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Fe leaking from orthodontic appliances affects buccal enamel more than lingual during in vitro experiment

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The presence of trace metals in the oral cavity, whether natural or treatment-induced, may influence enamel properties and health. Evidence suggests that metals released from orthodontic appliances affect enamel integrity, though the underlying mechanisms remain unclear. This study examined whether Fe ions from orthodontic devices preferentially incorporate into either the buccal or lingual enamel. For this, six human molars underwent a 12-month in vitro simulation involving metal appliances and cyclic pH fluctuations. Bacterial and enzymatic effects were not assessed. Lingual–buccal enamel cross-sections were analysed using LA-ICP-MS pre- and post-experiment. Fe concentration increased over time, varying among teeth. Fe accumulated significantly more (Student's t-test, $p = 0.000000$) and penetrated deeper (U–Mann–Whitney, $p = 0.000000$) on the buccal side. Unlike the lingual side, the buccal Fe increase correlated with a Ca decrease (Pearson, $r > -0.79$), while final Fe levels correlated with initial Zn on both sides ($r > 0.68$). Findings suggest greater Fe susceptibility in buccal enamel with an assimilation mechanism different from lingual enamel. Further research should examine its and Zn's roles in post-treatment alterations, with proposed clinical trial outlines. The in vitro method used may help optimise buccal and lingual systems for enamel preservation and post-appliance care.

Minerals that form human enamel incorporate a certain amount of trace elements into their structure[1,2]. The type, concentration, and depth of assimilation of these elements affect specific features of the enamel, determining its chemical resistance and functionality in the oral cavity[3–6]. The incorporation of trace elements occurs during the daily demineralization and remineralization cycles of enamel; thus, it is a lifelong process[7]. During orthodontic treatment, the enamel might be particularly prone to chemical changes due to metal-containing parts present in the oral cavity for an extended period[8,9].

Some parts of orthodontic appliances are made of alloys consisting of metals, such as: Fe, Ti, Ni, and Cr[10]. Ions from this devices can leak in small amounts and may be incorporated multi-elementally into the enamel as trace components[9,11]. Among these metals, the Fe ions have the greatest potential impact on the enamel in terms of their assimilation concentrations and their influence on the growth of Sulphate Reducing Bacteria (SRB) in the oral cavity[9,12]. Furthermore, iron is a key and cost-effective component of various orthodontic devices, including metal bands used across different appliance types and retention systems, which stabilise patients' teeth—typically on the lingual surface—for years following treatment completion[13,14].

There are several types of orthodontic systems including conventional metal or aesthetic brackets placed buccally and customised lingual or 3D-printed systems[15,16]. The relationship between the material of orthodontic devices, their placement on the tooth surface, post-treatment enamel condition, and the effectiveness of different techniques has been the focus of numerous studies[17–21]. Despite the fact that the hygiene of the lingual enamel is more demanding for orthodontic patients, some researchers recommend lingual systems as the primary option during treatment[17,22]. It has been reported that the buccal site is more prone to post-appliance disease changes,

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such as white spots lesions[17]. On the other hand, lingual systems are more expensive due to the advanced manufacturing technologies required[21].

Despite extensive research comparing lingual and buccal systems, no method reliably enables the non-invasive and definitive prediction of enamel changes following orthodontic treatment. This results from the incomplete understanding of the mechanisms underlying the varying enamel response to these systems. While analytical methods for assessing microbiome composition, saliva components involved in demineralisation, and metal ion leakage from orthodontic appliances are well established[23], reliable in situ techniques for direct enamel examination remain lacking. Enamel stability is determined by its atomic composition and crystallographic structure, yet, in situ dental analytical techniques, including X-ray diffraction (XRD) and laser-based methods, lack the resolution required for real-time, trace-level analysis of enamel composition and structure[24,25]. As a result, fundamental questions remain unanswered. For instance, it remains unclear whether the preferential formation of white spot lesions on the buccal surface is associated with local elemental substitutions in enamel or whether structural or elemental markers could predict specific enamel responses to different orthodontic systems, providing patients with clear guidance on the associated risks.

The limitations of in situ analyses prevent clinical studies thus for now, in vitro research remains the only viable approach for systematically investigating these mechanisms. Despite its constraints, it provides a controlled environment to test hypotheses and gradually uncover the processes underlying clinically observed enamel alterations[9,26]. Therefore, the aim of this study was to determine whether the incorporation of Fe ions, representing metals released from orthodontic appliances, is preferentially enhanced on a specific tooth surface in an in vitro experiment. The null hypothesis (H_0) tested herein assumed that the Fe ions do not accumulate more in lingual nor buccal enamel in terms of their maximum concentrations and assimilation depth. This null hypothesis was tested against the alternative hypothesis (H_1) of a difference between enamel sides. Our aim was to elucidate the accumulation process of metal ions leaking out the appliances from a materials science perspective—specifically, the local propensity of enamel to incorporate ions leaking out the metal appliances. This remains a largely unexplored factor within the complex network of interactions occurring in the oral environment during orthodontic treatment.

Results

In this in vitro study, six healthy human molars were subjected to cyclic pH fluctuations using metal appliances over a period of 12 months. The concentration of Fe, Ca, Ni, Ti, Cr, Cu and Zn in the enamel was thoroughly analysed before and after the experiment using the LA–ICP–MS technique. Comparative Fe distribution maps and statistical analysis of its assimilation depth and maximum concentrations within the lingual and buccal parts of the enamel were prepared. The main manuscript presents the Fe and Ca concentration results for Tooth #1, along with summary tables and correlation analyses. The results for Teeth #2–6 and the Control Tooth, structured similarly to those for Tooth #1, are provided in the Supplementary Materials to provide data for statistical interpretation. Data for all other metal analyses are available in the Supplementary Excel files titled *Analysis Before Experiments* and *Analysis After Experiments*.

Figure 1 and Supplementary Figs. S1–S5 present Fe distribution in enamel before and after the experiment. The metal concentrations in enamel before the experiments were negligible in all analysed sites, usually not exceeding 630 µg/g locally. Following exposure to orthodontic appliances, all samples exhibited increased Fe levels. The penetration depth and post-experimental Fe concentrations varied between individual teeth, with metal incorporation occurring heterogeneously within the enamel surface layer. Different tooth regions (lingual, buccal, lateral, and crown) displayed distinct Fe assimilation depths and maximum concentrations. However, no such increase was observed in the control sample, where Fe distribution after the experiment remained comparable to the initial state (Supplementary Figs. S6 and S7).

Table 1 presents the maximum enamel depths at which Fe was detected in the lingual and buccal enamel using LA–ICP–MS after the experiments, along with the corresponding statistical analysis. Data from the coronal regions were not subjected to statistical interpretation, as variations in the chemical composition of the crowns were beyond the scope of this study.

The deepest Fe penetration (539 µm from the tooth surface) was observed in the lateral region of the buccal side of Tooth #3, whereas the shallowest Fe incorporation was recorded in the lingual enamel of Tooth #1 (15.7 µm from the tooth surface). When considering data from all profiles, Fe assimilation depth in the lateral enamel regions was significantly greater on the buccal side than on the lingual side (U–Mann–Whitney, $p < 0.000000$).

Table 2 shows the maximum Fe concentrations recorded in the lingual and buccal enamel sides of the studied samples along with the statistical analysis of the results. The maximum Fe concentrations on the lingual side of the enamel ranged from 490 µg/g to 7171 µg/g, and on the buccal side from 2612 µg/g to 27,364 µg/g, depending on the tooth. Statistical analysis showed that the differences between the lingual and buccal parts of the enamel were significant (Student's t-test, $p < 0.000000$, $df = 52$). Generally, the Fe concentration decreased with enamel depth, with maximum values occurring within the outermost ~ 50 µm of the enamel surface for most samples.

Figures 2 and 3, along with Supplementary Figs. S8–S17, present a detailed distribution of Ca and Fe concentrations in individual lingual and buccal analytical profiles, both before and after the experiments. These data allow for precise localisation of enamel depths at which maximum metal assimilation occurred, as well as a comparative analysis of Ca and Fe distribution. The highest initial Fe concentrations, reaching 0.16 wt%, were observed in Teeth #3 and #4. In Tooth #4, Fe accumulated at the surface, while in Tooth #3, it was more dispersed. Before the experiments, Fe exceeded detection limits more frequently on the buccal side and tended to concentrate at the enamel surface, though occasional deeper peaks were noted. Similarly, in the control sample, small Fe amounts were detected at varying enamel depths depending on the test site.

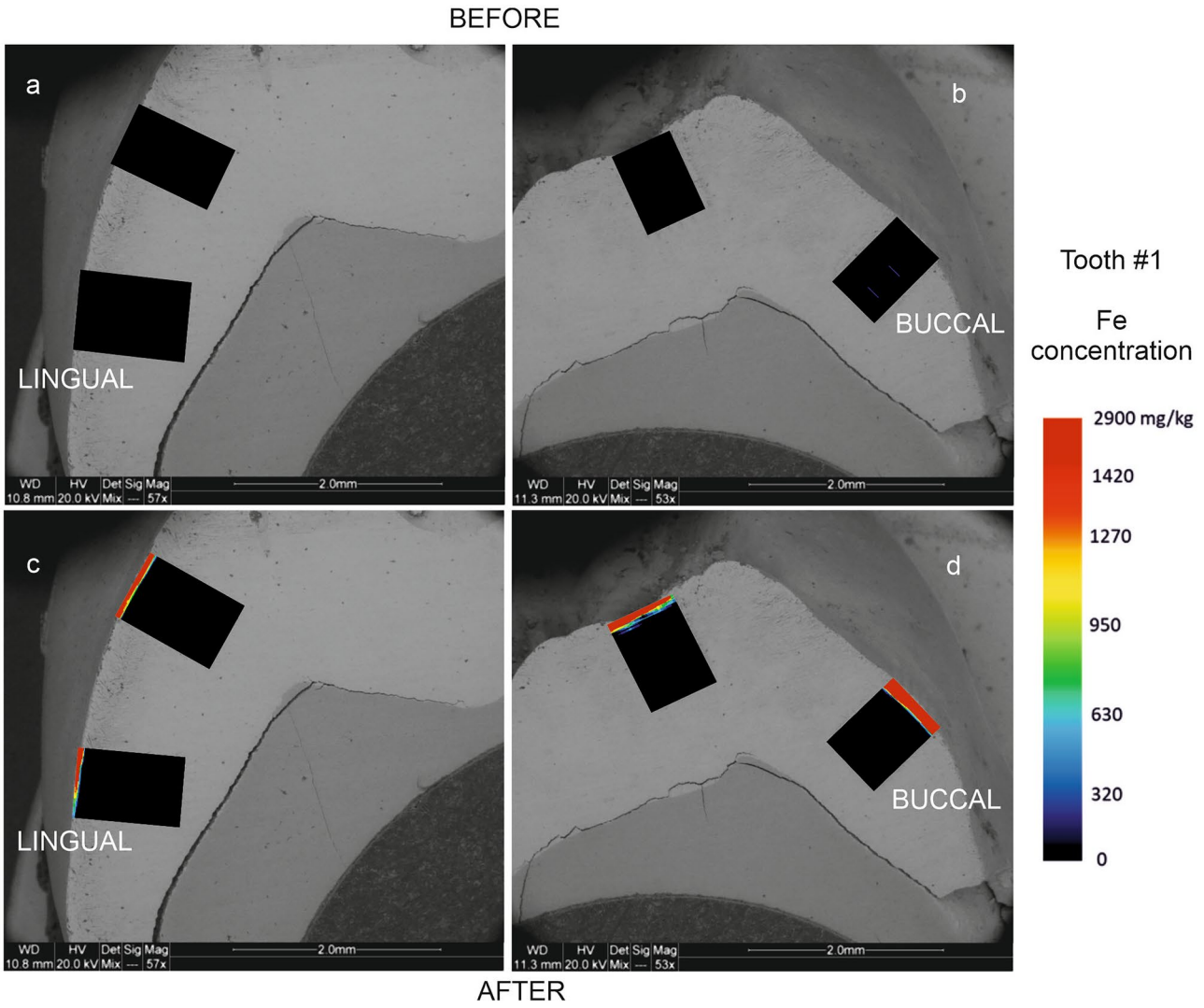

Fig. 1. Distribution maps (LA–ICP–MS) of Fe concentration in the enamel of Tooth #1 before and after experiments using metal orthodontic appliances. Maps are presented on the SEM images of the tooth after the experiment. (**a**) Lingual enamel before the experiment; (**b**) Buccal enamel before the experiment; (**c**) Lingual enamel after the experiment (**d**) Buccal enamel before the experiment.

Ca analyses are presented as concentration values and absolute counts per second (CPS) (Figs. 2 and 3, along with Supplementary Figs. S8–S17.) Pre-experimental fluctuations were typical for healthy enamel. After the experiments, Ca concentration decreased towards the surface for some analytical sites, though CPS reductions were inconsistent across profiles. The buccal side showed more pronounced declines, with 21 of 27 profiles linking lower Ca concentrations to CPS reduction. On the lingual side, this occurred only in three profiles of Tooth #4. In 24 lingual profiles, CPS increased towards the surface despite up to a 1 wt% overall concentration drop in some cases.

Tables 3 and 4 show correlations between post-experimental Fe concentrations and other metals before and after the experiments, focusing on the outer enamel layer (~600 µm). A consistently strong correlation was observed between post-experimental Fe and Zn across all profiles, regardless of measurement site or time. Notably, Fe and Ca showed a strong negative (Pearson, $r > 0.80$) correlation on the buccal side for 20 profiles, unlike the lingual side. In some post-experimental profiles, Fe also correlated with Ni, Cr, Cu or Ti—common components of orthodontic appliances.

Discussion

The experiments conducted for this study confirmed our previous observations that the presence of a fixed metal appliance can chemically alter the enamel surface[9]. After the experiments in the presence of metal parts of orthodontic appliances, the Fe content in the enamel samples increased considerably and this metal accumulated mainly in the superficial layers of the enamel (Fig. 1 and Supplementary Figs. S1–S5). Fe exceeded the detection limits of LA—ICP—MS in all tested places. The control sample that underwent 360 pH cycles without the

Tooth sample	Analysed enamel site	Enamel depth from the surface (µm)		Statistics	
		Mean*	SD	Test	Significance
Tooth #1	lingual	36.1	14.3	Student's t-test	$p = 0.00004$, df = 8, t = —11.0967, n = 5
	buccal	133.8	13.6		
Tooth #2	lingual	38.6	6.3	U-Mann–Whitney	$p = 0.029402$, Z = −2.17807, n = 4
	buccal	156.1	25.7		
Tooth #3	lingual	81.4	22.2	U-Mann–Whitney	$p = 0.011926$, Z = −2.50672, n = 5
	buccal	510.1	20.6		
Tooth #4	lingual	93.4	13.7	U-Mann–Whitney	$p = 0.834532$, Z = −0.208893, n = 5
	buccal	95.5	7.1		
Tooth #5	lingual	23.9	9.78	Student's t-test	$p = 0.000007$, df = 8, t = —10.2791, n = 5
	buccal	96.5	12.4		
Tooth #6	lingual	63.8	8.8	U-Mann–Whitney	$p = 0.046302$, Z = −2.17807, n = 3
	buccal	151.2	4.3		
		Median*	IQR		
All profiles	lingual	53.7	48.4	U-Mann–Whitney	$p = 0.000000$, Z = −5.61513, n = 27
	buccal	132.2	66.7		

Table 1. Depth of Fe penetration into the enamel after the in vitro experiment with metal appliances. *SD*: Standard Deviation of the data at a particulate site, *IQR*: Interquartile Range. *Mean or Median values calculated for all analytical profiles at a particular site of the tooth. Number of profiles at each site is equal to *n*. $p < 0.05$ are statistically significant (bolded).

Tooth sample	Analysed enamel site	Enamel depth from the surface (µm)		Statistics	
		Mean*	SD	Test	Significance
Tooth #1	lingual	2879	2753	U-Mann–Whitney	$p = 0.012186$, Z = −2.50672, n = 4
	buccal	13,682	965		
Tooth #2	lingual	1815	177	U-Mann–Whitney	$p = 0.029402$, Z = −2.17807, n = 4
	buccal	8915	1196		
Tooth #3	lingual	5690	1414	U-Mann–Whitney	$p = 0.012186$, Z = −2.50672, n = 5
	buccal	10,864	2474		
Tooth #4	lingual	7960	2619	U-Mann–Whitney	$p = 0.012186$, Z = −2.50672, n = 5
	buccal	3412	977		
Tooth #5	lingual	1119	212	U-Mann–Whitney	$p = 0.012186$, Z = −2.50672, n = 5
	buccal	10,195	1448		
Tooth #6	lingual	2849	682	U-Mann–Whitney	$p = 0.080857$, Z = −1.74574, n = 3
	buccal	15,597	10,293		
All profiles	lingual	2527	2127	Student's t-test	$p = 0.000000$, df = 52, t = —7.29024, n = 27
	buccal	10,119	4975		

Table 2. Maximum Fe concentration in the enamel after the in vitro experiment with metal appliances. *SD*: Standard Deviation of the data at a particulate site, *IQR*: Interquartile Range. *Mean values calculated for all analytical profiles at a particular site of the tooth. Number of profiles at each site is equal to *n*. $p < 0.05$ are statistically significant (bolded).

presence of orthodontic appliances did not show changes in relation to the initial state at a similar level as the experimental samples (Supplementary Figs. S6 and S7).

While the process of metal leakage from orthodontic devices is well recognized[11,27], knowledge about the impact of this phenomenon on the physicochemical parameters of enamel during and after orthodontic treatment is limited. In our previous work, we detailed the possible mechanisms of metal assimilation (Fe, Ni, Cr, Ti, Cu) from orthodontic appliances by enamel and its causes[9]. We also discussed the potential positive and negative effects of this phenomenon on dental and oral health. Therefore, the following discussion focuses on the differences between the lingual and buccal sides of the tooth concerning potential changes in Fe concentrations in enamel that may occur following treatment with metal devices.

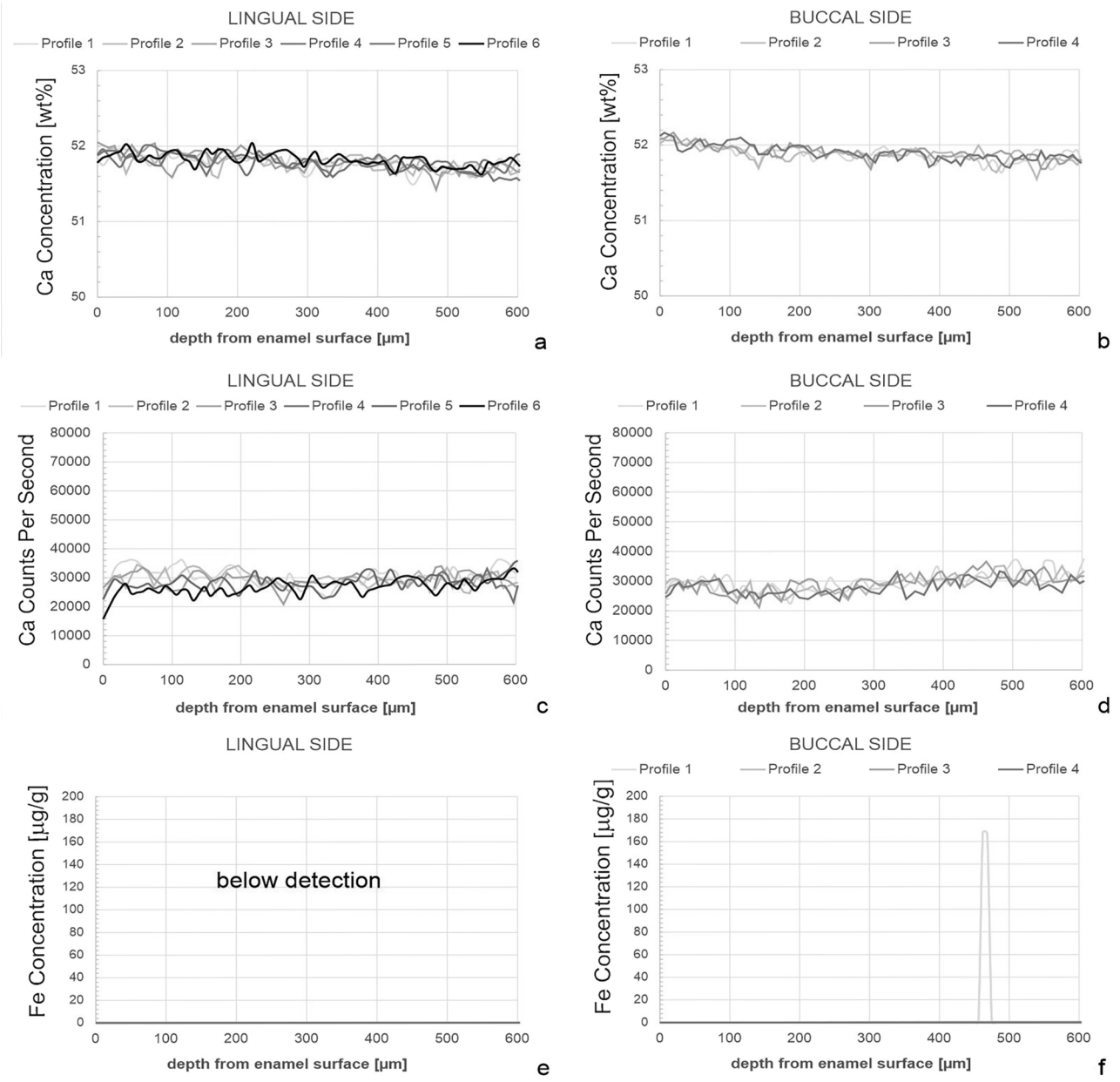

Fig. 2. Ca and Fe concentrations (LA-ICP-MS) in enamel of Tooth #1 before the experiment using metal orthodontic appliances vs. precise enamel depth. (**a**) Lingual enamel - Ca concentration; (**b**) Buccal enamel - Ca concentration; (**c**) Lingual enamel - Ca in Counts Per Second (**d**) Buccal enamel - Ca in Counts Per Second; (**e**) Lingual enamel - Fe concentration; (**f**) Buccal enamel - Fe concentration.

The conducted research and statistical analysis allowed for the rejection of the null hypothesis of no differences between Fe concentration in the buccal and lingual enamel after an experiment with orthodontic wires. The buccal side was much more susceptible to metal assimilation than the lingual side. Fe penetrated deeper and in larger quantities on the buccal side (Tables 1 and 2). The lack of statistically significant difference in Fe maximum concentration for Tooth #6 is due to the small number of profiles performed for this sample. Differences between lingual and buccal sites in both depth and Fe concentrations are visible in the graphs (Supplementary Fig. S17).

During our experiments, teeth were randomly arranged in reactors, and brackets were not attached, eliminating methodological bias in Fe incorporation. All samples were molars but from different donors, excluding patient-specific influences. The in vitro design ensured the absence of bacteria, enzymes, and mechanical stress, ruling out these factors as contributors to the observed changes. Thus, the buccal side of molars appears to have inherent material properties that increase Fe affinity from orthodontic devices. These may be primary (genetically determined structure and function) or secondary (post-eruptive enamel recrystallisation influenced by trace elements).

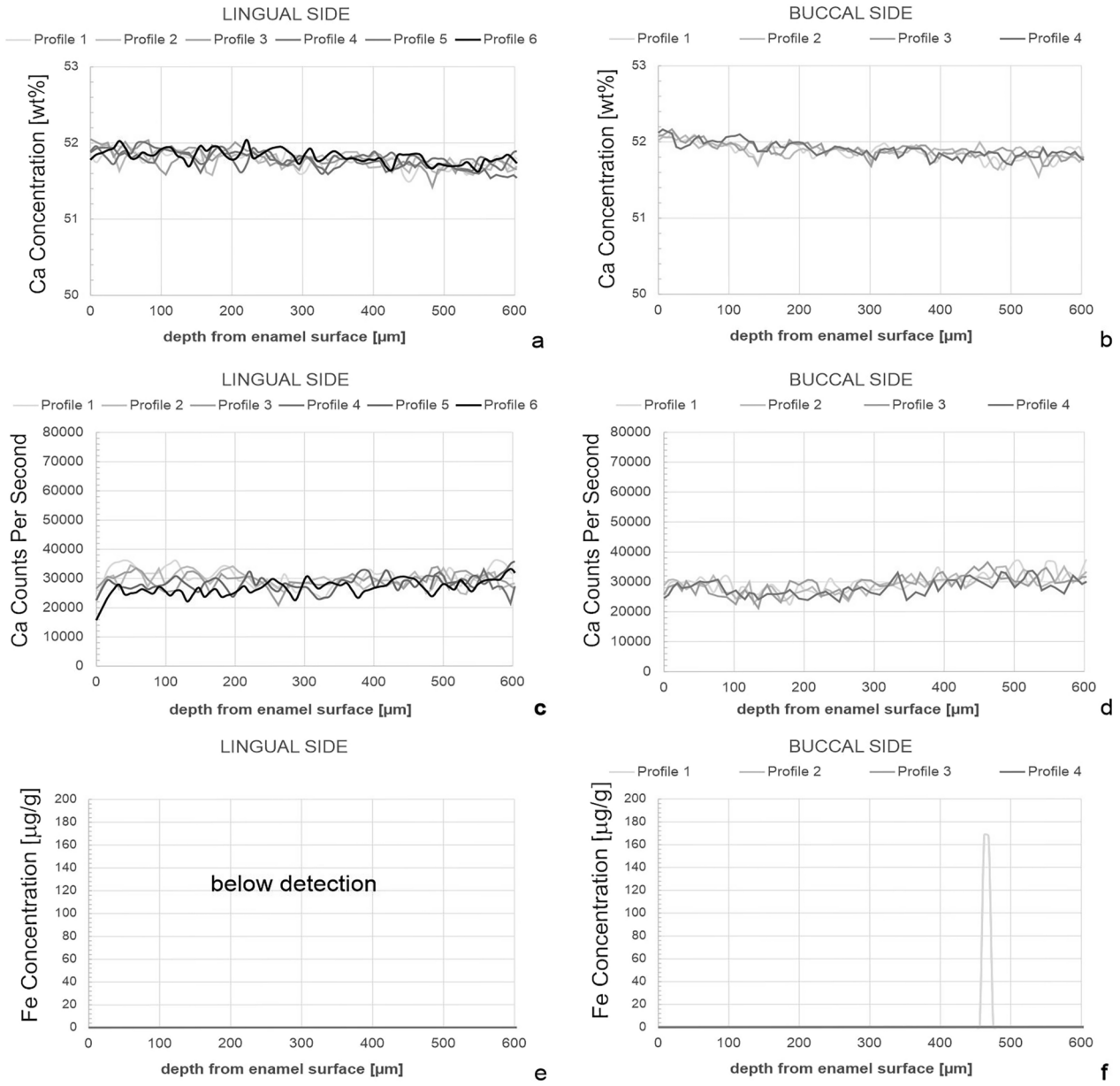

Fig. 3. Ca and Fe concentrations (LA-ICP-MS) in enamel of Tooth #1 after the experiment using metal orthodontic appliances vs. precise enamel depth. (**a**) Lingual enamel - Ca concentration; (**b**) Buccal enamel - Ca concentration; (**c**) Lingual enamel - Ca in Counts Per Second (**d**) Buccal enamel - Ca in Counts Per Second; (**e**) Lingual enamel - Fe concentration; (**f**) Buccal enamel - Fe concentration.

Differences in the enamel structure, including the chemical composition of its constituent minerals, their spatial orientation, density, and degree of crystallinity between individual sides of the tooth, have been studied for years[28]. Understanding these differences has improved with advances in the resolution of analytical methods, but many parameters have yet to be mapped and framed statistically. For example, it has been reported that the content of enamel components such as F, Na_2O, Mg_2O or CO_3^{2-}, and hydroxyapatite (HAP) density do not differ between lingual and buccal sites[5,28–30]. However, concentrations of CaO and P_2O_5, and Knoop hardness (KHN) are higher on the buccal side[5,30].

The elemental composition and mechanical stress affect the a and c parameters of the HAP unit cell, which generally decrease with depth from the enamel surface but exhibit larger values in the buccal side[4]. It is worth mentioning, that the enamel surface on the buccal side releases more calcium during abiotic demineralization than the lingual side of the same area[31], suggesting deeper and more extensive dissolution on this side. This was also observed in our studies (Fig. 3 and Supplementary Figs. S8—S17). Hence, it can be preliminary concluded that the increased affinity of the buccal enamel for Fe leaking from the metal appliances may be attributable to the strained parameters of the hydroxyapatite (HAP) unit cell which facilitate dissolution and ionic substitutions during remineralization[32–34]. Furthermore, in our study, after the experiment, the Fe increase on the buccal side

Tooth sample	Enamel site	Element													
		Ca		Ni		Ti		Cr		Fe		Cu		Zn	
		*Mean***	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*
Tooth #1	lingual	0.28	0.11	-	-	-	-	-	-	-	-	0.51	0.34	**0.56**	0.09
	buccal	0.64	0.07	-	-	-	-	-	-	-	-	-	-	**0.70**	0.05
Tooth #2	lingual	0.33	0.04	-	-	-	-	-	-	-	-	-	-	**0.60**	0.06
	buccal	0.62	0.07	-	-	-	-	-	-	-	-	-	-	**0.91**	0.02
Tooth #3	lingual	0.26	0.17	-	-	-	-	-	-	-	-	-	-	**0.65**	0.15
	buccal	0.66	0.07	-	-	-	-	-	-	-	-	-	-	**0.81**	0.04
Tooth #4	lingual	0.39	0.09	-	-	-	-	-	-	-	-	0.35	0.41	**0.56**	0.22
	buccal	0.47	0.03	-	-	0.71	0.10	-	-	0.66	0.18	0.35	0.41	**0.70**	0.07
Tooth #5	lingual	−0.10	0.24	-	-	-	-	-	-	-	-	-	-	**0.51**	0.07
	buccal	−0.15	0.28	-	-	0.56	0.12	-	-	-	-	-	-	**0.72**	0.08
Tooth #6	lingual	0.29	0.11	0.61	0.04	-	-	-	-	-	-	0.19	0.09	**0.58**	0.12
	buccal	0.42	0.04	0.11	0.07	-	-	-	-	-	-	0.67	0.04	**0.77**	0.03

Table 3. Pearson correlation *r* parameter for Fe concentration in the enamel AFTER the experiments and selected metal concentration in the enamel BEFORE the experiment*. *SD*: Standard Deviation of the correlation data at a particulate site. *Correlations are provided for the outer enamel layer (~600 µm),. **Mean values calculated for correlations among all profiles at a particular site of the tooth,. ***A gap in a table cell indicates that at the correlated element was below the detection limit for most of the profiles.

Tooth sample	Enamel site	Element											
		Ca		Ni		Ti		Cr		Cu		Zn	
		*Mean***	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*
Tooth #1	lingual	−0.32	0.35	0.48	0.33	0.90	0.04	- ***	-	0.81	0.09	**0.78**	0.06
	buccal	**−0.85**	0.04	0.86	0.07	0.91	0.07	0.95	0.02	0.98	0.01	**0.86**	0.03
Tooth #2	lingual	−0.12	0.08	−0.02	0.06	0.91	0.02	-	-	0.83	0.09	**0.71**	0.04
	buccal	**−0.94**	0.01	0.62	0.09	0.87	0.03	0.75	0.08	0.94	0.04	**0.91**	0.01
Tooth #3	lingual	−0.74	0.10	0.74	0.26	0.81	0.05	0.92	0.05	0.94	0.05	**0.75**	0.04
	buccal	**−0.96**	0.01	0.19	0.05	0.75	0.07	0.91	0.03	0.93	0.01	**0.94**	0.01
Tooth #4	lingual	0.33	0.12	0.48	0.29	0.52	0.29	-	-	0.69	0.18	0.45	0.10
	buccal	−0.26	0.37	-	-	0.92	0.05	-	-	0.85	0.14	**0.61**	0.16
Tooth #5	lingual	−0.16	0.22	0.42	0.45	0.57	0.24	-	-	0.33	0.27	**0.63**	0.10
	buccal	**-0.80**	0.11	0.91	0.03	0.71	0.17	0.85	0.12	0.95	0.04	**0.74**	0.07
Tooth #6	lingual	−0.74	0.01	0.42	0.04	0.85	0.02	-	-	0.92	0.05	**0.69**	0.02
	buccal	**−0.92**	0.04	0.48	0.02	0.94	0.01	0.83	0.09	0.93	0.03	**0.83**	0.02

Table 4. Pearson correlation *r* parameter for Fe concentration in the enamel AFTER the experiments and selected metal concentration in the enamel AFTER the experiment*. *SD*: Standard Deviation of the correlation data at a particulate site. *Correlations are provided for the outer enamel layer (~600 µm),. **Mean values calculated for correlations among all profiles at a particular site of the tooth,. ***A gap in a table cell indicates that at the correlated element was below the detection limit for most of the profiles.

correlated with a Ca decrease (Table 4) and was typically accompanied by a CPS reduction. This pattern was inconsistent on the lingual side, suggesting distinct assimilation mechanisms.

It remains unclear whether the preferential presence of Fe in buccal enamel has positive, negative or neutral effects on tooth reaction for treatment. Enamel surface chemistry influences its mechanical properties and biocompatibility[4,5,30,33]. Fe in apatite reduces the mineral thermodynamic stability[35]; yet some studies link Fe presence to lower enamel degradation[36]. However, Fe salts contribute to discoloration, increased cracking, and porosity[37]. It is also worth mentioning, that Stainless steel in the form of fixed appliances in the oral cavity may also promote sulphate-reducing bacteria (SRB) colonisation[12], potentially enhancing microbial enamel degradation via nutrient scavenging. Nevertheless, given the oral cavity's complexity, enamel metal substitutions are typically multi-elemental, with potential counteracting effects[1,9].

In the context of the above, Tooth #4 warrants special attention. While Fe concentrations differed significantly between sides of this sample, the penetration depths were similar. Initially, Tooth #4 had the highest surface Fe concentration among all samples, however this was not remained after the experiments. Unlike other teeth, it also exhibited a post-experimental decrease in calcium CPS on the lingual side. Our findings align with previous in vitro studies showing Fe can disrupt enamel demineralisation and remineralisation[38]. The initially high Fe

content in Tooth #4 likely altered its interaction with experimental solutions, resulting in a distinct response compared to other teeth.

The in vitro nature of this study and the limited number of samples preclude direct clinical conclusions. It is also possible that Fe assimilation is overestimated, as in vitro conditions may not precisely reflect metal concentrations in the oral cavity during treatment. However, our primary aim was to experimentally demonstrate differential chemical reactivity of the lingual and buccal enamel surfaces during orthodontic treatment, which we achieved.

While speculative, the observed increase in Ca loss on the buccal side, coupled with deeper and greater Fe assimilation, may have clinical implications. This could potentially contribute to increased bacterial adhesion[12] and further demineralisation on this surface. Our previous work also demonstrated that metal accumulation, particularly Fe, is highest around brackets. Variations in Fe assimilation between the lingual and buccal sides may influence enamel response during debonding, affecting crack formation, unintended debonding, or adhesive system wear. These findings may aid in optimising adhesive effectiveness and developing metal-based protective strategies for enamel[39].

An intriguing aspect of this study is the correlation between Fe assimilation and initial Zn content in enamel. Zn, present in saliva and involved in enzymatic processes, has been already linked to enamel demineralisation processes[40]. If Fe assimilation from orthodontic appliances reflects potential chemical changes in enamel during treatment, initial Zn levels in patients' enamel may serve as a marker for some post-treatment alterations, susceptibility to pathological changes, or other treatment-related challenges. Zn concentrations in tooth enamel are higher than those of Fe, allowing for more precise determination using various analytical methods. However, further clinical research is needed to validate these findings. as it remains unconfirmed whether the potential remineralisation of enamel mediated by metals released from orthodontic appliances has any clinical significance. Nevertheless, given the limitations of in situ enamel analysis[24], such studies would be challenging but feasible. They could involve patients undergoing tooth extraction before treatment, allowing pre-treatment enamel composition—particularly Zn content—to be correlated with treatment-related difficulties, and other unwanted post appliance effects.

Conclusions

As demonstrated by the in vitro abiotic experiments, Fe released from orthodontic appliances alters enamel composition under pH cycling. The buccal side of molars exhibits greater Fe assimilation than the lingual side, both in terms of maximum ion concentrations and penetration depth. The highest metal concentrations occur in the outer enamel (up to several dozen micrometres) and decrease with depth, though assimilation varies between teeth.

Correlations between Ca and Fe suggest distinct assimilation mechanisms: Fe substitution on the buccal side and enriched precipitation on the lingual side. Initial Fe content in enamel may influence further Fe uptake, while Zn appears to be a potential marker for enamel changes after appliance exposure.

The in vitro nature of the study and the limited number of analysed samples preclude direct clinical conclusions. However, the findings highlight processes that may contribute to differences in lingual and buccal enamel reactivity across treatment systems. To further investigate these mechanisms, a clinical study framework is proposed, addressing the limitations of in situ enamel analysis.

Methods

The protocol used in this study was partially published in our previous paper[9], nevertheless all necessary details to are describe herein as well. The proposed method, like any in vitro approach, has inherent limitations due to the model-based nature of the study. The modified pH cycle employed was designed to simulate acid exposure experienced by the metal components of orthodontic appliances while preventing decalcification, as demonstrated in our previous work [9]. The rationale behind sample selection, including tooth size and type, has also been detailed elsewhere [26]. In this study, enzymatic and bacterial activity within the oral cavity was deliberately excluded. While clinical studies account for microbiome variability and enzymatic activity in saliva, they overlook the direct chemical alterations in enamel caused by metal ion release from orthodontic appliances. These aspects necessitate simplified in vitro investigations.

As we state in the Introduction section, our aim was to elucidate the accumulation process of metal ions from a materials science perspective—specifically, the local propensity of enamel to incorporate ions. Therefore, in the experiments described below, no orthodontic appliances were mounted on the teeth. As demonstrated in our previous study[9], adhesive systems provide a tight seal that prevents enamel from assimilating metal ions released from orthodontic appliances, making direct observation of such changes under the brackets impossible. Furthermore, given that brackets are typically bonded at the centre of the tooth, their attachment to either the buccal or lingual surface, as per standard practice, would preclude a direct comparative analysis of these two regions which is the main objective of this study. During orthodontic treatment, however, certain areas of the tooth surface remain uncovered by brackets and are thus exposed to metal ion release. For instance, in buccal systems, the lingual surface remains unprotected, while in all appliance types, some molars retain exposed enamel on both sides. Therefore, we consider the model employed in this study to be appropriate for the defined research objectives.

Tooth samples

A total of 7 teeth extracted for orthodontic purposes were used for the experiments. All teeth were undamaged, unbleached molars, with no signs of caries or other diseases. The samples set consisted of four upper left molars (Tooth # 1, #2, #3 and #4), two lower left molars (Tooth #5 and #6) and one upper right molar (Control). All were

obtained randomly from nonsmoking men and woman aged 25–45 years. Each sample came from a different donor. To understand the variability of chemical composition among samples, they were tested for Ca, Zn, Cu, Fe, Ni, Cr, Ti before experiments as provided in section "Experiments" and section "Control Experiments".

Sampling and further experiments' procedure was approved by Bioethics Committee at the District Medical Chamber in Krakow, Poland (approval number: 154/KBL/OIL/2016). All methods were performed in accordance with the guidelines and regulations provided by the Committee. As agreed, teeth were granted by the Department of Dental Surgery at the University Dental Clinic in Krakow (Poland), after patients' informed consent. After extraction, the teeth were stored in formalin (10% p. Chempur, Piekary Śląskie Poland). They were then cleaned of soft tissues with 2% papain (Merck, Darmstadt, Germany), deionized water, and fluoride toothpaste. After the samples were thoroughly rinsed, they were dried in a laboratory oven (Heratherm; Thermo Fisher Scientific, MA, USA) for 16 h at 60 °C and stored in this form in a refrigerator (4 °C) until the experiments were conducted.

Orthodontic appliances

One set of orthodontic braces was used to conduct the experiment. It consisted of the following: 20 brackets (Batch number: 901.2032. LOT#072,717; Orthoclassic OR, USA), 4 bands (size: 37; Batch number: MSDS-054; Orthodontic Design and Production, CA, USA), 1 Ni-Ti archwire (Batch number: 61.40.580.01725. LOT#302,127; Orthoclassic OR, USA) and 1 Ni-Cr archwire (Batch number: 61.40.520.02125. LOT#071,111; Orthoclassic OR, USA). The orthodontic archwires were replaced in the reactors every 30 cycles simulating daily changes in oral pH.

Solutions and reagents

As part of the experiments, the solutions in the experimental reactors were changed daily. The solutions were prepared according to the modified recipe used in pH cycling experiments[9]. Remineralizing solution composition: 1.5 mmol $CaCl_2 \times 2\,H_2O$ p.a. (Chempur, Piekary Śląskie Poland), 0.9 mmol KH_2PO_4 p.a. (Chempur, Piekary Śląskie Poland), 130 mmol KCl p.a. (Chempur, Piekary Śląskie Poland), 20 mmol HEPES bufor p.a. (4-(2-hydroxyethyl)−1-piperazine ethane sulfonic acid) (CARL ROTH, Karlsruhe, Germany),

and KOH p.a. to adjust pH = 7.0 (Chempur, Piekary Śląskie Poland). Demineralizing solution composition: 1.5 mmol $CaCl_2 \times 2\,H_2O$ p.a. (Chempur, Piekary Śląskie Poland), 0.9 mmol KH_2PO_4 p.a. (Chempur, Piekary Śląskie Poland), acetic acid p.a. (50 mmol), and KOH p.a. to adjust pH = 4.3 (Chempur, Piekary Śląskie Poland).

Fluoride (0.0047 mmol NaF 99 + % (ACROS ORGANICS, Thermo Scientific Chemical, Delphi, India)), was added to the remineralization solution as it plays a crucial role in both enamel remineralization and metal corrosion[41–43]. The solutions were prepared using deionized water with a conductivity of 5 µS/cm produced by a R5Uv device (Hydrolab, Straszyn, Poland). In addition, a commercially available oral hygiene liquid (ELMEX; Colgate-Palmolive, Świdnica, Poland), recommended for oral hygiene during orthodontic treatment, was used in the daily pH cycles. The pH of the fluid, measured using a CPI-505 device with an EPS-1 electrode (Emeltron, Zabrze, Poland), was 4.50 ± 0.05.

The daily sequence of the solutions in the reactors was as follows: step 1. – demineralization (30 – 45 min); step 2. – hygiene: mouthwash (2 min.), tap water (10 s.), deionized water (10 s.); step 3 – remineralization (3 – 6 h); step 4 – demineralization (30—45 min.); step 5. – hygiene: mouthwash (2 min.), tap water (10 s.), deionized water (10 s.); step 6. – remineralization (16 h – 19.5 h). Demineralization and remineralization solutions' volume was 100 ml, while mouthwash solution volume was 50 ml.

Experiments

After washing and cleaning the soft tissues (description in section "Tooth samples"), the lingual–buccal cross-sections of the experimental teeth (1 – 6) were exposed using sandpaper made of SiC and 1 micron Poly-Top-DUO Diamond slurry (Microdiamant, Lengwil, Switzerland) and analysed at selected sites (lateral lingual and buccal and crown lingual and buccal) using a scanning electron microscope with an energy dispersive spectrometer (SEM-EDS) and a laser ablation inductively coupled plasma mass spectrometer (LA-ICP-MS) for: Ca, Zn, Cu, Ni, Ti, Cr, and Fe concentration. The results for Fe and Ca, the primary focus of this study, are presented in Figs. 1, 2, 3 (Tooth #1) and Supplementary Figs. S1–S17 (Teeth #2–#6). The analysis results for the remaining elements are available online in the Supplementary Excel file titled *Analysis Before Experiments*. For analytical details please see section "Control Experiments". After the analyses, the exposed cross-section was secured with stickers (Color Coding Dots 3010; Avery Dennison Zweckform, München-Grünwald, Germany). This method effectively protects the enamel surface in experimental studies using a pH cycle and has been confirmed previously[44]. The stickers were replaced every 15 solution cycles.

The teeth were then placed in the reactor, along with the appliance set and underwent 360 cycles of pH changes. After termination of the pH cycles, the teeth were rinsed thoroughly with tap water (2 min) and deionized water (2 min). The exposed tooth cross-section was polished with 1 micron Poly-Top-DUO Diamond slurry (Microdiamant, Lengwil, Switzerland) to a depth of approximately 50 µm and then cleaned again with tap and deionized water and a cotton swab soaked in concentrated nitric acid (65% ultrapure; Merck, Darmstadt, Germany).

The samples prepared in this way were reanalysed using LA-ICP-MS at the same sites of the cross-section as before the experiments and then imaged using SEM-EDS. The results for Fe and Ca, the primary focus of this study, are presented in Figs. 1, 2, 3 (Tooth #1) and Supplementary Figs. S1–S17 (Teeth #2–#6). The analysis results for the remaining elements are available online in the Supplementary Excel file titled *Analysis After Experiments*. For analytical details please see section "Analytical methods" and Supplementary Table S1.

Control experiments

The core objective of the experiment was to examine changes in the enamel's chemical composition, specifically the accumulation and spatial distribution of Fe ions released from orthodontic appliances over time. To ensure rigorous experimental control, the initial chemical composition of each tooth was analysed before the experiment and re-examined at the same locations post-experiment, accounting for the laser penetration depth within the margin of error.

Control for the potential impact of pH cycling on enamel composition was based on a previously published experimental set conducted on 54 teeth divided into 107 samples[9]. These studies demonstrated no statistically significant differences in enamel composition between the control groups subjected either to pH cycles without appliances or to no exposure at all. However, to confirm additionally that Fe local distribution observed after the experiments in present experimental set primarily resulted from the presence of the orthodontic appliance component in solution, one tooth from the control group of the previous study[9] was analysed using LA-ICP-MS profiling. The analysis was conducted on both the labial and crown lingual and buccal sites.

The selected tooth, originating from the control group (of previous study) titled *Experiment #2*, was bisected along the lingual-buccal plane[9]. One half of the tooth underwent 360 pH cycles, while the other remained unexposed, serving as a reference for pre-experimental conditions. The procedures for profile preservation, analysis, and pH cycling were identical to those applied to the experimental samples[9]. Results of the analysis are presented in Supplementary Figs. S6 and S7 and in Supplementary Excel files titled *Analysis Before Experiments* and *Analysis After Experiments*.

Analytical methods

Imaging of the dental samples and analysis of the alloy composition of the parts of the orthodontic appliances were performed using a variable pressure field emission scanning electron microscope coupled with an FEI Quanta 200 FEG energy dispersive spectrometer (Thermo Fisher Scientific, OR, USA) at 20 kV (SEM-EDS).

The analysis of the spatial distribution of Fe in the enamel was performed using LA-ICP-MS. For this purpose, an ICP-MS NexION 300 spectrometer (Perkin Elmer, MA, USA) coupled with an LSX-213 laser ablation system (CETAC, NE, USA) was used. The operating parameters of the ICP-MS measurement system and laser ablation conditions along with detection limits for Fe are listed in Supplementary Table S1. NIST SRM 1400 and NIST SRM 610 (Sigma–Aldrich, Merck, Steinheim am Albuch, Germany) were used as reference materials[45]. The method of making maps of element distribution has been described in previous publications[46–48]. Supplementary Table S1 presents detection limits for analysed elements.

Statistics

TIBICO Statistica version 13.3. and 14.0.0.15 (TIBICO, Palo Alto, CA, USA) was used for all statistical analyses in this study. The normal distribution was verified with a use of Kolmogorov–Smirnov (K–S) test with Lilliefors correction (K–S–L) and Shapiro–Wolf test (S–W). The Levene's test was used to check the homogeneity of variances. The normal distribution data with homogeneous variances were subjected to Student's t-test; a nonparametric U Manna-Whitney test with discontinuity correction was applied, otherwise. The statistical parameters including significance p values are presented in Tables 1 and 2. The correlation between metal concentrations within the first 80 laser measurement points from the enamel surface (approximately the first 600 µm in depth) was assessed using the Pearson test. Results are presented in Tables 3 and 4. Statistical analysis was conducted on the results obtained for the labial-lingual and buccal regions. Data collected from the crown regions were not included in the interpretation as they were beyond objectives of this study.

Data availability

The data underlying this article are available in the article and in its online supplementary material.

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Author contributions
All authors were responsible for manuscript preparation. Apart from this: J.M.T.: funding,: leading conceptualization, experiments and interpretation; A.J.: LA-ICP-MS analysis, validation and mapping; G.A.K.-B.: sample preparation, experiments and SEM-EDS analysis; B. W.: LA-ICP-MS analysis, validation and supervision; S.M.: orthodontic sample preparation and methodology.

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Declarations

Competing interests
The authors declare no competing interests.

Ethical approval
This study protocol was reviewed and approved by the Bioethics Committee at the District Medical Chamber in Krakow, approval number [154/KBL/OIL/2016]. The Department of Dental Surgery at the University Dental Clinic in Krakow supervised the study participants in terms of the collection of their teeth for the experiments. Teeth were extracted for orthodontic purposes after written informed consent was obtained.

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