Spectroscopic examination of enamel staining by coffee indicates dentin erosion by sequestration of elements

Sinai H.C. Manno, Francis A.M. Manno, Irfan Ahmed, Rafay Ahmed, Lei Shu, Li Li, Shisan Xu, Fangjing Xie, Vincent Wai Li, Johnny Ho, Shuk Han Cheng, Condon Lau

1. Introduction

Teeth are susceptible to staining by drinks such as coffee, one of the most popular beverages consumed worldwide. There are studies describing the advantages that coffee offers, for instance, preventing periodontal disease [1] and caries [2,3]. However, the pigmentation of most popular beverages consumed worldwide. There are studies demonstrating the mechanism of coffee altering the structure of teeth. Coffee has a high risk of eliciting erosion [5–7], but the mechanism is not fully understood. Currently, a lack of evidence exists concerning the structural changes induced by coffee staining on teeth. In the present study, our aim was to determine the structural changes induced by coffee on teeth and determine how teeth are affected by coffee pigmentation.

Dentin and enamel are two mineralized tissues with an important function in the external protection of teeth. Dentin shares similar properties to bone in matrix composition and possesses anisotropic qualities, providing resistance to mechanical stress [8]. Although harder than bone, comprising a 65% inorganic and 35% organic matrix, dentin has the capacity to prevent fractures due to its elasticity and flexibility overlying the inelastic enamel [9,10]. Enamel is followed by dentin, one of the most popular beverages consumed worldwide. There are studies demonstrating the mechanism of coffee altering the structure of teeth. Coffee has a high risk of eliciting erosion [5–7], but the mechanism is not fully understood. Currently, a lack of evidence exists concerning the structural changes induced by coffee staining on teeth. In the present study, our aim was to determine the structural changes induced by coffee on teeth and determine how teeth are affected by coffee pigmentation.

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which is the principal component of the tooth and often considered to be the hardest tissue in vertebrata body [11]. Its hardness is attributed to a high level of hydroxyapatite which is deposited over the organic matrix, rich in proteins, principally collagen. Enamel contains the organic matrix and water, 1% and 4% composition, respectively, but 95% of enamel derives from mineral compounds [12]. The main elements forming the structure of hydroxyapatite are calcium (Ca), phosphorus (P) and other elements in minor amounts [13]. Enamel is the outermost layer of teeth, therefore vulnerable to damage. The staining of enamel could lead to demineralization causing erosion [14,15]. We have postulated that coffee deposition could lead to subtle erosive effects on teeth by altering enamel composition. Erosion is a progressive localized loss in the main elemental components of enamel by the effect of acid or chelation without bacterial action [15–17]. Decalcification is the first step in enamel destruction; therefore, variation in the level of Ca and P detected by spectroscopy and/or microscopy techniques could serve as markers for erosion of teeth [15].

Analytical sample processing and analyses are techniques used to investigate elements in biological specimens [18–21]. Spectroscopic analyses are popular techniques because of their capacity for specific elemental distinction in tissues and materials of interest [13,17,22–31]. In Table 1, we have summarized recent spectroscopy techniques used to determine elements in teeth enamel and dentin [15,22,23,31,32]. For example, Laser-induced Breakdown Spectroscopy (LIBS) possess high sensitivity and spatial resolution in real time allowing one to identify and sort out the nature of solids, such as teeth [13,28,30]. The LIBS technique has the potential to recognize changes in the composition of enamel in healthy teeth, carious teeth and teeth treated by bleaching. The main advantages of LIBS include easy sample preparation and relatively no sample alteration or destruction [13,26–31]. Here, we have used LIBS to measure the surface (e.g. in the enamel) erosion effect induced by coffee. We subsequently utilized a second spectroscopic technique, X-ray Fluorescence Spectroscopy (XRF), to assess dentin elemental components, due to the higher penetration, i.e. the XRF technique analyzes a sample deeper than LIBS (hence the ability to assess dentin). With XRF it is easy to identify the elemental content by depth of penetration [25,30,32]. The XRF technique provides excellent analyses of underlying tissues such as dentin. Both LIBS and XRF in combination provide an exact measure of surface (enamel) and depth (dentin) of teeth during biological changes, such as the effect of coffee. Although there are studies using spectroscopy in dental research, little evidence exists about alterations induced by coffee staining to the enamel structure and the mechanism of coffee pigmentation to dental tissues.

In the present study, our objective was to identify the changes in the enamel surface elicited by coffee immersion through LIBS, due to the high sensitivity of detection of elements. Secondly, we complemented our LIBS surface analysis with XRF instrumentation as a standard technique to compare the spectrum of nine elements from every specimen. Additionally, we performed morphological analysis using Scanning Electron Microscopy (SEM) and histological assessment by Hematoxylin/Eosin (H&E) and Masson’s Trichrome staining to report the histological changes induced by coffee. Lastly, we have identified the quantity of metals contained in coffee by mass spectroscopy. In summary, we report that coffee elicits a loss of mineral content in teeth induced by superficial staining of the enamel, resulting in structural erosion of the underlying dentin.

2. Material and methods

This study used 24 unilateral jaws immersed in coffee or water during two weeks. The samples were dried in a freeze dryer prior to the start of the experiments using LIBS, XRF, SEM and histology techniques. Fig. 1 summarizes the experimental design and logical flow of our study.

2.1. Back-of-the-envelope calculations

In the style of E. Fermi we wanted to roughly estimate the effects of coffee on teeth by simulating the deposition of coffee over a long duration. Methodologically, we followed a protocol similar to previous immersion procedures [33]. Here, we have some back-of-the-envelope calculations to estimate drinking coffee for a long period duration. We choose a decade to simulate our coffee drinking period. We choose young (4 weeks) and old groups (6 months) because we anticipated older rats would have changes in their inorganic matrix, causing more prominent erosion compared to younger rats. We wanted to replicate the deposition of coffee which takes place over 3650 days (decade), but in an accelerated format; therefore, we calculated if an individual drinks coffee for 5 min a day, every day a year for a decade: 5 min × 365 days = 1825 min a year or 18,250 min in a decade. To simulate this type of deposition, we placed our samples in coffee groups (old and young) in liquid coffee for 18,250 min (Fig. 1a). Since there are 10,080 min in a week and 20,060 min in a two week period, we simulated a decade of deposition by placing the samples in coffee for two weeks. Roughly a fortnight of teeth in coffee should be equivalent to drinking coffee for approximately a decade or more. Each daily coffee dose consisted of approximately 30 g brewed coffee. All coffee was donated from a Starbucks coffee shop located on the University campus.

2.2. Animals

Twenty-four unilateral jaw bones from male Sprague-Dawley (SD) rats of one month or six months of age, considered as young or old group (N = 12, 250–350 g or N = 12, 500 g, respectively) were used. Animals were provided by the accredited Laboratory Animal Unit of the University of Hong Kong. The present study was approved by the animal research ethics committees of the City University of Hong Kong, the University of Hong Kong, and the Department of Health of the Hong Kong Special Administrative Region. The animals were housed under a constant 25 °C temperature and 60–70% humidity at the Laboratory Animal Research Unit of the City University of Hong Kong. Animals were housed in 12/12 h light/dark cycles with access to food and drinking water, ad libitum. Animals were acclimated to the housing environment for at least one day prior to euthanasia and surgical extraction of jaw bones. The entire bilateral jaw was extracted and used for the experiments, separated from the adjacent soft tissue and cleaned with fresh water. Teeth were conserved on jaws. Twenty-four unilateral jaws were divided into 4 groups of six samples per tube consisting of 50 ml distilled water or coffee (Fig. 1a). The control water group or the coffee groups were changed every 24 h for two weeks duration. The coffee was 30 mg/50 ml steamed coffee (coffee groups) or 50 ml distilled water. Six jaws were grouped in tubes and labeled as follows: young control, young coffee, old control, old coffee.

2.3. Coffee preparation

We used Arabica coffee which was changed every 24 h during the 2-week period. The pH of coffee was evaluated using Eutech-Instruments-700 pH meter in water at room temperature. We prepared 30 g of coffee

<p>| Table 1 Spectroscopic methods used to investigate dental tissues. |
|-------------------------|-------------------------|-------------------------|</p>
<table>
<thead>
<tr>
<th>Dental tissue</th>
<th>Spectroscopy method</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enamel</td>
<td>LIBS</td>
<td>Gazmeh et al. [31], Imam et al. [13].</td>
</tr>
<tr>
<td>Dentin</td>
<td>LIBS</td>
<td>Gazmeh et al. [31], Imam et al. [13].</td>
</tr>
<tr>
<td>Dentin</td>
<td>XRF</td>
<td>Gomes et al. [32], Djoméri et al. [22], Imam et al. [13].</td>
</tr>
<tr>
<td>Dentin</td>
<td>XRF</td>
<td>Djoméri et al. [22], Beres et al. [23].</td>
</tr>
</tbody>
</table>
in hot water shaken vigorously. Jaws in the coffee groups were immersed and placed at room temperature \( \approx 22 ^\circ C \). After two weeks, all samples were washed in distilled water and dried in a freeze dryer for 48 h (Fig. 1a).

### 2.4. Determination of caffeine content and metal present in coffee

A quantity of 90 g of coffee powder was tested by High Performance Liquid Chromatography (HPLC) to determine the caffeine content, in addition to principle elements. The quantitative caffeine content was determined using Deutsches Institut für Normung (DIN) ISO 20481 standard. The content of metals such as: K, Mg, Fe, Mn, Zn, Sr, Ni and Cd was based on the AOAC method 999.10 in Lead, Cadmium, Zinc, Copper, and Iron in Foods Atomic Absorption Spectrometry after Microwave digestion. An Inductively Coupled Plasma Mass Spectrometer (ICP-MS – Agilent 7500cs) or Atomic Absorption Spectrometer (AAS) was used to assess concentrations. Here, products were digested with HNO3 and H2O2 under pressure. The procedure followed was previously described [34,35].

### 2.5. Laser-induced breakdown spectroscopy (LIBS)

We adopted the LIBS procedure from our previous publication using biological material [36]. Twelve bilateral jaws were used for the LIBS analysis (3 from each group). Three teeth were extracted from jaws to avoid background spaces among the occlusal grooves and adhered to a glass slide placed in a sample holder for LIBS emission (Fig. 1b). A 1064 nm pulsed laser (CFR200, Quantel) emitting 8 ns, 200 mJ pulses focused to a 10 \( \mu \)m spot was used to shoot teeth samples. The sampling depth of LIBS was superficial, and examined only the outermost 0.5 \( \mu \)m surface of the enamel in teeth. The optical emission from the ablated tissue was collected by a six channel fiber (2000 \( \mu \)m diameter) bundle positioned 35 mm from the focal point at 45\(^\circ\) from the laser beam. The fibers relayed light to six spectrometers spanning 200–800 nm with 0.1 nm resolutions (MX2500+, Ocean Optics). The spectrometer was triggered to acquire 0.9 \( \mu \)s after the laser firing and with 1 ms acquisition. The experiments were performed in standard atmospheric air. A PC processed spectrum acquired by the spectrometers and produced a graphical presentation of spectral intensity against the corresponding wavelength. At the time of acquisition, the slide was placed on the sample holder with the dental region of interest at the focal point, three laser pulses were fired, and the emission spectra recorded. The slide was then moved to measure subsequent locations with three more acquisitions performed, with three laser pulses fire per location. The LIBS system is represented in Fig. 1b.

### 2.6. X-ray fluorescence (XRF) spectroscopy

We adopted the XRF procedure from our previous publication in bone [37]. For XRF analysis, a x-ray fluorescence spectrometer was used (Eagle-III \( \mu \)Probe – EDAX) with a power of 40 kV, 40 W X-ray tube (Mo), 1 mm Mono-capillary, and 80 mm\(^2\) SapphireTM Si(Li) detector. The XRF allowed the detection of elements in the interior structure of the tooth, approximately 10–20 \( \mu \)m depth of penetration. The equipment has a microscope integrated where it was possible to obtain images at 0.5 mm, 100 \( \times \) zoom. Three different regions per sample were selected with a dwell time of 20 s each (Fig. 1c). Nine elements were obtained from the spectrum according to the principle mineral contents of enamel and calculated as a factor of 100% weight. Here, elements were calculated as percentage of 100% (9 elements) and represented the whole spectrum acquired from XRF. The classification was considered as major or minor constituents as follows:

- **Major constituents**: Calcium (Ca), Phosphorus (P), Potassium (K), Manganese (Mn) and Chlorine (Cl),
- **Minor constituents**: Iron (Fe), Zinc (Zn), Sulfur (S) and Strontium (Sr).

Fig. 1. Experimental design. (a) Jaws from young or old groups were immersed in coffee or in distilled water during two weeks. (b) The exodontia of three teeth was used to perform LIBS spectral analysis to determine surface alterations due to coffee. (c) For XRF, three different regions of the clinical crown were selected and analyzed for dentin structural effects due to coffee. (d) Jaws were analyzed with SEM. (e) Hematoxylin/Eosin and Masson’s trichrome staining was used for histological analysis.
2.7. Scanning electron microscopy (SEM)

Each sample was cleaned and subsequently placed in a freeze dryer prior to collecting scanning electron microscopy (SEM) images (Phenom Pro. Generation 5) (Fig. 1d). Previous to detailed assessment, specimens were scanned using a standard microscope 62× magnification (Fig. S3) coupled to the equipment. From this point, the magnification proceeded to 205×, 760×, 2800× and 13,500× operated with an acceleration voltage of 10 kV beam irradiation of secondary electrons. The distance of magnification oscillated from 1 mm to 5 µm, according to the size of the specimen. Five digital non-overlapping images of first, second and third tooth were obtained identifying the occlusal surface band on the mesial slope or distal facet of each tooth.

2.8. Histology

Two jaws per group were selected and histologically processed (N = 8). The decalcification and fixation of tissue samples was performed using an ethylenediaminetetraacetic acid (EDTA) solution with fixative, incubated and gently shaken during fifteen days (17% EDTA/10% Formalin). After decalcification and fixation, samples were embedded in paraffin. Titrations of ethanol changes starting at 75% ethanol to 100% ethanol were administered hourly, followed by xylene immersions and paraffin wax at 60 °C for two hours. The slices were cut ≈ 5 µm thick in the central section using a microtome (Leica RM 2125RT). Slides were dehydrated in 100% alcohol, cleared in xylene 2 × 5 min and mounted with Permount™ mountant (Fischer Scientific). Every section was mounted on silica-coated slides (Fig. 1e). Teeth were stained with hematoxylin & eosin or Masson’s trichrome. Images were taken with an Olympus BX61 microscopy coupled to an Olympus DP72 camera. The protocol was adopted from previous reports [38,39].

3. Results

In coffee groups, we observed ratios of Ca and P decreased using spectroscopy techniques. Microscopy and histology analyses indicated coffee caused demineralization and irregularities on the superficial enamel, in addition to substantial damage to internal structures of the tooth (i.e. dentin).

3.1. Caffeine and element content of coffee

The pH of coffee used in this study was pH of 6.3. The HPLC indicated that the concentration of caffeine was 738.3 mg per tube (90 g) where teeth were immersed every day. As determined by ICP-MS or AAS, the major metal elemental contents in coffee was K (1102.49 mg), followed by Mg (318.96 mg), Fe (16.36 mg) and Mn (8.7 mg). Whereas the minor contents in coffee were Sr with 16.5 mg and Zn with 3.41 mg and Ni and Cd, with values under 0.08 mg (Table 2).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total content (mg/kg)</th>
<th>Content tested (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caffeine</td>
<td>2215</td>
<td>738.3</td>
</tr>
<tr>
<td>K</td>
<td>3307.49</td>
<td>1102.49</td>
</tr>
<tr>
<td>Mg</td>
<td>956.90</td>
<td>318.96</td>
</tr>
<tr>
<td>Fe</td>
<td>49.08</td>
<td>16.36</td>
</tr>
<tr>
<td>Mn</td>
<td>26.02</td>
<td>8.67</td>
</tr>
<tr>
<td>Zn</td>
<td>10.23</td>
<td>3.41</td>
</tr>
<tr>
<td>Sr</td>
<td>4.51</td>
<td>16.5</td>
</tr>
<tr>
<td>Ni</td>
<td>&lt; 0.25</td>
<td>&lt; 0.08</td>
</tr>
<tr>
<td>Cd</td>
<td>&lt; 0.25</td>
<td>&lt; 0.08</td>
</tr>
</tbody>
</table>

The total content refers to the amount obtained from 90 g of coffee powder. The content tested was weight used from 30 g of coffee daily immersed during two weeks.

3.2. Loss of main elements in enamel identified by LIBS

The principal elements in the tooth structure were identified with a range from 200 to 800 nm by spectroscopy. Elements with major intensity were Ca (I and II), P I, Na I, Mg II and K I and minor intensity were Zn II, C I, Mn II, and Sr II (S1). Tables 3 and 4 summarize the relative wavelengths and intensity spectra from each element identified from young and old groups, respectively, with their statistical differences marked by asterisk. The tables indicate the average for every group obtained from different points per sample as detailed in the methods. Fig. 2 illustrates the contrast intensity between young-control (black) and young-coffee (red) and the main elements from enamel (Fig. 2a). The ranges include (Fig. 2b–e): 200–234 nm, 230–310 nm, 365–475 nm and 565–785 nm. The results indicate a generalized decrease in the levels of calcium (Ca I and Ca II), phosphate (P I), sodium (Na I), manganese (Mn), zinc (Zn II) and strontium (Sr II) in coffee groups. In young groups, the elements which experienced a statistical significant decrease were Ca I (t = 4.281, p < 0.001) and Ca II (t = 4.748, p < 0.001), P I (t = 5.02, p < 0.001) and Na II (t = 11.62, p < 0.001). In contrast, statistically significant increases were observed in the spectra of Mg II (t = 8.339, p < 0.001), Fe I (t = 4.216, p = 0.001), and K I (t = 17.61, p < 0.001). Although some elements were not statistically significant, the intensity of Mn, Ni and Zn decreased after coffee immersion, whereas emissions lines in C increased after coffee immersion (Table 3 and Fig. 2). Similarly, Fig. 3 indicates the merge of old-control (blue) and old-coffee group (green), showing the differences in the principal elements of the dental surface (Fig. 2a). The minor element ranges were from 200 to 234 nm, 230–310 nm, 365–475 nm and 565–785 nm (Fig. 3b–e). The levels of Ca I (t = 9.451, p < 0.001) and Ca II (t = 5.081, p < 0.001), P I (t = 4.75, p < 0.001), Mn II (t = 5.768, p < 0.001), Sr (t = 8.234, p < 0.001), Na (t = 9.57, p < 0.001) and Zn II (t = 4.177, p < 0.001) decreased significantly in the coffee group in almost all wavelengths identified. On the other hand, a statistical significant increase in Mg II (t = 7.604, p < 0.001), K I (t = 8.934, p < 0.001), and Fe I (t = 3.96, p < 0.01) after coffee immersion was observed for old teeth. The Sr II and C I decreased after coffee immersion, but did not reach significance (Table 4 and Fig. 3). Overall, the old-coffee group was affected more after coffee immersion while young-coffee had a greater retention of magnesium, potassium and iron (Fig. S2). Statistical results from the ANOVA are listed with an asterisk (*) in LIBS figures. Generally, the ANOVA indicated that young-control and young-coffee groups were statistically different, with wavelength differences dependent on the element (Fig. 2). Generally, the ANOVA indicated that old-control and old-coffee were statistically different, with wavelength differences dependent on the element (Fig. 3).

3.3. XRF: coffee immersion altered elemental composition of teeth

The analysis of XRF indicated average value percent weight (wt%) of elemental concentration as assessed by spectroscopy. The main elements considered were Ca, P, K, Mn, Zn, Sr, S, Cl, and Fe. All elements showed considerable variabilities among controls and coffee groups. Fig. 4 summarizes the average intensity of elements found from young-control, young-coffee, old-control and old-coffee groups. Calcium was significantly decreased in young-coffee compared with young-control (t3 = 3.208, p < 0.05). The levels found in phosphorus for young-coffee remained nearly identical; in contrast, the old-coffee group had a slight increase. Furthermore, the Ca/P ratio of the four groups was evaluated. Subsequently, groups were compared with the standard reference material Bone Ash to determine how coffee affected this metric (Tables S1 and S2). Bone Ash resulted in a ratio of 2.102, while coffee group ratios were reduced compared to their respective controls. In general, a decalcification was noted compared with their respective control groups based on the decrease in ratio found in both coffee groups (Fig. 4 and Tables S1 and S2).
To follow-up the qualitative analysis, we performed a two-way ANOVA to determine if groups (young-control, young-coffee, old-control, old-coffee) were different in terms of elements with follow-up t-tests to determine elemental significance. We report that groups were significantly different (F3,27 = 10,740, p < 0.001) most likely due to use of total percent composition as the underlying factor. However, a follow-up t-test on percentage found Ca significantly different between young-control and young-coffee (t3 = 3.08, p < 0.05). Therefore the sensitivity of XRF was considerably lower than LIBS when using the ultrastructural variations in all groups and identify ultrastructural concentrations of Fe, Zn, and Sr as detected by XRF.

### 3.4. Scanning electron microscopy: decomposition of structures in enamel and EDJ due to coffee

Scanning electron microscopy was performed to determine the topographical variations in all groups and identify ultrastructural differences in coffee groups. Fig. 5 illustrates occlusal or transversal surfaces of specimens at magnification from 100 to 3 μm. Generally, a change of brown coloration in young and old coffee groups was noted, but the coloration was substantial in the old group (Fig. S3). Young-control images demonstrated a pattern of parallel organization well-defined in the mature enamel region (Fig. 5a and b). In old-control

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| Table 3 |

Principal elements in enamel structure determined by LIBS in Young groups.

<table>
<thead>
<tr>
<th>Element</th>
<th>Group</th>
<th>Relative wavelength (nm)</th>
<th>Relative intensity (a.u.)</th>
<th>Element</th>
<th>Group</th>
<th>Relative wavelength (nm)</th>
<th>Relative intensity (a.u.)</th>
<th>Element</th>
<th>Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zn II</td>
<td>Control</td>
<td>203</td>
<td>240.9*</td>
<td>383.18</td>
<td>Fe I</td>
<td>Control</td>
<td>1166*</td>
<td>643</td>
<td>Ca I</td>
</tr>
<tr>
<td>Zn II</td>
<td>Coffee</td>
<td>206</td>
<td>118.9*</td>
<td>213</td>
<td>Ca II</td>
<td>Control</td>
<td>1294*</td>
<td>671</td>
<td>Ca I</td>
</tr>
<tr>
<td>Mg II</td>
<td>Control</td>
<td>247</td>
<td>2693</td>
<td>374</td>
<td>Ca I</td>
<td>Control</td>
<td>18,990*</td>
<td>468</td>
<td>Sr II</td>
</tr>
<tr>
<td>Mg II</td>
<td>Coffee</td>
<td>253</td>
<td>1951</td>
<td>393</td>
<td>Ca II</td>
<td>Control</td>
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<td>733</td>
<td>Sr II</td>
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<td>715</td>
<td>6534*</td>
<td>396</td>
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<td>Control</td>
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<td>Control</td>
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<td>744</td>
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<td>609*</td>
<td>446</td>
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<td>29,774*</td>
<td>746</td>
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<td>Mg II</td>
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<td>K I</td>
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<tr>
<td>Mg II</td>
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<td>4304*</td>
<td>554</td>
<td>Ca I</td>
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<tr>
<td>Mg II</td>
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<td>3500*</td>
<td>1316</td>
<td>Ca I</td>
<td>Control</td>
<td>7157*</td>
<td>7157*</td>
<td>K I</td>
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Wavelengths are expressed in nanometers and their respective intensity found in young control and young coffee groups. * indicates p < 0.001.

| Table 4 |

Principal elements in enamel structure determined by LIBS in Old groups.

<table>
<thead>
<tr>
<th>Element</th>
<th>Group</th>
<th>Relative wavelength (nm)</th>
<th>Relative intensity (a.u.)</th>
<th>Element</th>
<th>Group</th>
<th>Relative wavelength (nm)</th>
<th>Relative intensity (a.u.)</th>
<th>Element</th>
<th>Group</th>
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<td>K I</td>
</tr>
</tbody>
</table>

Wavelengths are expressed in nanometers and their respective intensity found in old control and old coffee groups. * indicates p < 0.001 and for Fe, p < 0.01.
group the pattern of parallel organization indicated the presence of
breakage and interruption in the continuity of enamel prisms
Fig. 5e and f). Both coffee groups (young and old) showed cracks,
craters and separations in the enamel and EDJ surfaces being most
abrupt in the old-coffee group. For the young-coffee group, the
presence of pores and craters was identified among disorganized prisms
over the mature enamel region (see Fig. 5c and b). Furthermore,
images obtained from the old-coffee group demonstrated a pattern of
separated lamella and considerable spaces with loss of continuity
along of the EDJ (Fig. 5g and h).

3.5. Histological evidence corroborates coffee erosion

The histology exhibited a lack of cell components due to the long
interim before staining and lack of fixation, which was part of the ex-
perimental design and not caused by coffee (Fig S4). In general, the
Hematoxylin/Eosin staining exhibited lack of mineral tissue in enamel
structures in both young-control and young-coffee groups. Demineralized
areas in the EDJ over the primary dentin were identified in the young
coffee group as well as a splashed zone with opalescent pigments in and
after the EDJ. This pattern was different from the young-control group
(Fig. S4c and d). The H&E staining made evident that the old-coffee
group had open spaces along the enamel and dentin tubules, being
different from their respective control (Fig. S4c and d).

Masson’s trichrome indicated exposure of the organic matrix as seen
by blue staining in the enamel and EDJ regions of both young-control
and young-coffee groups. However, the young-coffee group showed an
organic matrix staining highly-colored, principally in the enamel, while
the young-control group had splashed areas of collagen in the EDJ (Fig.
S4c and d). The old-control group had exposed collagen, principally
throughout the dentin region. The Masson’s trichrome staining did not
detect organic matrix in the old-coffee group, but there were open
spaces along the enamel and dentin as a result of the demineralization
process (Fig. S4g and h). In summary, histological analysis demon-
strated coffee immersion elicited demineralization in enamel and
change in the dentin structure.

4. Discussion

In the present study we examined the effect of coffee on teeth with
two spectroscopy techniques and they were complemented with elec-
tron microscopy and histological analysis. Our results demonstrate
coffee promotes loss in the mineral phase of teeth and demineralizes
areas causing open spaces among the enamel prisms and dentinal

Fig. 2. The LIBS analysis for young-control and young-coffee groups. (a) The
merge of emission lines from young-control and young-coffee groups obtained
by LIBS demonstrated decreases of the main elements present in enamel and
increases of metals present in coffee. (b) Magnification of emission lines of zinc,
calcium and phosphorus decreased after coffee immersion. (c) Phosphorus and
manganese decreased while levels of magnesium were considerably elevated
in the coffee group. (d) Elevated levels of iron and magnesium and decreases in
calcium and strontium after coffee immersion. (e) The levels of sodium, calcium,
calcium, phosphorus and strontium were decreased while potassium intensity in-
creased under coffee immersion. Statistical significance in young-control (black)
and young-coffee (red) are represented with an asterisk (*) where p
values < 0.001.

Fig. 3. The LIBS analysis for old-control and old-coffee groups. (a) The
merge of emission lines from old-control and old-coffee groups obtained by LIBS de-
monstrated decreases of the main elements present in the enamel and increases
of metals present in coffee. (b) Magnification of emission lines of zinc, calcium
and phosphorus decreased after coffee immersion. (c) Phosphorus and man-
ganes decreased while levels of magnesium were considerably elevated in the
coffee group. (d) Elevated levels of iron and magnesium and decreases in cal-
cium and strontium after coffee immersion. (e) The levels of sodium, calcium,
calcium, phosphorus and strontium were decreased while potassium intensity in-
creased under coffee immersion. Statistical significance in old-control (blue) and old-
coffe (green) are represented with an asterisk (*) where p values < 0.001 and
for Fe, p values < 0.01.
tubules. The degree of damage was found to be larger in old-coffee compared with the young-coffee group. Coffee possesses low pH, which could lead to reduction in concentrations of Ca and P by sequestering these elements [5,7,40]. According to our results, coffee not only stains the external enamel but also affects the internal structures of teeth (i.e. dentin).

Fig. 4. The XRF analysis demonstrates considerable variability in the principal components of enamel and dentin among the groups treated with coffee. The y-axis is intensity percent weight (wt%). Levels of calcium were statistically different in the young group after coffee immersion. (a) Calcium. (b) Phosphorus. (c) Potassium. (d) Manganese. (e) Zinc. (f) Sulfur. (g) Strontium (h) Chlorine. (i) Iron. All values were determined according to weight of each element. Statistical significance is represented with an asterisk (*) where p value < 0.05.

Fig. 5. Coffee induces decalcification of the enamel and primary dentin surfaces identified by SEM. (a) Enamel (black arrow) and dentin exposure (asterisk) on the occlusal surface of first molar from young-control. (b) Parallel organization of prisms from the immature enamel in young-control. (c) Enamel and EDJ (black arrow) in the cusp of first molar from young-coffee. (d) Craters and depressions among prisms with irregular organization in the enamel and EDJ in young-coffee. (e) Enamel (black arrow) and dentin exposure (asterisk) on the occlusal surface in cusp of the first molar of old-control group. (f) Parallel organization of enamel prisms and EDJ with some slight cracks (yellow head-arrows) from old-control group. (g) Enamel-dentin-junction (black arrow) in the longitudinal section from old-coffee group. (h) Wide spaces interspersed and loss of continuity along the EDJ and in the dentinal tubules (yellow head-arrows). Circles indicate the magnification regions. Images were taken in magnifications from 100 µm, 30 µm and 3 µm. Scale bar = 100 µm.
4.1. Erosive and staining effects of coffee on the superficial enamel surface

Laser-induced breakdown spectroscopy is used for quantitative analysis of surfaces by identifying different emission lines of elements in real time [26–30]. The high sensitivity of LIBS for the external structure of teeth such as enamel is demonstrated by the specificity for the main elements of the mineral phase in teeth such as Ca and P, in addition to the spectra of Zn II, Ca I and II, C I, P I, Mg II, Mn II, Sr II, Na I, Fe I, and K I. Elements were matched to emission lines by the NIST database and corroborated by previous studies by Imam et al. [13] and Samek et al. [28]. These authors indicate LIBS is a useful tool for detecting acute loss in the main elements in enamel and dentin, i.e. calcium and phosphorus. Although these researches did not include the emitted lines of Fe in their studies, we considered Fe as important for our objectives and reported Fe since it is an important element in teeth [37] and changes considerably after coffee immersion, consistent with our results from ICP-MS. Here, ICP-MS results indicate elements present in coffee with high concentration (Table 2). Nevertheless, we experienced difficulty in discerning the lines of Fe, due to its proximity with Mg. According to Nsullah et al. [41], the emission lines of Fe are often difficult to identify because of similarities with Mg and Ca. Moreover, for both coffee groups we noticed the intensity in K, Mg and Fe increased. Interestingly, studies by Prskalo, et al. [42] and De Sousa, et al. [43], have reported elements such iron, manganese, nickel and others in liquid solutions can change pigmentation of enamel to colors such as brown, black or green. Specifically, Prskalo [42], demonstrated that in tooth discoloration there are trace presences of calcium, magnesium, iron, copper and zinc. In the current results, it was clear coffee elicited a strong change in coloration and increased concentrations of Mg, K, and Fe in both coffee groups. Currently, not enough evidence exists concerning the specific elements in coffee and this justifies further investigation. Jãlevik et al. [44] has described levels of Mg and K increases in regions toward the surface of dentin to enamel eliciting hypomineralization. Enamel is comprised of ridges which facilitate penetration by drinks with high staining properties, such as coffee. These ridges could assist in the changing of coloration, and more importantly the erosive effect. Although the levels of carbon in the young group did not show a statistical difference, these increases correlate to the exposure of the organic matrix, and it is carbon that is an important element for organic compounds [45]. Therefore, the effect induced by coffee was a decrease in inorganic elements crucial in dental structure and increase of other elements such as Mg and K which most likely facilitating erosion in enamel. The LIBS emission results indicate an alteration in elements eliciting staining of enamel surfaces most likely involved in hypomineralization.

4.2. Erosive effects of coffee on the deep structure

The minerals forming teeth include trace elements that are distributed along the crown (in enamel and dentin). The quantification and distribution of those essential components assessed using spectroscopy techniques provide specific information on the dental composition [27]. To identify differences in the structure of teeth at depth, i.e. dentin, we performed XRF analysis, selecting nine inorganic elements: Ca, P, K, Mn, Zn, S, Sr, Cl, and Fe. We hypothesized, that due to the degree of penetration of XRF, the percent weight of these elements would vary in dentin between our coffee and control groups. Here, we report a Ca decrease, changing of the Ca/P ratio, and increases in P, Mn, K, Cl, Fe, S after coffee immersion. A recent study performed by Gomes, et al. [32], used spectroscopy techniques similar to the current manuscript, and the authors reported the erosive effect of some medications on the enamel utilizing micro-energy-dispersive XRF. The authors did not find statistically different losses of calcium and phosphorus; however, they described a Ca/P ratio decrease for enamel after erosive treatments. Similarly, we observed a decreased Ca/P ratio after coffee treatment in young-coffee and old-coffee group. In contrast, some elements showed increases such as P, Mn, K, Cl, Fe, S, after coffee immersion in young-coffee and old-coffee groups. Although the difference was not statistically significant, it could suggest these alterations correspond to the presence of coffee, since the assessment of coffee by ICP-MS indicated the presence of K, Fe, Mn, and Sr in high concentrations. Here it is important to point out; the differences found with XRF among elements should be considered to have taken place on the dental structure deep to the surface. Therefore, the significant decrease of calcium in the young-coffee group as measured by XRF indicates the erosive effect of coffee alters dentin, with a slight difference due to age noted. Furthermore, a decrease of calcium in old-coffee group was statistically significant, further supporting coffee eliciting these deleterious effects. In summary, the levels of calcium reported in LIBS and XRF after coffee immersion suggest an erosive effect not only in enamel but also in dentin. Although some studies have considered that coffee is an extrinsic stain [46,47], here we found that the staining compromises the intimate layer of dentin with an intrinsic pigmentation.

4.3. Ultrastructural correlations between erosion and demineralization

An important factor inducing erosion is related to the low pH of coffee. The pH of the coffee used in this study was 6.3, considered acidic. Generally, coffee contains a pH around 5.8 [48]. As a result, decalcification is correlated with erosion primarily caused by acidity. Dental erosion is induced by decalcification, meaning the degree of mineralization [17]. Nevertheless, in the oral cavity the saliva is a key element protecting dental tissues or dissolving the enamel to induce erosion, according to the Ca/P concentrations [30,47]. In the present study we did not include salivary conditions since our goal was to determine through analytical spectroscopic methods the direct effect of coffee on teeth. However, saliva plays a buffer role to regulate acidity [33] and it should be considered for further studies. After loss of mineral elements, we found that the enamel surface had cracks more pronounced for coffee groups. The spaces we found in coffee groups were similar to those found by Miranda et al. [49], particularly in the young-coffee group. These authors investigated erosion induced by bleaching procedures and described the appearance of craters and pores as a consequence of erosion. They reported irregularities in the enamel with a marked increase of depth and depressions. In the current SEM analysis, the demineralization effect of coffee was marked in our old group, where the appearance of separated lamella was clearly observed with wide spaces in the transversal view of the tooth, indicating a weakness in the enamel (Fig. 5h). A similar pattern was found in the conclusions made by Dinçer et al. [17]. These authors stated that demineralization occurs frequently when some etched regions of the enamel are exposed to acid drinks, such as coffee. Being an acidic drink, coffee can dissolve even some resin for obturator material in dental operative or for orthodontic treatments. Therefore, the effect of coffee in the most outer layer of teeth cannot be ignored, the ultrastructure evidence found by SEM demonstrated clear change due to coffee immersion. Overall, coffee most likely facilitated penetration and absorption in the primary dentin layer, thus eliciting its erosive effect.

4.4. Macroscopic relationship between erosion and demineralization

The cellular component of enamel is ameloblasts, which excrete organic proteins for the matrix in which calcium and phosphate are deposited and crystallized into hydroxyapatite [9,50,51]. For histology, identified by H&E and Masson’s trichrome, the layer of enamel in coffee groups was slightly thinner than controls. Despite our in vitro model lacking fixatives leading to denucleation of tissue, we performed histology to determine if gross macroscopic differences were apparent due to coffee staining. Consequently, the loss of mineral content exposed the organic matrix components, which in the present study was corroborated by a subtle change in Masson’s trichrome staining. Masson’s staining has been used mainly for identifying mineralized tissues and
tissues rich in collagen proteins [51,52]. The H&E and Masson’s trichrome demonstrated a demineralized area in enamel and EDJ loss of continuity in the enamel of young and old coffee groups, respectively. In the young-coffee group, decalcification of enamel has been reported, where Masson’s trichrome stain the organic matrix corresponding to a blue staining [52]. In this regard, it is interesting to note that the old-coffee group demonstrated a dark-pink staining where we identified loss of continuity in the enamel prisms and open spaces indicating generalized decalcification. In summary, SEM and histology analysis demonstrated decalcification in coffee groups which indicates coffee weakens the first layer of teeth, and coffee metals undermine the EDJ and therefore staining and erosion follow.

4.5. Susceptibility of teeth to damage influenced by age

In this study, two different age groups were included as we anticipated older teeth would be more fragile to the effects of coffee. In the aged human population, attrition and dental erosion occur more frequently than in young people. Primarily, wear with increasing age is observed for cervical and occlusal/incisal tooth surfaces [53]. Dugmore and Rock [5] investigated erosion factors in young ages, and their studies did not show significant differences between 12 and 14 years old patients. Nevertheless, coffee consumption is preferably consumed by older adults, whom drink 1–7 cups per day [53–56]. As has been reported previously [9,57–59] with age, enamel becomes progressively worn in regions of masticatory attrition and where sensitivity due to dentin exposure occurs. Our rats of six months demonstrated progressive occlusal tooth wear mimicking the aged human condition, as has been previously reported [57]. Therefore rodent animals are a suitable model to study gerodontontology research. Here, our results demonstrate that old age groups are more susceptible to erosion than young age groups.

4.6. Agents offering protection are necessary to prevent the erosive effect of coffee

Erosion is considered irreversible. The mineral content in erosion is mainly lost in the surface, rather than in the underlying layers [60,61]. Studies supporting the consumption of cheese and/or milk assisting in preventing erosion due to calcium and phosphate salts have been known for some time [62]. Although the morphology of the dental wear caused by erosion is never regenerated completely, improvements in diet after acid drinks/food, such as brushing, are necessary for the protection of teeth [61,62]. Furthermore, using agents containing fluorides (either by means of tablets or mouth-rinses) enhance the rapid recovery of elements crucial in enamel and dentin i.e. incorporation of ions in hydroxyapatite crystals [60,63]. In fact, stannous ions are more stable to the dental surface creating a barrier against erosive acids, such as coffee. As a result, the usage of dentifrices containing tin provides better protection against erosion compared with sodium fluorides [61,64,65]. Using additives after the consumption of coffee may provide protection and reduce the effect caused by coffee.

5. Study limitations and future directions

In vivo studies of human dental tissues are ethically difficult; as a result, rodent models are required and provide substitutes to study cavities, fluorosis, etc. Although some natural conditions such as the role of salivary buffering are lost in this in vitro design, the characteristics of mammalian teeth (i.e. in rodents) are consistent with human teeth and their results are applicable to human dental structures. A clear drawback in the present research was the in vitro model as we were ethically unable to feed coffee to rodents due to the ancillary effects of caffeine, e.g. hyperactivity. Investigating in vivo coffee effects in short periods in rodents could provide information concerning the effects of coffee erosion. Furthermore, individuals who consume coffee often may be causing significant damage to their teeth; therefore, implementing new strategies combining nanotechnology, tissue engineering and regenerative medicine [50] might be useful to create new agents to battle the erosive effect of coffee on teeth after its consumption.

6. Conclusions

Our results demonstrate coffee induces a loss in the main elements of hydroxyapatite which are calcium and phosphates. Coffee elicits weakening of the enamel and these decalcified tissues easily expose the dentin to damage. The change in color of teeth is due to the high concentrations of metals that coffee contains. The erosive effect of coffee also has differences depending on the fragility of teeth due to aging. Here we report the elemental changes due to coffee staining and demineralization on teeth.

Acknowledgments

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Conflict of interest

The authors declare no competing financial interests.

Author contributions


Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.talanta.2018.07.032.

References


Author contributions


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