significant differences.

4.6.2.3 Summary

Incorporation of different compounds in pulp capping cements can alter the hydration and chemical properties of the materials. Overall, there was a positive correlation of the calcium hydroxide leaching profile with the cytotoxicity and antimicrobial effectiveness.

In most commercial materials, the effect of compound incorporation was masked by several optimisations in their formulation, such as presence of calcium chloride which enhanced leaching. The Bio-C Pulpo exhibited extended hydration after 28 days. The material's 28-day leachate presented significant reactivity, appearing cytotoxic but also strongly antibacterial, albeit calcium release plateaued at early stages. Similarly, optimisations that have been carried out in Biodentine have improved its leaching profile and accelerated the hydration of the material. However, only minor differences were evident in comparison with its chemically relevant prototype TCS/ZO in the long-term in terms of biocompatibility and antibacterial activity, as neat 28-day eluates of Biodentine appeared cytotoxic but also effective in inhibition of bacterial growth. TotalFill RRM exhibited an enhanced leaching profile with escalating calcium release and reactivity.

The inclusion of a resin-matrix in the cementitious phase as observed in Theracal, affected negatively the hydration process of the cement and the calcium release. The material exhibited overall acceptable biocompatibility values and presented increasing antibacterial activity with time, despite the compromised leaching profile. Leaching out of monomers from the resin matrix of Theracal should be further investigated.

Activa induced an initial cytotoxic effect possibly due to degradation of non-polymerised monomers which was decreased over time. Negligible calcium release and alkalinisation was reported in the material correlating with a minor antibacterial potential only against *L. casei* in the 28-day leachate.

In the prototype cements, inclusion of silicon oxide modified the leaching pattern, as it reacts back with calcium and inhibits long-term calcium release and formation of calcium hydroxide. The inclusion of 20% micro-silica resulted thus in a neutralised cytotoxic response and a decreased antibacterial potential. Incorporation of 15% CP in the cementitious phase of TCS did not have a significant effect on the leaching profile and reactivity of the cement, albeit a stronger initial bacterial inhibitory effect was induced.

In conclusion, biological and antimicrobial properties of hydraulic TCS-based cements are directly linked to calcium hydroxide. The effect of different additives in the formulation of new generation of these cements should be thoroughly examined to establish how they affect their leaching profile.

CHAPTER 5

CONCLUSIONS

Part of the conclusions are included in an article accepted for publication in Dental Materials (Koutroulis A, Batchelor H, Kuehne SA, Cooper PR, Camilleri J). These sections have been written by the author.

The present project assessed how modifications in the composition of the pulp capping cements affect their chemical, biological and antimicrobial properties and the role of calcium hydroxide release in hydraulic cements' properties. The following conclusions can be drawn:

- Replacement of tricalcium silicate cement components with chemicals affects differently the water demand and the hydration profile of the cement.
- Adjustment of the w/p ratio in modified hydraulic cements can be accomplished by establishing similar rheological properties with a well-characterised reference material.
- Modifications in the w/p ratios of hydraulic cements affect the physicochemical properties of the hydraulic cements in comparison with mixing with the standard 0.35 ratio.
- Commercial materials based on pure tricalcium silicate or Portland cement contain optimisations, such as calcium chloride, calcium carbonate and water reducing polymers that alter significantly their chemical profile.
- A positive correlation of calcium ion release with antibacterial effectiveness and a moderate relationship with cytotoxicity was observed, which is also affected by hydroxyl ions.
- TCS-based commercial and prototype materials remain reactive throughout a 28-day period by leaching out elements that can induce an escalating cytotoxic and antimicrobial effect.
- Inclusion of calcium phosphate in 15% in TCS-based materials did not alter significantly the leaching pattern of the cement. Higher CP replacement in TCS

should be investigated to observe potential alterations in leaching and biological and antimicrobial reactivity.

- Incorporation of micro-silica (10% and 20%) in TCS-based cements can compromise the formation of calcium hydroxide, especially in the 20% inclusion and consequently alter significantly the hydration reaction, inducing a neutralised cytotoxic effect but compromising also the antimicrobial performance. The leaching pattern of calcium in these materials is mainly characterised by rapid calcium release and a consequent plateau. The latter was also observed in the Bio-C Pulpo, which contained silicon oxide, albeit it reported a highly reactive profile, due to other compounds included in its formulation.
- The resin-matrix in Theracal alters significantly the chemical properties, namely hydration and calcium release of the material in comparison with the commercial and prototype TCS-based cements. The moderate alkalinisation effect results in better biocompatibility values.
- Alterations in the traditional formulation of a radiopacified hydraulic cement in an effort to improve physical properties or handling characteristics, should be investigated well not to diminish any of their existing chemical, biological and antimicrobial characteristics.
- Further research should be conducted on how modifications of pulp capping cements can balance the induction of a mild biological stimulus with a strong bactericidal effect.

CHAPTER 6

FURTHER WORK

The current investigation highlighted that multiple factors should be taken into consideration when an alteration of the original formulation of a radiopacified TCS is introduced. Further research is needed in order to conclude robust results regarding materials' characteristics that will balance between desirable physicomechanical and biological and antibacterial properties.

6.1 Development of prototype materials

The hydration profile and the chemical properties of a prototype material with higher amounts of CP incorporation, namely 25% should be investigated in order to elucidate if the speculated pattern of modified reactivity will be verified.

Additionally, prototype TCS-based materials with a resin matrix could be developed to compare their performance with Theracal and identify if the glass phase which is also added to the latter affects the cement's hydration. The role of release of unreacted monomers in the extract solution could be monitored further.

6.2 Chemical analyses

In the current project, the chemical analysis of the leachates for the specified time-periods was performed in the initial soaking medium (HBSS). Most materials appeared to elevate significantly the pH shortly after immersion. In an effort to identify a clearer trend in the leaching profile and avoid the saturation effect in the pH of the solution, further chemical analyses could be performed by re-immersing the

material pellet in fresh HBSS after each test period. A potential plateau in the calcium release and pH values could be thus identified.

6.3 Biological assays

In Chapter 4, it was highlighted that an initial necrotic zone and a mild inflammatory effect caused by the application of a material in proximity with the pulp tissue and mainly by the elevation of pH is desirable as it can lead to a series of regenerative events. The balance between chronic destructive inflammation and healing is fine in this system [9].

The cytotoxic effect of the eluates of pulp capping materials should therefore be investigated following exposure to cells for varying contact periods, in order to assess if a recovering in cell proliferation occurs after the initial 'shock' in the metabolic activity of cells. Evidence in the literature indicates that cell cultures exposed to Biodentine, TotalFill RRM and Theracal exhibited improved proliferation during longer incubation periods [216, 244].

Additionally, the limitations of the MTT assay that were discussed in Section 4.5.5.1 suggest that further cytotoxicity assays should be carried out in order to compare the results from the current experiments.

The events that trigger odontoblastic differentiation following a mild inflammatory stimulus have been well described [6, 351]. In brief, pulp fibroblasts secrete specific growth factors that attract stem cells to the site of injury that will further differentiate into odontoblast-like cells and produce tertiary dentine [352, 353]. It is therefore important to evaluate the cytokine and other growth factor release

of dental pulp cells as well as secretion of differentiation markers by the HDPC cell cultures following incubation with each leachate. Finally, the mineralisation potential of each leachate should be investigated further and identify if there is a direct correlation with the calcium leaching profile of each material as has been previously suggested and identify a potential dose-response effect. This is routinely tested by the expression of a mineralisation-related protein marker in cells responsible for hard tissue formation, alkaline phosphatase [354].

6.4 Antimicrobial assays

An initial screening of the antimicrobial effect of eluates was carried out by assessing their ability to inhibit the growth of a bacterial suspension. However, dental caries and endodontic pathology are biofilm associated diseases [331, 332]. Thus, in a more clinically relevant scenario, it would be interesting to investigate further the antibacterial potential of eluates against a biofilm model. A protocol to assess the effect of eluates against a 3-day old monospecies biofilm has been developed and could be carried out in future projects. Pilot experiments were conducted for the cultivation of monospecies biofilms and an indirect test with a similar concept with the MIC assays in planktonic bacteria is described below.

6.4.1 Assessment of activity of eluates against biofilms

Intact extracted human teeth were collected from the Birmingham School of Dentistry Tooth Bank under ethical approval no: 14/EM/2811. Teeth were sectioned horizontally at the cementoenamel junction and in the apical third with the aid of a

saw with a diamond disc; then, dentine slices of an average of 1 mm thickness were created by longitudinal sections. Removal of the smear layer of specimens was carried out by immersion in a 17% EDTA solution; substrates were then rinsed with distilled water for 1 min, autoclaved, and transferred to 24-well plates. Random specimens were selected and viewed under the SEM in order to assess the condition of dentinal tubules (Figure 6.1).

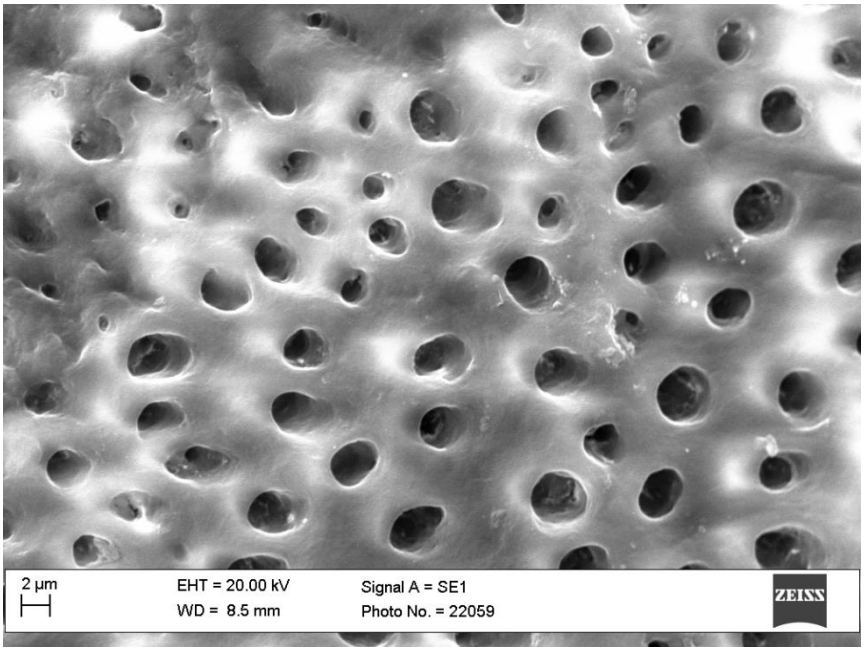


Figure 6.1 Secondary electron micrograph of a dentine disc (5000× magnification) following treatment with 17% EDTA. Dentinal tubules are open and thus the samples can be used as substrates for biofilm cultivation.

Consequently, bacteria were grown overnight as described in Section 2.3.6.1 and standardised to an OD of 0.1 (600 nm). One ml of the bacterial inoculum was transferred to the well and samples were incubated at 37^{o}C, 5% CO_2 supplemented

atmosphere. The growth medium was replaced each day by carefully pipetting, taking care not to disrupt the growing biofilm.

After an incubation period of 3 days, dentine discs were retrieved and immersed in 1 ml solution containing 500 µl sterile growth medium and 500 µl of the material's test leachate. For positive control, pure HBSS with sterile growth medium was used in the solution. Further incubation of the samples took place for 24 h. After the contact period, the dentine discs were retrieved and stained with 200 µL of staining solution. The solution was prepared by adding 3 µL of SYTO 9 stain and 3 µL of propidium iodide (PI) stain to 1 mL of filter-sterilized water (Filmtracer™ LIVE/DEAD™ Biofilm Viability Kit, Thermo Scientific, Waltham, MA, USA) in a foil-covered container. SYTO 9 is a green fluorescent stain, labelling all bacteria in a sample; PI, which is a red-fluorescent nucleic acid, stains bacteria with damaged membranes.

Following a 25-min staining period protected from light at room temperature, the dentine discs were mounted inside a glass bottom dish (µ-dish 35 mm, high Glass Bottom, ibidi GmbH, Martinsried, Germany) and viewed under an inverted confocal laser scanning microscope (CLSM; LSM 700 Carl Zeiss Ltd., Cambridge, UK). Six randomly selected microscopic scans of 25 µm depth were obtained for each sample. The 40× oil immersion lens was used with a 1 µm step-size and a 1024 x 1024 pixels format. This area in the microscope corresponds to 159.9 x 159.9 µm and the scanning was performed from the top of the biofilm to the biofilm substrate. Consequently, assessment of the live/dead population of bacteria was performed utilising an imaging programme (Image J). The total biovolume and the percentage of red cells could be therefore assessed.

The biofilm forming ability of *S. mutans* after 3 days of cultivation was assessed in the CLSM using the staining and mounting procedure described above. As is evidenced in Figure 6.2, the biofilm formation was apparent.

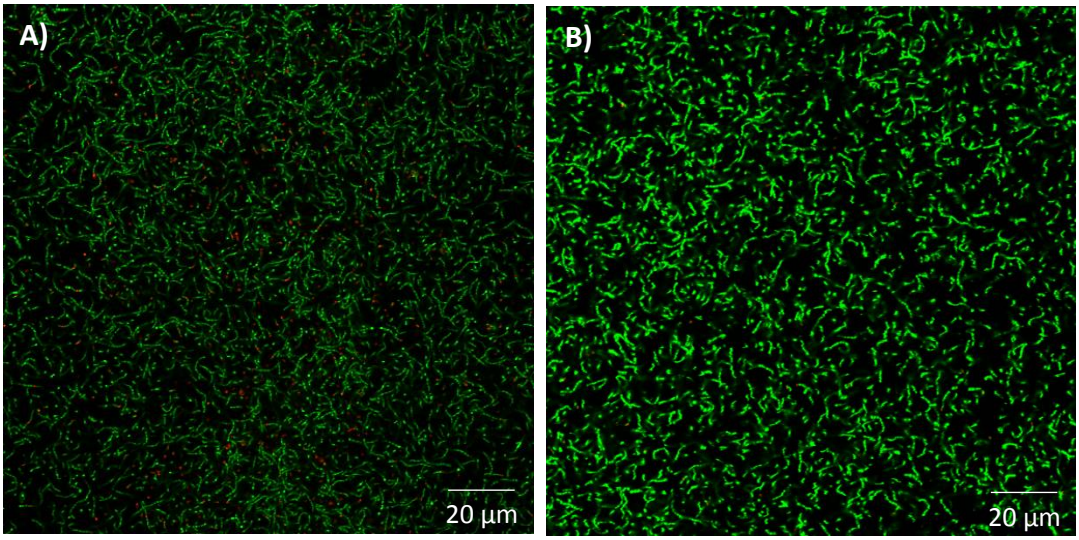


Figure 6.2 Representative CLSM images of a 3-day biofilm of *S. mutans* (40× magnification). For the experimental assay, substrates were subsequently immersed in a solution of equal volume of broth medium and test leachate.

In conclusion, experiments performed for leachates against bacteria organised in biofilms can provide a holistic insight of the antibacterial dynamics of the test leachates, following their evaluation against a bacterial inoculum.

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Appendix

Publication

Koutroulis A, Batchelor H, Kuehne S, Cooper P, Camilleri J. Investigation of the effect of the water to powder ratio on hydraulic cement properties. Dent Mater (Accepted/In press).

Research poster

Koutroulis A, Batchelor H, Kuehne S, Cooper P, Camilleri J. Determination of water demand and calcium release in hydraulic cements. *To be presented at the* 97th General Session & Exhibition of the IADR, Vancouver Canada.