A laboratory study to assess the formation of effluent volatile compounds and disinfection byproducts during chemomechanical preparation of infected root canals and application of activated carbon for their removal

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A laboratory study to assess the formation of effluent volatile compounds and disinfection by-products during chemomechanical preparation of infected root canals and application of activated carbon for their removal

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ABSTRACT

Aim To assess in a laboratory setting using extracted teeth the formation of volatile compounds (VOCs) and disinfection by-products (DBPs) in effluent aliquots, during chemomechanical preparation of artificially infected root canal specimens, and determine the role of silver-impregnated activated carbon (Ag-AC) in their removal.

Methodology Single-rooted human teeth were decoronated to obtain 15mm-long root specimens and a nutrient-stressed multispecies biofilm was grown in the root canals. Specimens were randomly assigned into three groups [Group 1; instrumentation with rotary files and irrigation with sterile saline, Groups 2 and 3; instrumentation with rotary files and irrigation with 2.5% NaOCl and 17% EDTA]. A portable medical suction device was used to collect the effluent aliquots during root canal irrigation. In Groups 1 and 2, the reaction products of the collected effluents were analysed by selected ion flow tube mass spectrometry (SIFT-MS). The effluents from Group 3 were treated with Ag-AC prior to SIFT-MS analysis, to assess the removal capacity of Ag-AC against the reaction products. The synthesis of Ag-AC was characterised with scanning electron microscopy/energy dispersive X-ray spectroscopy (SEM/EDS). Two-way analysis of variance (ANOVA) with post hoc Tukey tests were used for data analysis and determination of a significant difference ($P<0.05$).

Results In Group 1, effluent VOCs and DBPs were detectable at very low levels. In Group 2, the collected effluent aliquots released high concentrations of methanol, propanol, ammonia, chloroform and formaldehyde, which were significantly greater compared to Group 1 ($P<0.001$). SEM/EDS analysis confirmed impregnation of Ag within the AC matrix. The treatment of effluent aliquots with Ag-AC (Group 3) resulted in a significant reduction in concentrations of acetone, acetic acid, propanol, acetaldehyde, acetonitrile and chloroform, compared to Group 2 ($P<0.001$). The concentration levels of ethanol, methanol, ammonia and formaldehyde remained unaffected ($P>0.05$).

Conclusions In this laboratory setting using extracted human teeth, the chemomechanical preparation of artificially infected root canals resulted in the formation of toxic volatile compounds and disinfection by-products as effluent suspensions. Their release during aspiration with dental suction indicates that potential environmental hazards should be investigated. The use of silver-impregnated activated carbon had potential for the point-of-use treatment of post-irrigation effluent aliquots.

Introduction

Apical periodontitis is aetiologically caused mostly by bacterial invasion of the pulp and growth within the root canal system of a tooth (Abbott 2004). The scope of root canal treatment is to eliminate bacteria from the infected root canal, prevent its reinfection and save the natural tooth, by preserving its function in the oral cavity (Siqueira *et al.* 2000). The use of NaOCl has been adopted universally as the main irrigant for the disinfection of infected root canals. A consensus exists that a concentration of 2.5% NaOCl is clinically acceptable both in terms of antimicrobial and pulp tissue dissolution capacity in Endodontics (Basrani & Haapasalo 2012). The use of a chelating agent, such as 17% EDTA, aids in the removal of the infected inorganic components (smear layer), during root canal instrumentation (Haapasalo *et al.* 2012).

Recent studies have reported that the chemical interaction of NaOCl with intracanal sources of natural organic matter (NOM), such as dentine, pulp, bacteria and serum albumin, results in the formation of toxic volatile compounds (VOCs) and disinfection by-products (DBPs) (Varise *et al.* 2014, Ioannidis *et al.* 2018). An *ex vivo* tooth model was used recently to measure the periapical extrusion of VOCs and DBPs, during chemomechanical preparation of artificially infected root canals (Ioannidis *et al.* 2020). The apical extrusion of methanol, propanol, acetaldehyde, ammonia and chloroform was quantified. In addition, the chemical interaction of NaOCl and EDTA resulted in the formation of high concentrations of formaldehyde, which is a known carcinogen (Ioannidis *et al.* 2020). The formation of chloroform and formaldehyde indicated that risk assessment of potential occupational hazards should be carried out. In addition, the risks from any environmental implications have not been reported.

To-date, dental professionals are under pressure to understand and adhere to clinical waste regulations (Department of Health, Environment and Sustainability, UK 2013). One characteristic example is the potential contamination of water systems, particularly through the production of dental amalgam waste (Shraim *et al.* 2011).

In a clinical setting, root canal irrigants are injected in the prepared root canals with the aid of syringes and endodontic needles. As a strong oxidising agent, NaOCl interacts with the infected and organic content of the root canal system, whilst instrumentation progresses and irrigation depth increases. Dentine chips, disrupted microbial biofilms, vital or necrotic cells, blood and plasma exudates, vital or necrotic pulp remnants comprise this multivariable content and provide a constant reservoir of natural organic matter (NOM), exposed to replenishing volumes of NaOCl (Torabinejad *et al.* 2002). The forming post-chlorinated biomass and the excess irrigant volumes are aspirated with the aid of dental surgical suction in order to ensure safe intracanal delivery, prevention of spillage, oral contact and ingestion of the effluent irrigant. A comprehensive literature search did not reveal any studies associated with the composition and

the fate of the post-chlorinated aliquots, which originate from dental unit disposal waterlines. Moreover, these aliquots have not been classified into any categories of biomedical or general dental office waste and the potential environmental hazards have not been determined yet.

During root canal irrigation and aspiration, the forming effluents flow through pipelines and water distribution systems, ending in drainage and sewage disposal networks, without any known point-of-use (POU) treatment or purification. The application of enhanced coagulation, ozonation and treatment with activated carbon are among the proposed scientific methods for the elimination of VOCs, DBPs and the reduction of NOM precursors, soluble microbial precursors and humic substances (Lou *et al.* 2009, Liu & Li 2015, Zhang *et al.* 2017). The use of activated carbon has also been advocated for the removal of organic constituents, impurities and residual disinfectants in water supplies, due to its adsorption capacity (Zhao *et al.* 2018). For the prevention of pathogens' growth on the surface of activated carbon and to exert antimicrobial activity, silver-impregnated activated carbon (Ag-AC) has been proposed for the POU treatment of water systems (Shimabuku *et al.* 2017). A comprehensive literature research showed no potential application of activated carbon in dentistry or dental units for the POU pre-treatment of effluent post-chlorinated solutions that emerge from the irrigation of infected root canals.

The aim of this study was to screen and quantify *ex vivo*, the formation of VOCs and chlorinated DBPs in effluent solutions, following the clinical simulation of instrumentation and irrigation of infected root canal specimens, with rotary NiTi instruments, 2.5% NaOCl and 17% EDTA. In addition, the application of Ag-AC was investigated for the potential treatment of the emerging effluents.

Materials and methods

Sample size calculation

A two-way repeated measures experimental design was employed. Sample size estimation was conducted *a priori* with G*Power 3.1.9.2 software (Franz Faul, Universitaet Kiel, Germany). To ensure that a standardized effect of size 0.23 would be detected by two-way ANOVA at 80% power and with a probability of alpha-type error of 0.05, a sample size of 42 specimens was required for three groups (n=3x14).

Specimen selection and preparation

Forty-two (N=42) freshy extracted teeth with single round canals, that were free of cracks, fractures, caries, external cervical root resorption, abrasions and discolouration were collected. Informed and written consent was obtained by medically-fit patients, who were referred by their dentists to have their teeth extracted in dental surgery premises. The collection and specimen storage procedures were approved and conducted in accordance with the protocol outlined by the

Research Ethical Committee (Wales REC 4, 14/WA/1004, UK). The crowns of the teeth were removed and the length of each root specimen was standardised to 15mm. Under magnification with a dental operating microscope (Global Surgical Corporation, St Louis, USA), apical patency was achieved with insertion of a sterile size 8 K-file (Dentsply Sirona, Weybridge, UK) 1mm beyond the foramen. The working length of each specimen was determined by apical insertion of a size 10 K-file (Dentsply Sirona) in the canal until its tip was detected through apical foramen, followed by 1mm subtraction (Ioannidis *et al.* 2020). The root canals were initially prepared up to working length, with ProTaper Universal (Dentsply Sirona) rotary files S1, S2 and F1 and disinfected with 5.25% NaOCl (Chloraxid, Cerkamed, Stalowa Wola, Poland), distilled water and 17% EDTA (Schottlander & Davis Ltd, Letchworth Garden City, UK) (Ioannidis *et al.* 2020). A final flush with 2mL distilled water was performed to remove any irrigant residues and the canals were dried with sterile paper points (Size F1, Dentsply Sirona).

Each root specimen was adjusted individually to a newly-proposed testing apparatus, fabricated to simulate mechanical preparation and intracanal irrigation under conditions of high-compliance periapical lesions and resistance to irrigant extrusion (supporting information S1) (Ioannidis *et al.* 2020). Prior to use, every surface of the testing apparatus elements was sterilised under UV irradiation for 2h.

Development of nutrient-stressed multispecies biofilm within root specimens

A stressed multispecies biofilm comprising five selected bacteria was developed in the root canal of each root specimen using a validated, published protocol (Niazi *et al.* 2014, Ioannidis *et al.* 2020). The selected endodontic bacteria in this biofilm included *Propionibacterium acnes, Actinomyces radicidentis, Staphylococcus epidermidis, Streptococcus mitis* -recovered from root canals of teeth with refractory endodontic infections- and *Enterococcus faecalis* strain OMGS 3202 (Dahlén *et al.* 2010, Niazi *et al.* 2010). The biofilm was grown under anaerobic conditions (supporting information S2) and the attachment against dentine substrate was verified with scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) (supporting information S3) (Ioannidis *et al.* 2020). After 14 days, 42 biofilm-containing root specimens were prepared for assignment to one control and two experimental groups (n=14 specimens/group), using a software (List Randomiser; https://www.random.org/lists/).

Protocols of chemomechanical preparation procedures

The root specimens were transferred into their assigned silicon indices. The glass vials were filled with 4mL sterile ultrapure water (Simplicity UV Milipore SAS, Molsheim, France), merged with the clamps and tightened with the aid of an electric screwdriver (Bosch IXO, Robert Bosch Holdings, Denham, UK), to ensure the performance of the apparatus as a single unit. Mechanical root canal preparation was performed with the use of rotary files Protaper Gold

Universal instruments F1, F2 and F3 (Dentsply Sirona). The irrigants used were sterile saline (NaCl 0.9%) (JFA Medical, Blackpool, UK), 2.5% NaOCl and 17% EDTA (Schottlander & Davis, Letchworth Garden City, UK). All procedures were performed in a Class II laminar flow biological safety cabinet (Nuaire, Plymouth, USA), to prevent cross-contamination of the specimens. The protocols of mechanical preparation, root canal irrigation and agitation are described in supporting information S4. The chemomechanical preparation was performed by an accredited Specialist Endodontist (K.I.). The sequence of instrumentation and irrigation procedures for Groups 1-3 is described in Fig. 1 and is briefly summarised below:

- Group 1 (n=14): Protaper F1+3mL saline, Protaper F2+3mL saline, Protaper F3+7mL saline, 1mL saline+UAI.
- Groups 2 & 3 (n=28): Protaper F1+3mL 2.5% NaOCl, Protaper F2+3mL 2.5% NaOCl, Protaper F3+3mL 2.5% NaOCl, 1mL saline/2mL 17% EDTA / 1mL saline, 1mL 2.5% NaOCl / UAI.

Collection of effluent aliquots

During irrigation, a portable medical suction unit (Armoline; Medical Import, London, UK) was used during irrigation to aspirate the effluent from the prepared root canals, with the aid of a sterile silicon tube (external diameter:10mm; Wall Thickness:2mm) (Ad Fontes Company, Hong Kong), connected with autoclavable polycarbonate liquid collection jars, assigned for each root specimen separately. The use of dental suction was contributory to the simulation of clinical conditions and standards of good endodontic practice. Once chemomechanical procedures were complete, the effluent liquid suspensions obtained from Groups 1 (irrigation with distilled water), 2 and 3 (irrigation with NaOCl and EDTA) were transferred to 20mL sterile Universal tubes and kept refrigerated at -80^{o}C until analysis.

Synthesis and characterization of silver-impregnated activated carbon (Ag-AC)

Steam activated and acid washed, highly purified activated carbon (AC) in powder form was obtained as a readily available formulation (Norit ®, LOT BCBK2016V, Sigma-Aldrich, Gillingham, UK). Particle size distribution was measured with a laser diffraction particle-size analyser (CILAS 1180, CILAS, Orléans, France). Five samples of AC (10mg) were collected, inserted into a water tank and ultra-sonicated for 30s. The median diameter (D50) of AC particles varied from 22.55μm to 30.36μm.

For the impregnation of AC with silver nanoparticles (AgNPs), an aqueous solution of 0.1M $AgNO_3$ (Sigma Aldrich) was prepared. Amber erlenmeyers were used and 1g of AC was weighted and 20mL of 0.1M $AgNO_3$ was added. A concentrated solution of 2mL NH_4OH (Sigma Aldrich) (estimated concentration of NH_4 at 20^0C= 32% w/v) as a silver (Ag) reducing agent (Tollens reaction) (Acevedo *et al.* 2014). The sample was stirred for 24h, then filtered and washed with distilled water and finally dried at 120°C for 4h.

For the elemental analysis of Ag-impregnated AC (Ag-AC), scanning electron microscopy/energy dispersive X-ray spectroscopy (SEM/EDS) (NeoScope JCM-6000plus, Tokyo, Japan) at 10kV was used to confirm the impregnation of AgNPs in AC matrix. The dried Ag-AC powder was attached to 12.5mm aluminium pin stubs (Agar Scientific Elektron Technology, Stansted, UK) using Leit C conducting carbon cement (Agar Scientific Elektron Technology) and gold- or carbon- coated for SEM or EDS imaging, respectively. The antimicrobial properties of Ag-AC were examined in a pilot study, which is described in supporting information S5.

Reduction of available chlorine content of NaOCl solutions after treatment with Ag-AC

Sodium hypochlorite (NaOCl) solutions (1%, 2.5% and 5% v/v) were initially prepared from a stock solution of NaOCl ≥10% v/v (Sigma Aldrich, Gillingham, UK) and their molarities (M) were verified with a standard iodine/thiosulfate method (iodometric titration) (Vogel 1962). Then different masses of Ag-AC (5, 10, 50, 100, 200 and 500mg) were added into amber erlenmeyers containing 10mL NaOCl of different concentrations (1%, 2.5% & 5% v/v). The solutions were stirred and homogenized with the aid of a magnetic stirrer for three different time periods of interaction (15, 30 and 60min). From each treated suspension, 9mL aliquots were collected and centrifuged (2000rpm, 22^0C) for 3min. The centrifuged aliquots were sub-divided into three 3mL-containing aliquots. Two mL of each was used, so that iodometric titration was performed in triplicates. Each sample was titrated with a standardized solution of 0.17M sodium thiosulfate ($Na_2S_2O_3$) and the residual chlorine was determined, according to the final stoichiometric measurements: $C_{NaOCl} \times V_{NaOCl} = C_{Na2S2O3} \times V_{Na2S2O3} / 2$.

Treatment of effluent liquid suspensions with Ag-AC

The effluent liquid suspensions obtained from Group 3 (n=14) were randomly sub-divided into 2 sub-groups ($n_a=n_b=7$), using software (List Randomiser; https://www.random.org/lists/). Based on the results obtained from the efficiency of Ag-AC to reduce the available chlorine content of NaOCl solutions, each of the two sub-groups was treated with Ag-AC under stirring at 60rpm (Tube Roller; Maple Scientific, Stone, UK), at different mass rates and reaction periods. After their interaction the carbon-treated effluent aliquots were centrifuged for 4min (G-force=672) and, similarly to Groups 1 and 2, 10mL were collected and transferred to 20mL sterile Universal tubes and kept refrigerated at -80^{o}C until analysis.

SIFT-MS analysis

The SIFT-MS technique has been extensively described elsewhere (Ioannidis *et al.* 2020) and a brief description is provided in supporting information S6. For analysis, the samples were defrosted in air and analysis of the headspace volatile compounds was carried out in real-time. The SIFT-MS instrument was operated in two modes: the scan mode, where the entire spectrum is captured over a desired mass-to-charge ration, and the selected ion mode, where individual

compounds were targeted according to the m/z value of their analyte ions and analysed individually. Quantification was carried out using the selected ion mode. Prior to analysis, three replicate 1ml aliquots of each sample were placed into a sample bag constructed from 50cm length, 65mm diameter Nalophan NA (Kalle, Foodpack, Colchester, UK), which was then filled with purified air and sealed prior to incubation at 37^{o}C (Fig. 2a). After equilibrium between the liquid and headspace above it (30min), the headspace was sampled directly into the SIFT-MS via a heated, calibrated capillary that defines the headspace sample flow rate, as is necessary for absolute quantification of VOCs (Fig. 2b). The analytical downstream quadrupole mass spectrometer was scanned over the range of mass-to-charge ratio, m/z, using the three reagent ions H_3O^+, NO^+ and O_2^+ independently. In addition, individual compounds of interest were selected and the analyte ions for these compounds were targeted and analysed, using the kinetics database stored in the instrument library (Figs. 2c,2d).

Statistical analysis

Two-way analysis of variance (ANOVA) with post hoc Tukey tests was used for data analysis of the forming VOCs and DBPs from the effluent as well as Ag-AC treated aliquots. The overall analysis was performed with SPSS software (version 22.0, IBM SPSS Inc., Chicago, IL, USA). The level of statistical significance was set at $P<0.05$.

Results

SIFT-MS analysis of effluent aliquots

The headspace of all samples contained several common compounds that are often seen in biological media headspace, including acetone, acetic acid, methanol, ethanol, propanol, acetaldehyde, acetonitrile and ammonia (Table 1) (Turner *et al.* 2008). The concentrations of all compounds in Group 1 headspace were detectable at very low concentrations. In Group 2 headspace, with the exception of ethanol, the concentration of all compounds increased significantly compared to Group 1 ($P<0.001$). Sample spectra using the H_3O^+, O_2^+ reagent ions for Group 2 are presented in Figs. 3 and 4, where analyte ions derived from chloroform (m/z 83, 85, 87) and formaldehyde (m/z 31), respectively, can be seen. In Group 2, a significant increase in concentration was seen for methanol, propanol, ammonia, chloroform and formaldehyde ($P<0.001$) (Table 1).

Characterisation of Ag-AC, reduction in chlorine content following interaction with NaOCl solutions and antimicrobial efficacy

SEM/EDS analysis confirmed the presence of Ag particles and their impregnation onto the AC mesopore surfaces (Figs. 5 and 6). The interaction of NaOCl with Ag-Ac revealed that NaOCl concentration, time of interaction and Ag-AC mass affected the availability of measurable

chlorine content (Table 2). Regardless of concentration, complete loss of chlorine ions from NaOCl, was observed when the aliquots interacted with 200mg Ag-AC for 15min. The minimum mass of Ag-AC for the complete dechlorination of NaOCl solutions was 50mg, when they interacted for a total period of 60min (Table 2).

The effect of Ag-AC treatment against VOCs and DBPs in the effluent aliquots

The chlorinated effluent aliquots of Group 3 were sub-divided and treated as shown below:

- Group 3a (n=7): Treatment of 10mL effluent with 50mg Ag-AC, for 60min, at room temperature.
- Group 3b (n=7): Treatment of 10mL effluent with 200mg Ag-AC, for 15min, at room temperature.

The treatment of chlorinated effluent aliquots with 200mg Ag-AC for 15min (Group 3b) caused a significant reduction in the detectable concentrations of acetone ($P<0.001$), acetic acid ($P=0.003$), propanol ($P<0.001$), acetaldehyde ($P<0.001$) and acetonitrile ($P<0.001$), compared to Group 3a (Table 1). The concentrations of the aforementioned compounds were also significantly reduced compared to the detectable concentrations in Group 2, where Ag-AC was not applied ($P<0.001$) (Table 1).

A significant reduction in chloroform concentrations was observed in both Groups 3a and 3b, compared to Group 2 ($P<0.001$) (Table 1). Both protocols of Ag-Ac treatment led to identical residual concentrations of chloroform ($P>0.05$).

Ethanol was the only compound that presented a slight but statistically significant increase in concentration in Groups 3a and 3b, compared to Group 2 (Group 2 vs Group 3a, $P=0.015$; Group 2 vs Group 3b, $P=0.005$) (Table 1). Methanol, ammonia and formaldehyde remained unaffected by Ag-AC treatments and their concentrations did not statistically differ from those in Group 2 ($P>0.05$) (Table 1).

Discussion

In this laboratory study using human extracted teeth, the formation of VOCs and DBPS was documented in sampled effluent solutions, originating from the simulation of root canal irrigation and aspiration procedures with the aid of a dental suction. A novel reproducible testing apparatus was fabricated to simulate the conditions of mechanical preparation and intracanal irrigation in a 'water-closed' apical system. The model was designed to maximise the simulation of periradicular tissue pressure and lead to efficient aspiration of the effluent with the dental suction (Ioannidis *et al.* 2020). The experimental root canal procedures were performed by an accredited Specialist Endodontist, to ensure that chemomechanical preparation was performed under conditions of

good clinical practice. The operational parameters of the disinfection protocol were subjected to conditions that predisposed minimum risk of extrusion. The volumes of the irrigants and the irrigation sequencing was adopted and justified in a recent study (Ioannidis *et al.* 2020).

The results of this study revealed that the chemomechanical preparation of infected root canal specimens in Groups 1 and 2 resulted in the formation of VOCs and DBPs in the sampled effluent solutions. When sterile saline was used as root canal irrigant (Group 1), only low concentrations of VOCs and DBPs were detectable in collected effluent, after chemomechanical preparation. SIFT-MS technology has been verified as a valuable tool for rapid detection of small quantities of a range of VOCs (Storer *et al.* 2011, Sovová *et al.* 2013). The formation of VOCs in Group 1 was proved to be relevant to the metabolic activity of the growing biofilm within root canal specimens (Sovová *et al.* 2013, Chen *et al.* 2016). The mechanical removal of root specimens' content among with flushing with a chemically inert solution, such as saline, resulted in their detection in low levels in effluent samples.

The detectable concentrations of methanol, propanol, acetonitrile, ammonia, chloroform and formaldehyde were significantly higher in Group 2, following the chemomechanical preparation of the infected root specimens with NaOCl 2.5% and EDTA 17%. These findings are in agreement with a previous study, in which the formation of VOCs and DBPs was confirmed in periradicular space of infected roots undergoing chemomechanical preparation, *ex vivo* (Ioannidis *et al.* 2020). These combined results reflect the dynamics of root canal preparation and irrigation procedures and shows that the formation of VOCs and DBPs is constant.

The use of NaOCl in root canal disinfection predominates, as the use of 10mL per canal has been considered practically acceptable (Basrani & Haapasalo 2012). Therefore, an assumption can be made that excessive chlorine availability, reactivity and replenishment is the chemical catalyst for the release of VOCs and DBPs. To the best of the authors' knowledge, dental units are not supplied with POU treatment or purification systems, as far as chemically derived effluents are concerned. The laboratory use of Ag-AC in powder form was proposed at different mass and time ratios, to assess firstly the effect of Ag-AC in chlorine availability with iodometric titration. The dechlorinating action of AC has been proved to be cost-effective and reliable for the removal of residual chlorine in the public water supply industry, since AC acts a reducing agent against chlorine because of its high surface area, high amount and rate of adsorption, and specific surface reactivity (Jaguaribe *et al.* 2005). Despite its chemical versatility, AC does not effectively dissociate bacteria and viruses, which may adhere on the surface and mesopores, using the latter as carbon source for their growth and metabolism (Kim & Park 2008). Hence, the synthesis of Ag-AC was reported to have substantial antimicrobial activity compared to pure AC. When tested against post-chlorinated effluent suspensions, the use of Ag-AC

effectively reduced the concentrations of acetone, propanol, acetonitrile and acetaldehyde, when the aliquots were treated with 200mg Ag-AC for 15min.

A significant increase in chloroform formation was noticeable in effluent suspensions in Group 2. In addition to its toxic properties, chloroform is not considered readily biodegradable in water systems and can be environmentally discharged in the receiving surface water undiluted (Rebelo *et al.* 2016). In view of the significant risk to or via the aquatic environment, chloroform was appointed as a priority substance under the European Water Framework Directive (European Commission 2013). The use of Ag-AC effectively reduced the concentration of chloroform, which is in line with current literature findings (Abea *et al.* 2001, Lou *et al.* 2009, Zhao *et al.* 2018). Therefore, Ag-AC has good potential for POU application in the disposal pipes and waterlines of dental units as a filtration system for the adsorption of DBPs and remediation of the post-chlorinated aliquots (Liu & Li 2015).

On the contrary, the use of Ag-AC did not have any selective adsorptive activity against methanol, ammonia and formaldehyde. Methanol has low toxicity to marine life and many of the effects of short-term exposure are temporary and reversible. Because of its properties, methanol readily mixes with water and evaporates quickly in the atmosphere and would rapidly dissipate into the environment (USEPA 2013a). Ammonia is one of several forms of nitrogen that exist in aquatic environments and it showed some toxicity for aquatic organisms (USEPA 2013b). As a VOC, formaldehyde is a natural component of the environment and the human body. The occurring toxicity in humans during exposure to formaldehyde, through breathing, gastro-intestinal digestion or by skin contact, from contaminated atmospheric air, indoors or occupational exposure has been well-documented (Zhu *et al.* 2017). Formaldehyde also showed some toxicity to aquatic life, but is not bio-accumulative (Haarstad *et al.* 2012, Lalonde *et al.* 2015). This study identified another potential source of formaldehyde emission via effluent aliquots of endodontic origin. Therefore, an early intervention at the POU may be beneficial and it might be necessary to consider additional treatment procedures to reduce or eliminate formaldehyde release in the environment.

One parameter that was not documented in this study was the fate of possible residual un-reacting EDTA suspensions. Whilst a recent study firstly proved that the interaction of EDTA with NaOCl results in the formation of formaldehyde (Ioannidis *et al.* 2020), previous studies confirmed that following their interaction, EDTA was still effective in removing the inorganic smear layer from prepared canal walls (Grande *et al.* 2006, Prado *et al.* 2013). This implies that residual EDTA solution may be released in the environment. Although the isolated molecule does not present a risk of bioaccumulation, the ligand-metal complexes may significantly increase the bioavailability of heavy metals that can potentially bind during the flow of the effluent into the waste-water

pipelines (Chen & Cutright 2001). In drinking water and waste-water plants, filtering through AC is ineffective to remove the chelate and additional methods such as pre-ozonation or photochemical oxidation systems are required (Rodríguez *et al.* 1999).

In clinical dental settings, variable volumes of root canal irrigants and post-chlorinated effluent solutions may result in pipelines and water distribution systems, drainage and sewage disposal networks and waste-water pipelines. Currently, there are no published data regarding the formation and release of VOCs and DBPs from dental practices or dental hospital facilities and the exact scale of contribution to environmental implications has to be studied further. Many of the VOCs and DBPs, identified in this study, are classified as hazardous chemicals. Various international organisations have proposed environmental quality standards (Table 3) and guideline values that are of health significance in relationship to the quality of water intended for human consumption (Table 4). Based on the results of this study, the measurable concentrations produced in dental effluents of endodontic origin appear to exceed some of the existing concentration thresholds, when analysed at the POU. However, without assuming any potential diluting effects when VOCs and DBPs enter the hydrologic system, direct mathematic comparisons cannot precisely justify the potential environmental impact from dental liquid waste. Future, specially designed multicenter studies, based on predictive modeling and analytics, are required to determine the exact environmental impact of dental effluent of endodontic origin.

Conclusion

Within the limitations of this *ex vivo* study, the mechanical preparation and irrigation of artificially infected root canals with rotary NiTi files, 2.5% NaOCl and 17% EDTA resulted in the formation of toxic volatile compounds and disinfection by-products in aspirated effluent aliquots. The adsorptive capacity of silver-impregnated activated carbon selectively reduced the concentration of chloroform, but had no effect against methanol, ammonia and formaldehyde. The use of silver-impregnated activated carbon may have potential for the point-of-use treatment of post-chlorinated effluent aliquots.

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Conflict of interests

The authors have stated explicitly that there are no conflicts of interest in connection with this article.

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FIGURE LEGENDS

Figure 1 Flow-chart of experimental procedures, including sample size calculation, allocation of root specimens, biofilm growth, protocols of root canal chemomechanical preparation and SIFT-MS analysis.

Figure 2 Stages of SIFT-MS analysis (a) Sample placement into Nalophan bag and incubation. (b) Application of heated calibrated capillary to sample headspace directly into the SIFT-MS. (c) Selection of reagent ions via computer software. (d) Activation of scanning process in mass spectrometer.

Figure 3 O_2^+ spectrum of Group 2 aliquot sample (irrigation with 2.5% NaOCl + 17% EDTA) obtained from collected effluent in air. Ions at m/z 83, 85, 87 indicating chloroform is shown on the spectrum.

Figure 4 H_3O^+ spectrum of Group 2 aliquot sample (irrigation with 2.5% NaOCl + 17% EDTA) obtained from collected effluent in air. Ion indicating formaldehyde at m/z 31 is shown on the spectrum.

Figure 5 SEM images of Ag-AC. (a) Prismatic rod AC particle (50μm scale; x600 magnification). (b) Deposition of Ag particles onto AC crevices (10μm scale; x3000 magnification). (c) Sponge-shaped AC particle (20μm scale; x1500 magnification). (d) Deposition of Ag particles onto AC surface hive (5μm scale; x5000 magnification).

Figure 6 EDS analysis of Ag-AC at 20μm scale and x2.2k magnification. (a) EDS spectra. (b) Elemental analysis of the sample obtained by EDS. (c) EDS image. (d-j) Elemental mapping of C(K), Si(K), K(K), Ag(L), O(K), Al(K), Na(K).

Table 1 Mean (SD) concentrations (ppb.V) of VOCs and DBPs after SIFT-MS analysis of aliquots obtained from effluent aliquots.

Aliquots in effluent (10ml)	**Group 1** **Chemomechanical preparation with Distilled Water (n=14)**	**Group 2** **Chemomechanical preparation with NaOCl 2.5% and EDTA 17% (n=14)**	**Group 3a** **Chemomechanical preparation with NaOCl 2.5% and EDTA 17% + Effluent treatment with Ag-AC 50mg /60min (n=7)**	**Group 3b** **Chemomechanical preparation with NaOCl 2.5% and EDTA 17% + Effluent treatment with Ag-AC 200mg /15min (n=7)**
Volatile compounds				
Acetone	11.65 (11.63) [a]	36.52 (23.22) *	22.85 (8.36)	13.13 (9.89) ***
Acetic Acid	38.25 (27.12)	139.25 (71.20) *	110.33 (51.92)	41.47 (7.92) ***
Methanol	118.87 (69.98)	3021.19 (986.56) *	3490.10 (568.99)	3056.58 (614.81)
Ethanol	265.53 (87.51)	258.20 (162.79)	418.03 (231.83) [+]	449.18 (275.18) [+]
Propanol	38.74 (23.82)	1654.21 (444.06) *	1042.26 (340.69)	184.31 (79.96) ***
Acetaldehyde	19.93 (15.98) [b]	635.56 (132.68) *	631.43 (78.73)	362.14 (62.56) ***
Acetonitrile	4.93 (3.02)	151.36 (59.55) *	125.71 (11.47)	82.71 (29.12) ***
Ammonia	661.10 (172.13)	4572.07 (1158.15) *	4131.14 (477.64)	4798.43 (1319.27)
Chloroform	62.21 (15.60)	1852.5 (312.33) *	244.14 (53.52) **	193.71 (46.22) **
Formaldehyde	97.76 (94.61)	12507.23 (2834.75) *	14413.88 (2606.11)	11896.18 (2847.95)

[a]: 4 samples presented 0 values; [b]: 6 samples presented 0 values;

*: Group 2- significant increase in concentration of each VOC and DBP compared to Group 1 ($P<0.05$);

**: Groups 3a & 3b- significant reduction in chloroform concentration compared to Group 2 ($P<0.05$).

***: Group 3b- significant reduction in VOCs' concentrations (acetone, acetic acid, propanol, acetaldehyde, acetonitrile) compared to Groups 3a and 2 ($P<0.05$).

+: Groups 3a, 3b- significant increase in concentration of ethanol compared to Group 2 ($P<0.05$).

Table 2 Mean (SD) chlorine availability following the interaction of 1%, 2.5%, 5% NaOCl with Ag-AC for different time intervals (15, 30, 60 min) and variations in Ag-AC mass (5-500mg).

			Remaining Chlorine Availability (M)						
			Interacting mass of Activated Carbon in powder form (mg)						
Irrigant (10mL)	**Molarity (M)**	**Time of interaction (min)**	5	10	20	50	100	200	500
NaOCl 1%	0.1473 (0.01)	15	0.1431 (0.007)	0.1417 (0.005)	0.1318 (0.007)	0.11133 (0.014)	**0.067 + (0.07)**	N/A	N/A
		30	0.1445 (0.004)	0.1431 (0.003)	0.136 (.000)	**0.0822 + (0.007)**	N/A	N/A	N/A
		60	0.1417 (0.003)	0.13 (0.014)	0.119 (.000)	N/A	N/A	N/A	N/A
NaOCl 2.5%	0.36 (0.01)	15	0.3457 (0.003)	0.3414 (0.002)	0.33 (0.003)	0.323 (0.004)	**0.254 + (0.033)**	N/A	N/A
		30	0.33 (0.005)	0.33 (0.007)	0.332 (0.004)	0.296 (0.005)	**0.144 + (0.024)**	N/A	N/A
		60	0.344 (.000)	0.3372 (0.007)	0.319 (0.004)	N/A	N/A	N/A	N/A
NaOCl 5%	0.696 (0.012)	15	0.6928 (0.008)	0.6928 (0.009)	0.6814 (0.005)	0.64 (0.003)	**0.581 + (0.007)**	N/A	N/A
		30	0.6871 (0.007)	0.7026 (0.011)	0.6828 (0.005)	0.6148 (0.002)	**0.5242 + (0.005)**	N/A	N/A
		60	0.6956 (0.005)	0.6715 (0.004)	0.6573 (0.003)	N/A	N/A	N/A	N/A

+: significant reduction of mean(SD) chlorine concentrations ($P<0.05$)

N/A: non-available

Table 3 Safety guidelines and thresholds for environmental emissions of VOCs and DBPs identified in this study: European Union -annual environmental quality standards (EQS) for priority substances; Scottish Environmental Protection Agency -annual Scottish pollutant release inventory (SPRI); United States Environmental Protection Agency – annual determination of reportable quantities (RQ) for hazardous substances.

International Organization	**Name of substance**	**Units of measurement**
		Annual average EQS within inland waters (rivers, lakes and related artificial or heavily modified water bodies) and other surface waters (µg/L)
European Union (EU) [a]	Chloroform	2.5
		SPRI emission reporting threshold to water and waste water (kg/year)
Scottish Environmental Protection Agency (SEPA) [b]	Ammonia	20
	Chloroform	5
		Annual determination of RQ for hazardous substances (kg/year)
United States Environmental Protection Agency (USEPA) [c]	Acetaldehyde	
	Acetic acid	454
	Ammonia	2270
	Chlorine	45.4
	Chloroform	4.54
	EDTA	4.54
	Formaldehyde	2270

Sodium Hypochlorite	45.4
	45.4

[a] Directive 2013/39/EU of the European Parliament and of the Council of 12 August 2013 amending Directives 2000/60/EC and 2008/105/EC as regards priority substances in the field of water policy.
(https://eur-lex.europa.eu/eli/dir/2013/39/oj).

[b] In the UK (including Scotland) releases of chloroform are controlled through regulations on pollution of surface waters (SI 1997/2560); Pollution, Prevention and Control (PPC) regulations; and as a VOC under the National Air Quality Strategy. European Directives controlling emissions of chloroform include those concerned with pollution of aquatic environments (76/464); evaluation of risks posed by substances (793/93); the marketing and use of certain substances (76/769/EEC); ambient air quality assessment and management (96/55/EEC); control of solvent use (99/13/EC); the hazardous wastes Directive; and is listed as a "priority substance" for the proposed Water Framework Directive.
(https://www.sepa.org.uk/environment/environmental-data/spri/).

[c] USEPA Clean Water Act (CWA) establishes the basic structure for regulating discharges of pollutants into the waters of the United States and regulating quality standards for surface waters. Under the CWA, USEPA has implemented pollution control programs, such as setting wastewater standards for industry and water quality standards for all contaminants in surface waters. Section 311(b)(2)(A) regulates discharges of hazardous substances, including formaldehyde.
(https://www.govinfo.gov/content/pkg/CFR-2013-title40-vol23/xml/CFR-2013-title40-vol23-part117.xml#seqnum117.1).

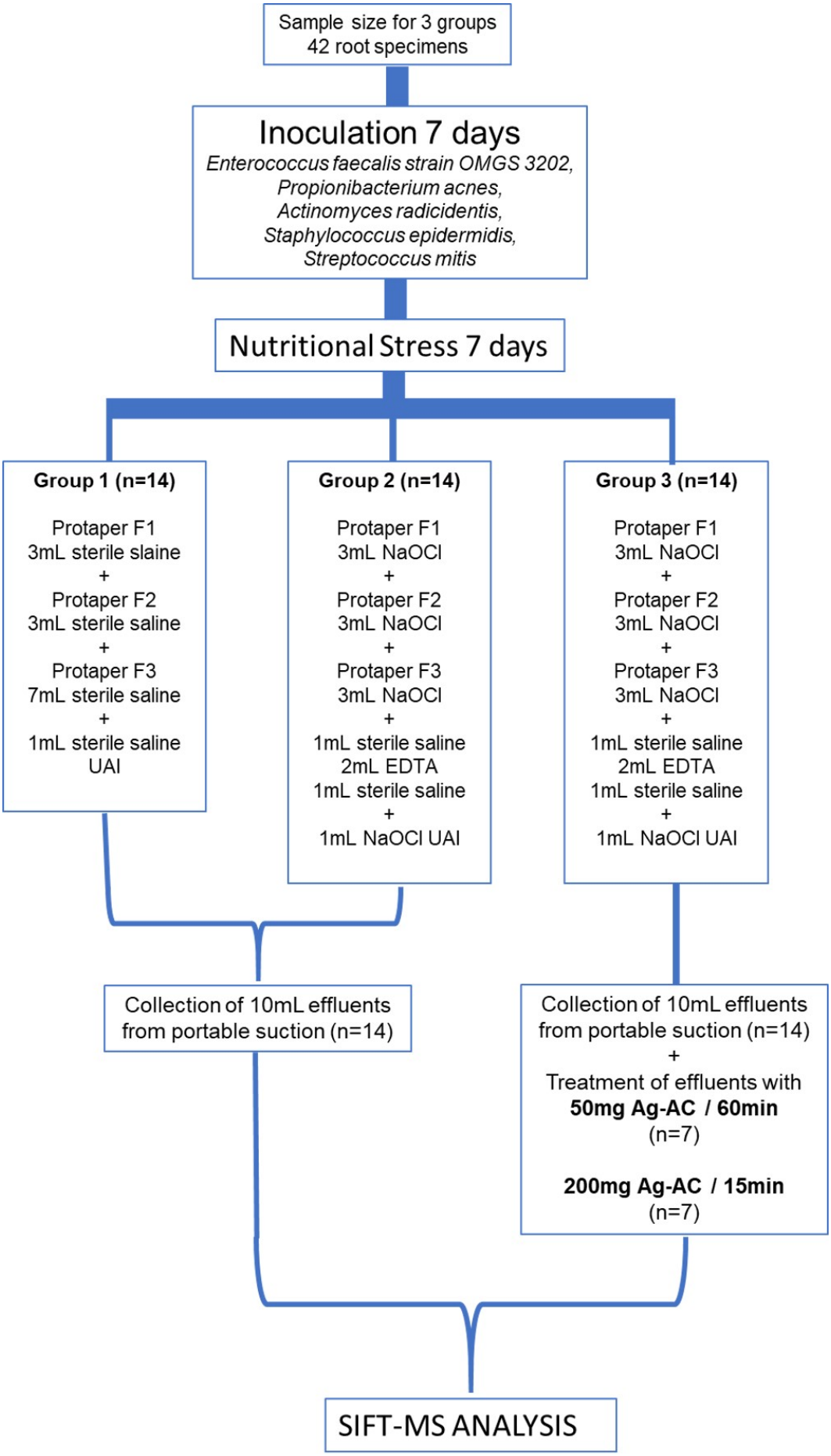
Table 4 Safety guidelines and thresholds in drinking water quality for regulated VOCs and DPBs identified in this study: United States Environmental Protection Agency – maximum contaminant level; World Health Organisation – Guideline value; European Union – Standard Value.

International Organization	**Units of measurement**
United States Environmental Protection Agency (USEPA) regulations [a]	**Maximum Contaminant Level (mg/L or ppm)**
Total Trihalomethanes (THMs) (including chloroform)	0.08mg/L or 0.08ppm
World Health Organization (WHO) guidelines [b]	**Guideline Value (mg/L or ppm)**
Chloroform	0.2mg/L or 0.2ppm
Formaldehyde	0.9mg/L or 0.9ppm
European Union Standards [c]	**Standard Value (mg/L or ppm)**
Total Trihalomethanes (THMs) (including chloroform)	0.1mg/L or 0.1ppm

[a] United States Environmental Protection Agency National Primary Drinking Water Regulations. (https://www.epa.gov/ground-water-and-drinking-water/national-primary-drinking-water-regulations).

[b] World Health Organisation- Guidelines for Drinking-water Quality, Geneva 2008. (https://www.who.int/water_sanitation_health/dwq/fulltext.pdf).

[c] Council Directive 98/83/EC of 3 November 1998 on the quality of water intended for human consumption. (https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:31998L0083&from=EN).


Sample size for 3 groups
42 root specimens
Inoculation 7 days
Enterococcus faecalis strain OMGS 3202,
Propionibacterium acnes,
Actinomyces radicidentis,
Staphylococcus epidermidis,
Streptococcus mitis
Nutritional Stress 7 days
Group 1 (n=14)
Protaper F1
3mL sterile slaine
+
Protaper F2
3mL sterile saline
+
Protaper F3
7mL sterile saline
+
1mL sterile saline
UAI
Group 2 (n=14)
Protaper F1
3mL NaOCl
+
Protaper F2
3mL NaOCl
+
Protaper F3
3mL NaOCl
+
1mL sterile saline
2mL EDTA
1mL sterile saline
+
1mL NaOCl UAI
Group 3 (n=14)
Protaper F1
3mL NaOCl
+
Protaper F2
3mL NaOCl
+
Protaper F3
3mL NaOCl
+
1mL sterile saline
2mL EDTA
1mL sterile saline
+
1mL NaOCl UAI
Collection of 10mL effluents
from portable suction (n=14)
Collection of 10mL effluents
from portable suction (n=14)
+
Treatment of effluents with
50mg Ag-AC / 60min
(n=7)
200mg Ag-AC / 15min
(n=7)
SIFT-MS ANALYSIS

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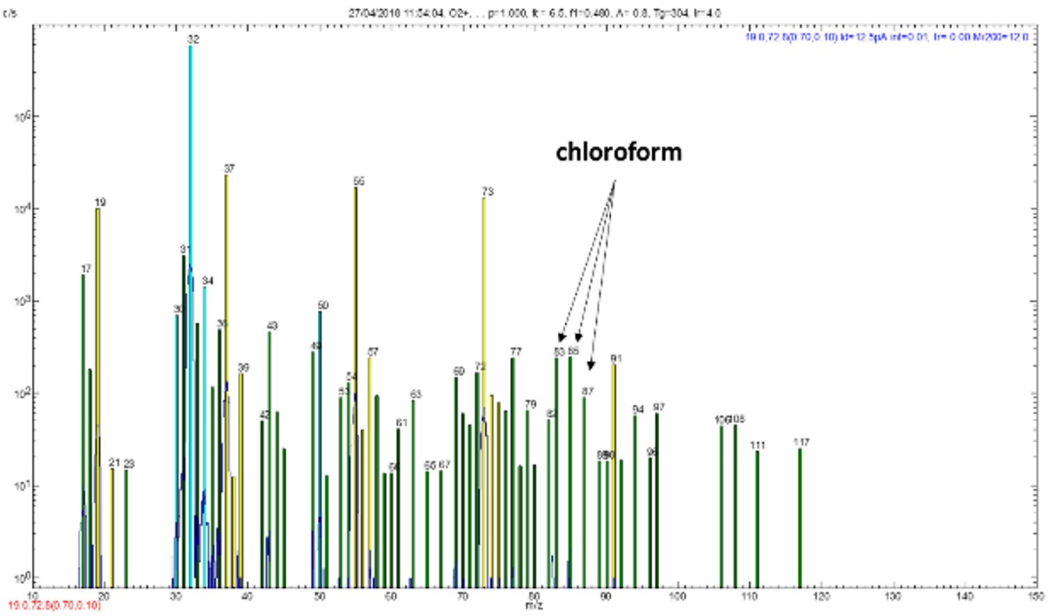

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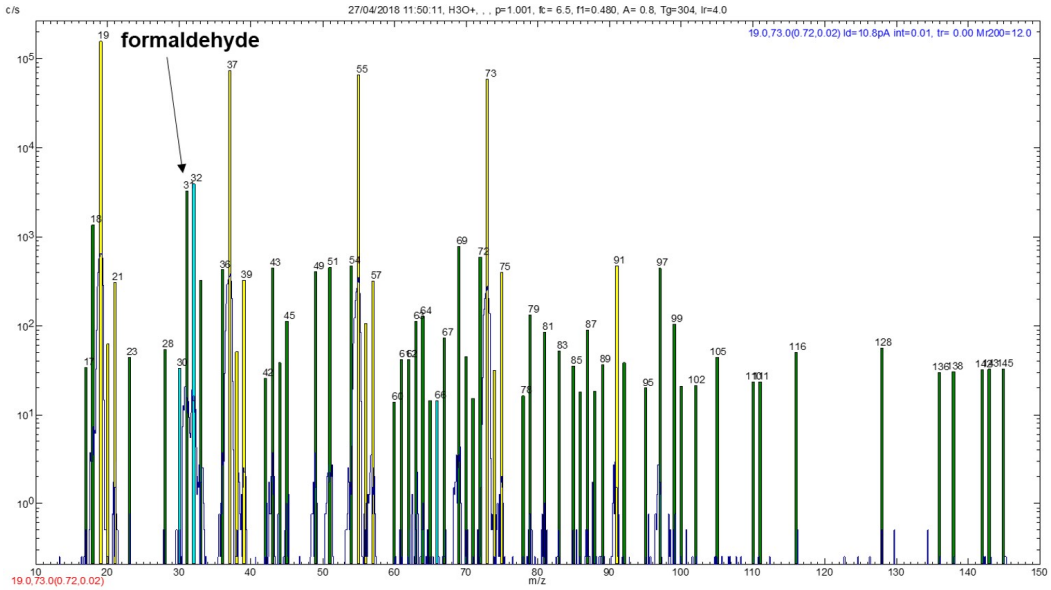

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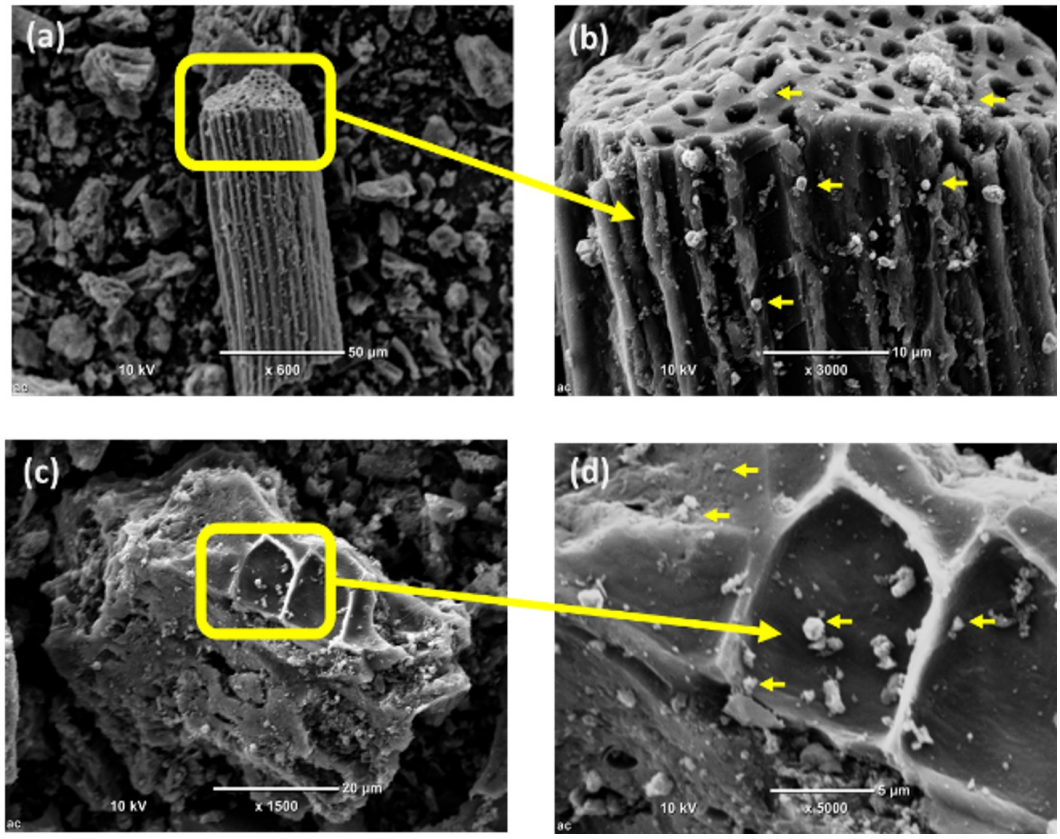

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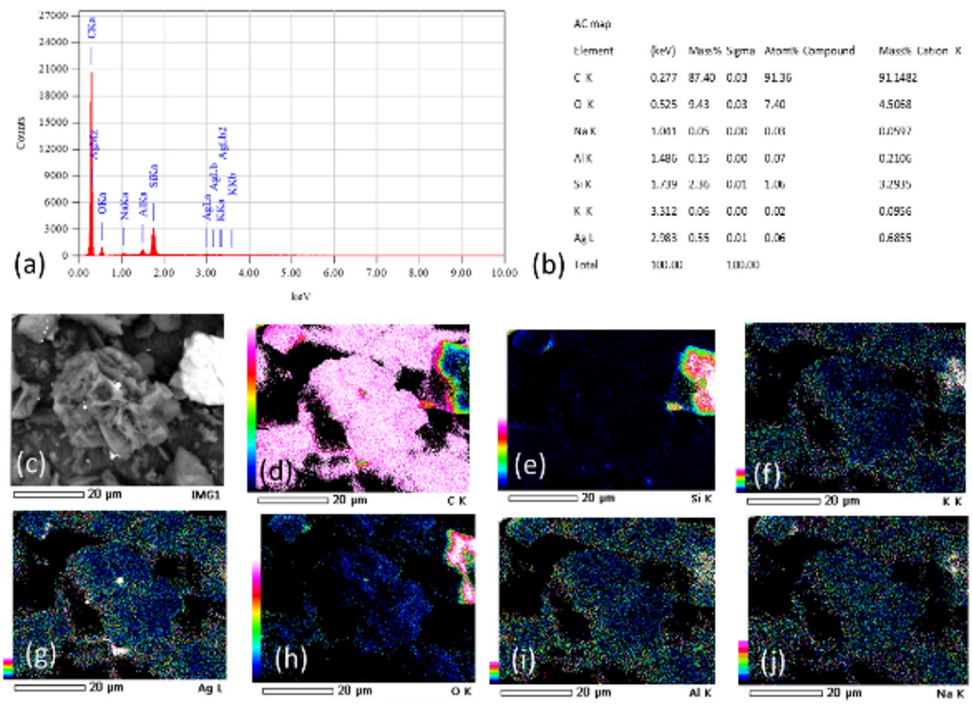

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