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Validation of a method to quantify titanium, vanadium and zirconium in oral mucosa cells by inductively coupled plasma-mass spectrometry (ICP-MS)

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ABSTRACT

The release of metal ions from fixed orthodontic appliances is a source of major concern. Various studies have evaluated the discharge of metals from these appliances in biological fluids, such as saliva or blood, overlooking the cells with prolonged contact with fixed appliances. The aim of this work is to develop and optimize an analytical procedure to determine Ti, V and Zr in oral mucosa cells in patients with and without orthodontic appliances by Inductively Coupled Plasma Mass Spectrometry (ICP-MS). The analytical procedure is based on an extraction and digestion of the samples and quantification of the elements. A suitable and practical procedure for assessing the trueness and precision of the proposed method has been applied by using validation standards. The method has been suitably validated: the regression equation was calculated from standards prepared in the same matrix without oral mucosa cells and the linear range was 0.5–50.0 ng/mL for Zr and 5.0–50.0 ng/mL for Ti and V. Limits of detection were 0.9, 2.8 and 0.4 ng/mL and limits of quantification 1.8, 3.4 and 0.7 ng/mL for Ti, V and Zr, respectively. The recovery percentages (%) obtained oscillated between 101 and 108 for Ti, 98 and 111 for V, and 92 and 104 for Zr. Intermediate precision (RSD%) data obtained were also adequate. The present method showed to be robust for the three factors considered: heating time, volume of the deionized water, and volume of PlasmaPure 65% HNO_3 used to dilute the samples, which permits its validation and application to oral mucosa cells from orthodontic patients.

1. Introduction

Orthodontic appliances biocompatibility is strongly related to ionic release. Currently there is an increasing research about the lixiviation of metal ions from biomaterials in several sites of the human body. Within the oral cavity, during an orthodontic treatment the oral cells are in full contact with metal appliances. Each orthodontic treatment lasts 24–30 months and during all this time, corrosion processes are usually present. Since the oral cavity has the proper conditions, such as humidity, pH and bacterial flora, the release of metal ions is facilitated, and that can cause adverse effects [1].

Fixed orthodontic appliances usually include brackets, bands, arch wires and springs. They are made of stainless steel, nickel–titanium or nickel–cobalt alloys [1]. Andreasen and Hilleman [2] first introduced nickel–titanium (NiTi) wires in orthodontics in the early 1970s. Such an alloy was characterized by 55% nickel and 43% titanium in terms of weight percent [3]. NiTi alloys are frequently used nowadays, especially during the levelling phase at the beginning of an orthodontic therapy with fixed appliances, because of their optimum mechanical properties [4]. Goldberg and Burstone [5] also highlighted that it is possible to make an orthodontic wire with interesting elastic properties, by processing 11% molybdenum (Mo), 6% Zr, and 4% tin beta titanium (βTi) alloys containing V. The super multifunctional titanium alloy "Gum metal" has been developed. This material, belongs to a beta-type titanium alloy having a body-centered-cubic structure and is fundamentally expressed as $Ti_3(Ta+Nb+V)+(Zr, Hf)+O$ [6]. The common criterion for all these fixed orthodontic materials is their permanent presence in the oral cavity for a long time without the ability to be removed by the own patient.

Several findings have been reported about the elemental release from many different dental casting alloys with different

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compositions. However, generalization of these statements for all dental casting alloys cannot be applied because of different reasons. First, multiple phases of the treatment will often increase the elemental release from alloys [7]. Second, certain elements have an inherently higher tendency to be released from dental alloys [7,8], and third, certain environmental conditions around the alloy will affect the elemental release [9,10].

Generally speaking for all fixed dental materials, elemental release from these materials plays a great role in their biocompatibility because they can induce adverse biological effects such as cytotoxicity, mutagenicity and allergy [11]. In this sense, various studies have evaluated the discharge of metal ions from orthodontic appliances in biological fluids, and most of them have concluded that they do not reach toxic concentrations in saliva and serum [12,13]. However, it cannot be excluded that even nontoxic concentrations might be sufficient to produce biological changes in the oral mucosa [14]. Occasionally, the host response to the elemental release differs in the nature and amount of the released elements. Moreover, classically allergic responses are characterized by dose-independence, this is, low doses that would not cause inflammation through toxicity but it would cause it by activating immune cells [15]. Also, mutagenicity and carcinogenic effects are not related with the dose of the toxicant. Therefore, knowledge about the elemental release from these materials into the oral cavity in regards to quantification is of great importance [11,16].

The release of elements from dental casting alloys has been mainly measured using either atomic absorption spectroscopy (AAS), inductively coupled plasma atomic emission spectrometry (ICP-AES), or inductively coupled plasma mass spectrometry (ICP-MS). Both techniques, ICP-AES and ICP-MS, are used for *in vivo* analysis of metals released in saliva [4,17].While ICP-AES was used with artificial oral saliva [18], ICP-MS was used with artificial oral saliva [19], cell culture medium [20], pH 3.5, pH 6 phosphate buffer solution, or pH 3.5 mixture of lactic acid and sodium chloride [21]. For many elements, the power of detection of ICP-AES is not sufficient to determine elemental background concentrations. In general, lower limits of detection (LODs) are possible to obtain by ICP-MS in comparison to ICP-AES [22]. Today, by application of ICP-MS the fast and accurate routine multi-element determination in biological samples has become possible due to improved sensitivity and robustness [22].

Compared with other biological samples, such as hair, serum, blood or urine, relatively scarce work has been done on methods for the multi-element determination in human saliva since today [4,17]. Some authors have indicated an increase in the salivary concentration of nickel (Ni) and chromium (Cr) following the insertion of fixed orthodontic appliances [12,23,24]. Saliva represents an easily accessible and useful body fluid for biomonitoring human exposure to environmental contaminants, although there is no consensus in its use for this aim [25]. Different advantages of saliva over blood collection are the following: it is non-invasive, it is the technique of choice for children and patients with limited coping abilities, its cost is lower, there is no risk of infection, and samples do not require special handling or preservation [26]. The disadvantage of saliva is related to its flow, which is influenced by many factors. Saliva flow does not influence all substance concentrations to the same degree, so it can still be a useful matrix for non-flow-dependent chemicals [25]. Moreover, saliva will give information at the moment of sampling only [27].

To the extent of our knowledge, the release of metals in oral mucosa cells, with prolonged contact with fixed appliances, has been scarcely investigated [1,27–30] and no previous validation data are available, although this matrix shows the same advantages than saliva samples previously mentioned. Moreover, no studies have been previously performed regarding Zr and V levels in this matrix. Therefore, more studies are necessary to elucidate optimal conditions to determine several metals in oral mucosa cells by ICP-MS, including robustness assays, which permit their validation, as it have been carried out in the present work. Classical approaches to analytical method validation rarely consider the stage corresponding to the robustness study, which is primary in the sense of "method transfer", according to harmonization purposes [31].

Taking all these data into account the aim of this work was to develop a rapid, sensitive and robust method, based on Inductively Coupled Plasma Mass Spectrometry (ICP-MS), suitable for simultaneously monitoring trace levels of Ti, V and Zr in oral mucosal cell samples from patients with orthodontic appliances. The procedure has been validated by using validation standards, according to González et al. [32]. Additionally, the metallic elements present in the orthodontic appliances employed were determined by micro-Xray fluorescence.

2. Materials and methods

2.1. Reagents and materials

High purity deionized water ($>$ 18 MΩ cm) obtained by a Milli-Q water purification system (Millipore, Bedford, MA, USA) was used throughout. All transfer pipettes, centrifuge tubes, plastic bottles, autosampler vials and glassware material were cleaned by soaking in 20% v/v HNO_3 analytical reagent grade for 4 h, rinsing three times with Milli-Q water, according to EPA method 200.8 [33], and drying in a laminar flow hood.

Blank solution consisted of 1% v/v HNO_3, prepared by diluting 65% PlasmaPure nitric acid (SCP Science, Courtaboeuf, France) with the appropriate volume of Milli-Q water. A tuning solution containing 10 ng/mL cerium (Ce), cobalt (Co), lithium (Li), thallium (Tl) and yttrium (Y) in 1% HNO_3 was prepared from single-element 10000 μg/mL stock standards (AccuStandard, Inc., New Haven, CT, USA), and was used to optimize ICP-MS parameters. Rhodium 1 μg/mL, prepared from a 100 μg/mL stock solution (AccuStandard, Inc., New Haven, CT, USA) was used as internal standard solution throughout the whole analysis.

A standard solution containing 1 μg/mL of V was prepared in 100 mL Pyrex glass volumetric flask by dilution of 10 μg/mL multi-element standard solution for ICP-MS (AccuStandard, Inc., New Haven, CT, USA). Similarly, Ti and Zr standard solutions (1 μg/mL) were prepared by dilution of 1000 μg/mL single-element standard solutions (High-Purity Standards, Charlestone, SC, USA). The standard solution of 1 μg/mL was subsequently diluted to obtain working solutions (100 ng/mL or 50 ng/mL) in order to spike the digestion extracts and prepare the validation standards.

2.2. Instrumentation

All ICP-MS measurements of metal contents were carried out in an Agilent 7500c ICP-MS (Agilent Technologies, Tokio, Japan), provided with and Octupole Reaction System and an Integrated Autosampler (Agilent Technologies, Tokio, Japan). Sample introduction was performed with a Babington PEEK (poly-eter-eter-ketone) nebulizer combined with a double-pass spray chamber (Agilent Technologies, Tokio, Japan). The spray chamber was water-cooled at 2 °C to ensure temperature stability and to reduce water vapor present in the nebulizer gas flow. The ICP torch consists of a three-cylinder assembly, with injector diameter 2.5 mm. Shield torch was used throughout the whole analysis. All instrument parameters were optimized daily while aspirating the tuning solution. Typical ICP-MS operating parameters are summarized in Table 1.

These parameters were optimized to obtain the highest signal-to-background ratio for ^{7}Li, ^{59}Co, ^{89}Y, ^{140}Ce and ^{205}Tl, as well as

minimizing the oxides ($^{140}Ce^{16}O^+/^{140}Ce^+$), hydrides ($^{140}CeH^+/^{140}Ce^+$) and doubly-charged ($^{140}Ce^{++}/^{140}Ce^+$) signals.

Micro-X-ray fluorescence (μXRF) measurements were performed in an EAGLE III [energy-dispersive analysis by X-rays (EDAX)] energydispersive micro-X-ray fluorescence spectrometer equipped with a Rh X-ray tube, 300-μm monocapillary optics, a charge-coupled device (CCD) camera, and an 80-mm^2 Si (Li) detector. Surface scans of 0.5 cm^2 were performed under a vacuum with a data acquisition time of 150 s. The quantification limit was 0.1%, and the elements that could be measured were those between Na and Pu. The apparatus was previously calibrated according to the manufacturer's specification using an aluminum–copper standard sample. Automated analyses were performed by using the fundamental parameter quantification routine.

2.3. Sample collection and sample preparation

Forty subjects were included in this study. Twenty patients required fixed orthodontic treatment (orthodontic group or test group), and 20 subjects served as the control group who were not undergoing orthodontic treatment. Both groups were similar regarding the sex of the components: 10 men and 10 women in the control group, and 13 women, 7 men in the orthodontic group. The ages range was 17–46 years in the control group and 12–53 in the orthodontic group. The time for orthodontic treatment of patients was 13–15 months. The orthodontic patients were all treated with fixed orthodontic appliances in both arches. The appliances consisted of 8 bands on the first and second molars, 20 brackets and 12 patients used 0.016 × 0.022 nickel–titanium archwires in upper and lower arches, whereas 8 patients used 0.016 × 0.022 stainless steel archwires in both arches. The archwires were fixed with 0.010 stainless steel ligatures and all the patients had long 0.012 stainless steel ligatures. The aims and the method of cell collection were explained to all subjects, and written consent to participate was obtained. Treatment was started after the institutional ethical committee of the University of Seville approved the protocol. The fixed of appliances consisted of an average of 4–8 bands or tubes and 20 bonded brackets. The material used was stainless steel alloy SAF2205, AISI316L and AISI303 for the brackets, tubes and bands (DM Ceosa; Madrid, Spain). The ligatures were made of stainless steel alloy AISI304. The archwires used in this study were nickel–titanium alloy (DM Ceosa, Madrid, Spain) or stainless steel (DM Ceosa, Madrid, Spain). The materials used in this study were analyzed by μXRF, and their compositions are shown in Table 2.

The inclusion criteria for subject selection included non smokers; no oral diseases, no systemic diseases, no oral restorations or prosthetic; clinically healthy oral mucosa; no previous orthodontic treatment; no occupational exposure to metals, and not receiving any medication or supplements. Subjects were initially screened with a questionnaire to check whether they fit the criteria of the study. Afterwards they were clinically assessed for normal oral mucosa [27].

The participants were asked to rinse their mouth with tepid water for 1 min to remove exfoliated dead cells. Epithelial cells of buccal mucosa from each patient were collected, using a mini-toothbrush (Difresh, Madrid, Spain), according to the method of Besaratinia et al. [34], by gentle brushing of the internal part of the oral mucosa in contact with the orthodontic appliances.

Once the samples were collected, they were digested and measured following the method of Natarajan et al. [29] with some modifications regarding the water and nitric acid volumes employed, the heating time (60 min instead of 30 min), and the use of an internal standard. Briefly, toothbrush was introduced into a previously cleaned (4 h in 20% v/v HNO_3) 50 mL centrifuge tube, together with 10 mL of deionized water and 100 μL of PlasmaPure 65% HNO_3. Then, samples were heated in a water bath at 80 °C for 60 min. Afterwards, samples were cooled lightly and sonicated in an ultrasonic bath for 5 min. Finally, samples were cooled down to room temperature, and the acid solution was separated from the brush. Acid solutions were stored in clean 20-mL polypropylene vials at 4 °C until analysis. 5 mL of the sample volume was required for the analysis. The amounts of Ti, V and Zr in the cells were quantitatively assessed by ICP-MS. The addition of internal standard (^{103}Rh), was performed on line.

Extraction efficiencies were performed in triplicate by spiking the matrix, clean toothbrush without oral mucosal cells submitted to the same extraction procedure, with the multi-element standard solution at three concentration levels: 1, 10 and 50 ng/mL for Zr, and 5, 10 and 50 ng/mL for Ti and V. Besides, a robustness study was carried out by spiking the matrix with a standard solution of 25 ng/mL of each analyte.

Table 1
ICP-MS instrument parameters.

Parameter	Setting
RF Power (W)	1500
RF Matching (V)	1.80
Sampling depth (mm)	4.6
Carrier gas (L/min)	1.15
Spray chamber temperature (°C)	2
Nebulizer pump (revolutions per second, rps)	0.1
Extract (V)	3.8
Einzel 1,3 (V)	−100
Einzel 2 (V):	22
Cell entrance (V)	−50
Cell exit (V):	−47
Plate bias (V)	−44
QP bias (V)	−4.5
OctP RF (V)	190
OctP bias (V)	−7.0

Table 2
Chemical composition of the orthodontic appliances used in the study.

Material – Product	Composition (wt%)
Stainless steel – Ligature.010	18.93 Cr, 0.50 Cu, 70.37 Fe, 0.39 Mo, 9.58 Ni, and 0.23 Rb
Stainless steel – Ligature.012	18.77 Cr, 0.30 Cu, 70.57 Fe, 0.21 Mo, 9.94 Ni, and 0.20 Rb
Band single tube	17.51 Cr, 0.60 Cu, 69.59 Fe, 2.06 Mo, 9.75 Ni, 0.29 Rb, and 0.19 V
Band double tube	18.66 Cr, 0.31 Cu, 68.80 Fe, 2.22 Mo, 9.63 Ni, 0.19 Rb, and 0.18 V
Bracket BioMesh	18.42 Cr, 0.37 Cu, 66.94 Fe, 2.47 Mo, 11.57 Ni, and 0.23 Rb
Tube	18.18 Cr, 0.50 Cu, 67.95 Fe, 2.30 Mo, 10.83 Ni, and 0.23 Rb
TMA arch	18.33 Cr, 0.53 Cu, 72.71 Fe, 0.27 Mo, 7.99 Ni, and 0.18 Rb
0.014 Nickel–titanium arch	0.03 Cr, 56.36 Ni, 43.49 Ti, and 0.12 Zr
0.016 Nickel–titanium arch	0.12 Cr, 56.33 Ni, 43.42 Ti, and 0.13 Zr
0.016x.022 Stainless steel arch (AISI302 alloy)	18.74 Cr, 0.61 Cu, 72.38 Fe, 0.24 Mo, 7.41 Ni, and 0.62 Co

2.4. Statistical criteria calculations for method validation

The study of intermediate precision and trueness was performed by applying an one-factor ANOVA (GraphPad InStat software Inc., La Jolla, USA) between days. Three validation standards covering the optimal working range (0.5–50 ng/mL) were used. Each validation standard was measured in quintuplicate for two different days. From the ANOVA results, as explained in "Section 3", both the intermediate precision and the recovery were obtained. The values have been compared with tabulated reference values.

The robustness study was carried out using an intermediate validation standard (25 ng/mL of each metal) according to the Youden procedure [35]. The influential factors (the heating time employed, the volume of the deionised water used to dilute the samples, and volume of PlasmaPure 65% HNO_3 employed) were tested according to the Student *t*-test as indicated below.

Data of metal content from oral mucosal cells of patients (control and orthodontic patients) are expressed as mean ± standard deviation. Data distribution was always found non-normal, and accordingly, non-parametric methods were applied. Dunn test was used for comparing the individual treatments. Statistical significance was inferred at $P < 0.05$ (GraphPad InStat software Inc., La Jolla, USA).

3. Results and discussion

3.1. General aspects

In order to develop the ICP-MS method for the detection of Ti, V and Zr in oral mucosa cells, commercially available calibration standards solutions of the three elements were prepared by diluting the appropriate volume of a 10 μg/mL mixed-element working standard with blank solution, to a final concentrations of 0.5, 1, 5, 10, 50, 100 and 250 ng/mL of each element. These calibration standards were used to assess the linear calibration range of the instrument,. It was found that, at least between 0.5 and 250 ng/mL range, the response of the ICP-MS was linear. The concentration of the internal standard was 300 ng/mL Rh in all sample and calibration standard solutions.

Selected isotopes were ^{47}Ti, ^{51}V and ^{90}Zr. Three-points-per-mass peak pattern was chosen, and measurements were carried out in three replicates. Integration times per point, and per mass, were 0.2 s and 0.6 s, respectively, for the three elements. Integration time for internal standard was 0.01 s per point and 0.03 per mass.

3.2. Method validation

3.2.1. Linear range

The response as a function of concentration of each metal was measured by at least 5-point calibration curve with a range within 0.5–50 ng/mL. In all cases, the ratios CPS analyte/CPS internal standard (being CPS counts per second) were recorded as signal. Response linearity was established according to Huber [36] by plotting the called response factors (signal response/analyte concentration) against their respective concentrations. Responses were obtained from five or six mini-toothbrush introduced in clean 50 mL centrifuge tubes and suitably spiked with working standards of Ti, V and Zr, and submitted to the digestion and ICP-MS proposed procedure in triplicate. The Huber plots obtained for Ti, V and Zr are shown in Fig. 1. The target line has zero slope and the intercept is just the median of the response factors obtained. Two parallel horizontal lines are drawn in the graph at 0.95 and 1.05 times the median value of the response factors in a fashion similar to the action limits of control charts. As no intersections with the lines were found in the case of zirconium, the linear range of the method applies to the full range studied, 0.5–50.0 ng/mL. In the case of Ti and V, the adequate linear range found for both elements was 5.0–50.0 ng/mL. These values are similar to those found by Natarajan et al. [29] when they analyzed nickel and chromium concentrations on oral mucosa cells in a range of 1–40 ng/mL of both elements, using ICP-MS. Fernández-Miñano et al. [30] evaluated in vivo metal ions release from three alloys, but they did not provide any validation data.

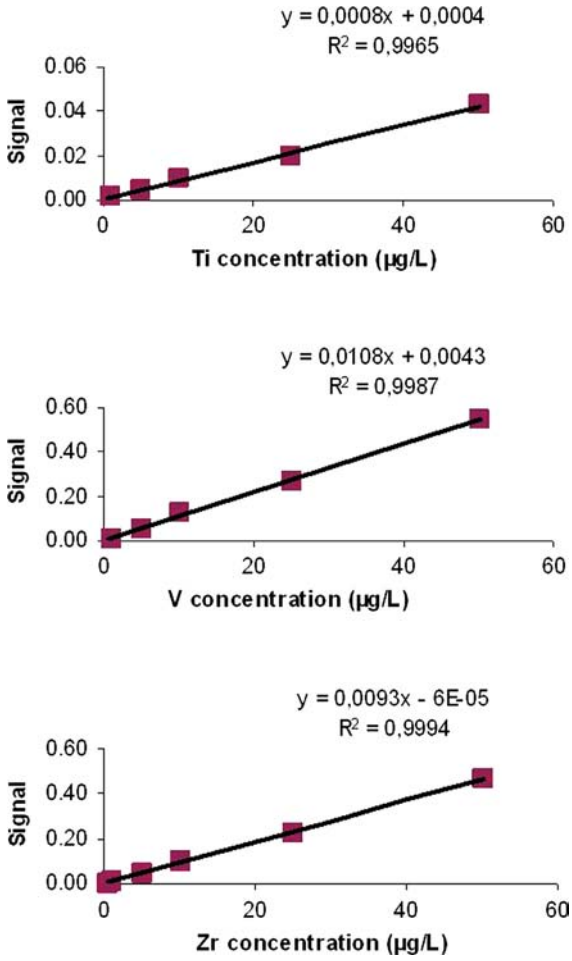

Fig. 1. Linear calibration functions for the proposed procedure.

3.2.2. Goodness of the fit

The linear calibration function was obtained by preparing five or six calibration standards in the digestion extracts resulting from the mini-toothbrush introduced in clean 50 mL centrifuge tubes (in triplicate) from 0.5 to 50 ng/mL of Ti, V and Zr, and recording the signal response according to the proposed digestion and ICP-MS procedure. Here, mini-toothbrush treated with 10 mL of deionized water and 100 μL of PlasmaPure 65% HNO_3 are taken as blank samples and the analytes (Ti, V and Zr) are spiked in order to obtain similar conditions for future samples. So, these calibration standards can be also considered as validation standards (VS). The calibration lines have correlation coefficients of 0.9965, 0.9987, and 0.9994 for Ti, V, and Zr, respectively (Fig. 2), and there is not lack-of fit and the calibration functions can be considered as linear.

3.2.3. Detection and quantitation limits

The limit of detection (LOD) and the LOQ were determined, by measuring 10 independent sample blanks. Limit of detection was

estimated using the expression $Y_{LOD}=Y_{blank}+3S_{blank}$, where Y_{blank} and S_{blank} are the average value of the blank signal and its corresponding standard deviation. Limit of detection values are then converted into concentration by using the calibration function. The procedure for evaluating LOQ was equivalent to that of LOD, but using the factor 10 instead of three for calculations. The LOD obtained were 0.9, 2.8, and 0.4 ng/mL for Ti, V and Zr, respectively. The LOQ for three elements assayed were 1.8, 3.4 and 0.7 ng/mL for Ti, V and Zr, respectively. To the extent of our best knowledge, no data of these parameters have been previously reported in the determination of trace metals in oral mucosa cells using this technique (ICP-MS). Similar detection limits (1 ng/mL) were found by Amini et al. [1] which analyzed nickel, chromium and cobalt in oral mucosa cells using atomic absorption spectrometry with graphite furnace (AAS-GF). As far as we know, no LOD and LOQ data were available for the three elements considered in the scientific literature.

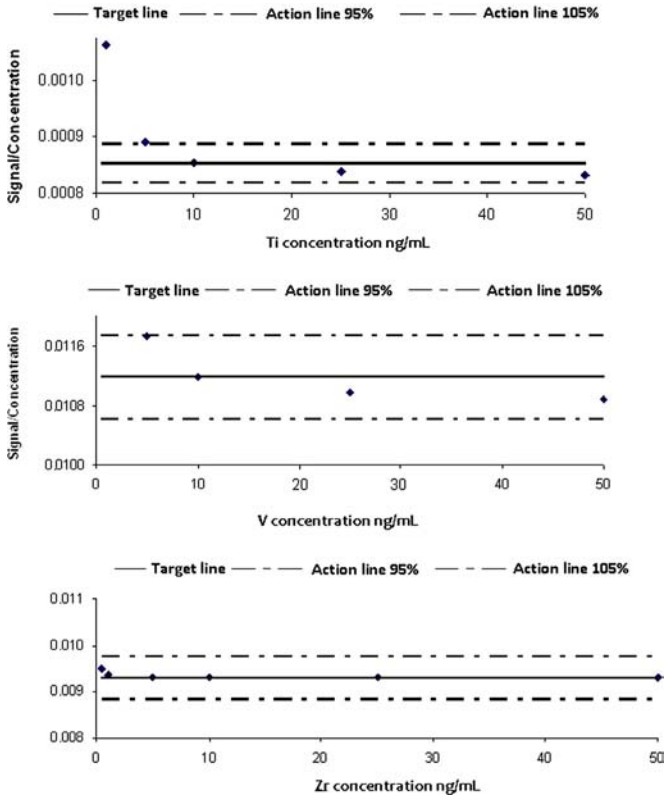

Fig. 2. Huber plots for assessing linear range.

3.3. Accuracy study

3.3.1. Intermediate precision and trueness studies

According to the International Conference on Harmonization guidelines [37], precision may be considered at three levels: repeatability, intermediate precision, and reproducibility. Repeatability expresses the precision evaluated under the same experimental conditions over a short time interval, and it is termed as intra-assay or within-run. Intermediate precision applies to within-laboratory variations: different days, different analysts or equipments and it is sometimes called between-run or inter-assay precision [32].

On the other hand, the trueness of an analytical procedure expresses the closeness of agreement between the mean value obtained from a series of measurements and the value, which is accepted either a conventional value or an accepted reference value like validation standards [32].

Repeatability and intermediate precision were calculated analyzing five replicates of mini-toothbrush spiked at three validation standards of the three metals considered (low, medium and high) covering the dynamic working range (0.5–50.0 ng/mL) on the same day and in two different days, respectively.

Considering two different days, as the main source of variation, an analysis of variance (ANOVA) was performed for each concentration, obtaining estimations of within-condition variance (S_w^2), also known as repeatability (S_r^2), and between-condition variance (S_B^2). Also, the intra laboratory reproducibility or intermediate precision, is obtained as $S_{IP}^2=S_r^2+S_B^2$ [31,32]. All these parameters are shown in Table 3.

From these data, the corresponding relative standard deviations, RSD_R were calculated and compared with the acceptable RSD percentages obtained from the AOAC Peer Verified Methods (PVM) program [32,36]. As a quick rule [32], the RSD_{IP} results should be compared with one-half the corresponding RSD values tabulated. Our results for Ti, V and Zr, at the three concentration levels considered, were lower or the same order than the one-half $\%RSD_{AOAC}$ tabulated for each element (Table 3).

The assessment of trueness can be performed according the same ANOVA results. Trueness can be expressed as the bias or recovery obtained for each validation standards assayed [38]. The recovery term has a more intuitive meaning and it has been tested in this work. The total recovery for any validation standards is defined as the ratio between the observed estimation of the validation standards concentration, and the "true" value T, expressed as percentage or as fraction. The recoveries (%) computed for the three validation standards considered for each element are shown in Table 3. We checked them for suitability by comparison with the published acceptable recovery ranges as a function of the analyte concentration [32,36]. In our method, as the Ti and Zr concentrations of the three validation standards ranged between 1 and 50 ng/mL,

Table 3
Estimations of within-condition (repeatability), between-condition, intermediate precision (intra laboratory reproducibility) and recoveries of titanium, vanadium and zirconium assayed at three validation standards, in two different days.

Paramenters	Ti concentration level			V concentration level			Zr concentration level		
	5 ng/mL	10 ng/mL	50 ng/mL	5 ng/mL	10 ng/mL	50 ng/mL	1 ng/mL	10 ng/mL	50 ng/mL
S_w	0.14	0.47	1.74	0.37	0.77	1.20	0.11	0.85	1.18
S_B	0.29	0.06	4.87	0.41	0.24	2.26	0.27	0.82	2.89
S_{IP}	0.20	0.39	3.15	0.38	0.64	1.63	0.18	0.84	1.93
RSD_{IP} (%)	10.00	3.55	6.20	6.90	5.80	3.30	15.00	8.10	3.90
½ RSD_{AOAC} (%)[a]	15	11	7.5–11	11–15	11	7.5–11	15	11	7.5–11
Recovery (%)	101 ± 9 Between (40–120)[b]	108 ± 1 Between (60–115)[b]	102 ± 3 Between (60–110)[b]	111 ± 3 Between (40–115)[b]	111 ± 1 Between (60–115)[b]	98 ± 1.5 Between (60–110)[b]	92 ± 8 Between (40–120)[b]	104 ± 2 Between (60–115)[b]	99 ± 2 Between (60–110)[b]

[a] RSD values obtained from the AOAC Peer Verified Methods program according to the concentration level of analyte ([32]).
[b] Acceptable recovery percentages according to the concentration level of analyte ([32]).

the recovery ranges (%) could oscillate between 40 and 120%, 60 and 115%, and 60 and 110%, for 1 ng/mL, 10 ng/mL and 50 ng/mL, respectively. The recoveries obtained oscillated between 101 and 108% for Ti, and between 92 and 104% for Zr. In the case of V the recoveries oscillated between 98 and 111%. All the recovery data fulfill the rule previously mentioned, and the method can be considered bias-free.

In summary, this procedure has been successfully assessed for trueness, intermediate precision and repeatability.

3.3.2. Robustness study

Robustness, considered in the sense of internal validation, deals with the effect of experimental variables, called factors, inherent in the analytical procedure (e.g., temperature, digestion conditions, pH, etc.) on the analytical result. A robustness study examines the alteration of these factors, as expected in a transfer between laboratories, so it is of the utmost importance in the uncertainty budget. The strategy for carrying out our robustness study is based on a landmark procedure suggested by Youden [35], according to the practical guide of González and Herrador [31]. Three influential factors in the sample preparation procedure were identified: (X_1) heating time employed; (X_2) volume of the deionized water used to dilute the samples, and (X_3) volume of PlasmaPure 65% HNO_3 employed. The levels are coded according to the rule: high value $=+1$($X_1=70$ min; $X_2=10.1$ mL; $X_3=200$ μL), and low level $=-1$($X_1=60$ min; $X_2=10.0$ mL; $X_3=100$ μL). The effect of every factor is estimated as the difference of the mean result obtained at the level $+1$ from that obtained at the level -1. Once effects have been estimated, to determine whether variations have a significant effect on the results, a significance t-test is used [39], and the t-values (X_k) are compared with the 95% confidence level two-tailed tabulated value with the degrees of freedom coming from the precision study for each concentration. In the present study, the experiments were carried out using validation standards spiked with 25 ng/mL of each metal considered (Ti, V and Zr), and each factor was analyzed by quintuplicate in two different days. So, for 9 degrees of freedom, the t-values obtained for X_1, X_2 and X_3 factors are shown in Table 4. In all cases, $t(X_k) < t_{tab}$ (2.262), and therefore the procedure can be considered as robust against the three factors considered (at the levels fixed in the study) for Ti, V and Zr determination.

3.4. Evaluation of titanium, vanadium and zirconium in patients with and without fixed orthodontic appliances

The cellular contents of the three elements from 40 patients, 20 of the control group and 20 of the orthodontic group, according to the proposed and validated method, were measured. The median values obtained for titanium concentration were 3.80 and 2.50 ng/g in orthodontic and control groups, respectively. Moreover, the mean value in control group (5.14 ± 3.90 ng/g) was similar to that found in orthodontic patients (5.23 ± 3.50 ng/g) and no significant differences were detected. Patients using NiTi arches showed slightly increased Ti values in comparison to patients wearing stainless steel arches, but no significant differences were found. Only traces of Zirconium were detected in the orthodontic group (0.54 ± 0.30 ng/g) and control group (0.32, lesser than the detection limit), and no significant differences were found between them. Vanadium was not detected in either the orthodontic group or the control group. These results are consistent with the minor presence of this metal in the composition of the orthodontic materials employed in this study (0.18–0.19% only in the case of bands), but the method would be suitable for monitoring emergent materials, such as Gum metal [6].

In comparison to other *in vivo* studies, our results are in agreement with the previous study by Natarajan et al. [29], in which the presence of Ni and Cr ions in the experimental group were not significantly higher than those in the control group. By contrast, Faccioni et al. [28] reported 3.4-fold and 2.8-fold increases in Ni and Co concentrations in oral mucosa cells of orthodontic patients. Fernández-Miñano et al. [30] reported that buccal cells that had been in contact with stainless steel showed higher concentrations of Ti^{47} and Mn^{55} than the control cells. Amini et al. [1], only found Ni contents significantly higher in mucosa cells of orthodontic patients compared with their non-appliance controls, and they did not report differences in chromium (Cr) and cobalt (Co) cell contents. Hafez et al. [27] reported that fixed orthodontic appliances for 6 months increased the Ni and Cr contents of the buccal mucosa cells. All these findings indicated that to ensure the safety of patients, further research would be needed to determine the long-term significance of metals release. Consequently, the development and validation of methods which permit their quantification in oral mucosa cells, which seemed advantageous because they are in direct contact with the appliances, is of great interest.

Table 4
Significance t-values (X_k) obtained in the robustness study assayed for the three elements.

Elements	X_1	X_2	X_3
Ti	0.737	0.169	0.614
V	0.101	0.157	0.095
Zr	0.667	1.428	1.985

Critical t-value = 2.262
X_1: heating time employed
X_2: volume of the deionized water used to dilute the samples
X_3: volume of PlasmaPure 65% HNO_3

4. Conclusions

In summary, we have developed and validated a method for titanium, vanadium and zirconium determination in oral mucosa cells from orthodontic patients in comparison to control patients, using a digestion procedure and quantification by ICP-MS. The procedure has been successfully assessed for trueness and precision, and can be considered as robust against the three factors considered in the digestion procedure, such as the heating time employed, the volume of the deionized water used to dilute the samples and volume of PlasmaPure 65% HNO_3 employed. The proposed method could be suitable for monitoring of these metals in buccal mucosa cells of orthodontic patients, as routine method to test the biocompatibility of fixed orthodontic appliances, and for *in vivo* studies focused in the discharge of metals from this kind of appliances.

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