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Comparative analysis of chromatic alterations of a Calcium Silicate-based Material: *in vitro* study

Integrated Master in Dental Medicine

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The noblest pleasure is the joy of understanding.

Leonardo da Vinci

ABSTRACT

Objective: The purpose of this study was to investigate and compare chromatic alterations of an inorganic silicate-based cement, also known as white mineral trioxide aggregate (WMTA), submitted to different environmental conditions using the CIE L*a*b* system evaluated by two different methods.

Methods: Twenty-four samples of WMTA (ProRoot® MTA, Dentsply Tulsa Dental, Johnson City, TN) were randomly distributed in four groups (n=4). In group 1 the samples did not undergo any additional treatment (negative control group). In group 2 WMTA samples were light irradiated for 60 seconds using a LED poliwave curing unit. In group 3 and 4 WMTA samples were coated with a layer of glycerine and adhesive, respectively and light irradiated for 60 seconds. A commercial spectrophotometer (VITA Easyshade® Advance 4.0, Vident™, California, USA) and calibrated photographic digital analysis were used to determine colour coordinates from the CIE L*a*b* system of each sample after three different time points: 30 minutes, 48 hours and 7 days. In order to understand the chemical alterations associated to colour variations of the material x-ray diffraction analysis was also conducted. Data was analysed using statistical software IBM® SPSS® Statistics, version 20.

Results: Consistency between the two colour measuring methods was not observed. Significant colour variation was observed for group 3. X-ray diffraction analysis showed no differences between compounds.

Conclusion: WMTA showed significant dark discolouration after irradiation with an LED poliwave curing unit in an oxygen-free environment promoted by glycerine although after 48 hours discolouration had faded. The same light irradiation protocol associated to an oxygen-free conditions achieved by an adhesive resin layer did not induce significant discolouration at the end of the experiment protocol.

Key-words:

White mineral trioxide aggregate, colour stability, discolouration, bismuth oxide

CONTENTS

1. INTRODUCTION

One of the growing concerns in clinical dentistry for both patient and clinician is the aesthetic appearance of teeth, in which colour plays a dominant role[1, 2] with a significant effect on patients' life quality.[2] Regarding this issue, one of the most common clinical situations, in which the aesthetic outcome is compromised, is when anterior teeth have undergone endodontic treatment, frequently leading to tooth discolouration.[3]

The diffusion of materials through dentinal tubules is suggested to be one of the primary causes of progressive tooth discolouration.[4] Nevertheless, material remnants in the pulp chamber in contact with dentin have also the potential to cause tooth discolouration, since they can diffuse through hard tissues.[5]

Considering the increasing demands for aesthetics, biomaterials used in different clinical situations involving pulp tissue management should focus on biological and functional aspects. These ought to be chromatically stable, present optical properties similar to dental structures and exhibit no staining effects on hard dental tissues over time.[6]

Material-induced tooth discoloration may be prevented to some extent by avoiding substances with a high staining risk.[7] Following the principles of conservative dentistry, the occurrence of exposed dental pulps requires a more weighed approach, maintaining as the primary goal the restitution of normal tissue architecture at the dentin-pulp interface.[8] In specific circumstances direct pulp capping may be performed and is considered as a treatment of choice.[9] The aim is to maintain pulp vitality and involves the placement of a biocompatible agent on pulp tissue by sealing off pulp pathways against bacterial penetration cell differentiation is stimulated enclosing exposure sites through the formation of a dentine bridge, and thus maintaining healthy pulp tissue.

A wide range of materials have been used to deal with pulp exposure.[10] For many decades calcium hydroxide has been considered as the material of choice for the maintenance of pulp vitality. In clinical and histological settings it has let to satisfactory results in both direct and indirect pulp capping, as it stimulates dentin bridge formation leading to pulp healing and thus providing high success rates in clinical procedures.[11]

Despite these advantages, calcium hydroxide presents some drawbacks, such as, poor bonding to dentin, material resorption and mechanical instability. As a result, calcium hydroxide does not seem to prevent microleakage in the long-term.[12] It causes tunnel defects of the newly formed hard tissue which may act as a gateway for microorganisms. These may cause secondary inflammation of the pulp tissue and are thought to be responsible for failed maintenance of tooth vitality. In addition, the high pH (12,5) of calcium

hydroxide suspensions causes liquefaction necrosis at the surface of the pulp tissue. Therefore, it may fail to provide an effective long-term barrier against bacterial penetration.[13]

A variety of new materials have recently been proposed as candidates for pulp exposure treatment. Calcium silicate-based materials (CSMs) such as mineral trioxide aggregate (MTA) have antibacterial properties and excellent biocompatibility properties, sealing ability, and regenerative capacities [14, 15]. These significant inherent advantages make them versatile materials that can be used in several treatment options.[16]

The original colour of MTA was grey (grey MTA or GMTA), thus having a high potential for tooth discolouration. Considering this drawback, white MTA (WMTA) was introduced for application in aesthetically sensitive areas.[17, 18] However, various studies have described tooth discolouration regarding the use of both kinds of MTA[19, 20]. Some authors have reported tooth discolouration when WMTA was applied in pulpotomies, in apical barriers or coronal biological barriers in the context of pulp revascularization[21, 22], while a recent, though single case report indicated that marginal gingival discolouration induced by GMTA was improved when replaced by WMTA.[23]

Notwithstanding, the colour of MTA is a key factor to the final aesthetic result of conservative treatment. In specific clinical applications, blood comes into contact with and often becomes incorporated into MTA during or after its placement. This contamination might have a detrimental effect on its physical properties.[24] In an *in vitro* study it was hypothesized that the presence or absence of oxygen plays an important role in the discolouration of white MTA[25], thus the selection of proper materials, regarding their staining properties is required.[4]

Several new CSMs have been developed, with the purpose of eliminating some of the main drawbacks of MTA such as its difficult handling, long setting time, and potential to discolouration. Biodentine™ [26], is a recently developed calcium silicate-based material (CSMs), which has overcome some of these shortcomings, still, to date, there is no available data on its colour stability.

The methods used to evaluate the colour of materials include subjective and quantitative analyses. It has already been shown that sphere spectrophotometers provide a more accurate assessment of colour change than subjective human evaluation. This technology measures the amount of visible radiant energy reflected or transmitted by an object, one wavelength at a time for each value, chroma and hue present in the entire visible spectrum. The change between the initial and the final colour (ΔE) specifies material colour alteration during a specific period of time.[20]

The aim of this study is to evaluate the over time chromatic alterations and colour stability of an inorganic cement (ProRoot® MTA, Dentsply Tulsa Dental, Johnson City, TN) under different oxygen-free conditions and poliwave LED curing unit, CIE L*a*b* system

evaluated by two different methods. The null hypothesis (H_o) is that there are no statistically significant differences in colour variation of the inorganic cement when submitted to different experimental conditions.

2. MATERIALS AND METHODS

2.1 Sample preparation

Twenty four samples of white mineral trioxide aggregate (WMTA) (ProRoot ® MTA, Dentsply Tulsa Dental, Johnson City, TN), were mixed according to the manufacturer's recommendations, and placed in a 6 mm wide and 4 mm high PVC blister. With a plastic cylinder (3mm diameter and 5mm height) amalgam carrier it was possible to fill the pellets with a standardized volume of material. The materials were then compressed with a compacter and a moist cotton pellet was temporarily placed in direct contact with the material until the first colour measurement was performed.

2.2 Experimental and control groups

The samples of WMTA were divided into four random groups: three experimental groups (n=6) and one control group (n=6) **(Table I)**. Group I was assigned as the negative control group and blisters were only filled with WMTA and left undisturbed.

Table I. Experimental groups

	Group 1	Group 2	Group 3	Group 4
Materials	ProRoot® MTA	ProRoot® MTA	ProRoot® MTA SR Gel	ProRoot® MTA Clearfil™ SE Bond
		LED light irradiated	LED light irradiated	LED light irradiated

MTA – mineral trioxide aggregate

LED – light emitting diode

In group 2, the samples of WMTA were light irradiated with a poliwave light emiting diode (LED) (Bluephase 20i, Ivoclar-Vivadent, Schaan, Liechtenstein) device, used in the high power mode. Light is characterized by a wavelength ranging from 380 nm to 515 nm and an intensity of 1200 mW/cm^2, for 60 seconds.

In group 3, the samples were coated with a standardized amount of glycerine (SR Gel, Ivoclar, Vivadent AG, Schaan, Liechtenstein) to generate an oxygen-free environment and were light irradiated with the same device and conditions, for 60 seconds.

In group 4, the samples were coated with a standardized amount of a hydrophobic resin (Clearfil SE Bond, Kuraray America, Inc., NY) to generate an oxygen-free environment

and light irradiated with the same device and conditions, for 60 seconds. Composition and batch numbers of materials are listed in **table II.**

Table II. Material description, manufacturers, compositions and batch numbers

Filling materials	Manufacturer	Composition		Lot and exp date
ProRoot® MTA	Dentsply Tulsa Dental, Tulsa, OK, USA	Chemical	wt%	12002493 2015/06
		CaO	44,23	
		SiO_2	21,20	
		$B_{i2}O_3$	16,13	
		Al_2O_3	1,92	
		MgO	1,35	
		SO_3	0,53	
		Cl	0,43	
		FeO	0,40	
		P_2O_5	0,21	
		TiO_2	0,11	
		H_2O+CO_2	14,49	
		Adapted from Asgary *et al.*[27]		
		75% Portland cement 20% bismuth oxide 5% calcium sulphate dehydrate		
SR Gel	Ivoclar, Vivadent AG, Schaan, Liechtenstein	Glycerine		S33793 2018/01
Clearfil™ SE Bond	Kuraray Medical Inc., Osaka, Japan	Bond: 10-MDP, Bis-GMA, HEMA hydrophobic aliphatic dimethacrylate, DET, silanated colloidal silica		041931 2014/05

Colour measurements were recorded at 3 time points: T_1: 30 minutes after placement of SR Gel and Clearfil™ SE Bond in groups 3 and 4, respectively; T_2: at 48 hours and T_3: at 7 days.

After the measurement in T_1, the specimens were stored in the dark, in a 100% humidity environment at 37°C with normal atmospheric gas levels, until 48 hours and 7 days upon retrieval, moment of the second and third measurements (T_2 and T_3), respectively.

2.3. Spectrophotometric Measurements

To evaluate and compare the specific degree of chromatic alterations in tooth crowns, induced by the materials, a spectrophotometer (VITA Easyshade® Advance 4.0, Vident™, California, USA) , with a white high power LED lamp type and with a temperature range from 15ºC to 40º C, was used by a single operator.

The measurements were performed by positioning the tip of the spectrophotometer in direct contact with the surface of the samples under constant laboratory light. The instrument was calibrated before the measurements for each group. Measurements were made following the manufacturer's recommendations.

For all groups, a spectrophotometric colour measurement of each sample was taken at time points 48 hours and 7 days. Measurements for the first time point were not possible to take since direct contact with the samples was not possible.

2.4. Digital photographic analysis

Calibrated digital macro photographs, at all evaluation periods were collected using a Single Lens Reflex (SLR) camera (Canon EOS 5D Digital; Canon Inc., Tokyo, Japan) with a 100 mm macro lens (Canon EF 100 mm f/2.8 Macro USM; Canon Inc., Tokyo, Japan) and a ring flash (Canon MR-14 EX; Canon Inc., Tokyo, Japan). Parameters used were aperture F32, velocity 1/200; ISO 200; automatic white balance and manual focus. Photographs were obtained as ".jpg" and ".raw" images with high resolution (3888x2592 pixels).

Each photograph was processed with Adobe® Photoshop software (Adobe® Systems Incorporated, San Jose, California, USA) and colour and space coordinates were extracted using java-based image processing and analysis ImageJ software (National Institute of Health, Bethesda, Maryland, USA).

2.5 Sample shade assessment

Instrumental measurements, including dental colorimeters and spectrophotometers, commonly utilize the International Commission on Illumination (CIE, from the french Comission Internationale de l'Éclairage's) L*a*b* system. CIE is an organization recognized by the International Organization for Standardization (ISO) as an international organization on the subject of light, vision, and colour.[28]

The total colour difference between two objects can be expressed numerically by their Euclidean distance, in ΔE values. Therefore, the difference between two colours, (L^*_1, a^*_1, b^*_1) and (L^*_2, a^*_2, b^*_2) is given by:

$$\Delta E = [(L_2^* - L_1^*)^2 + (a_2^* - a_1^*)^2 + (b_2^* - b_1^*)^2]^{\frac{1}{2}}$$

In this study, the colour measurements were taken directly in CIE L*a*b* colour space, where ΔL is the change in luminance [from 0 (black) to 100 (white), Δa* is the change in the red (positive a*) to green (negative a*) parameter, and Δb* is the change in the yellow (positive b*) to blue (negative b*) parameter **(Figure 1).**

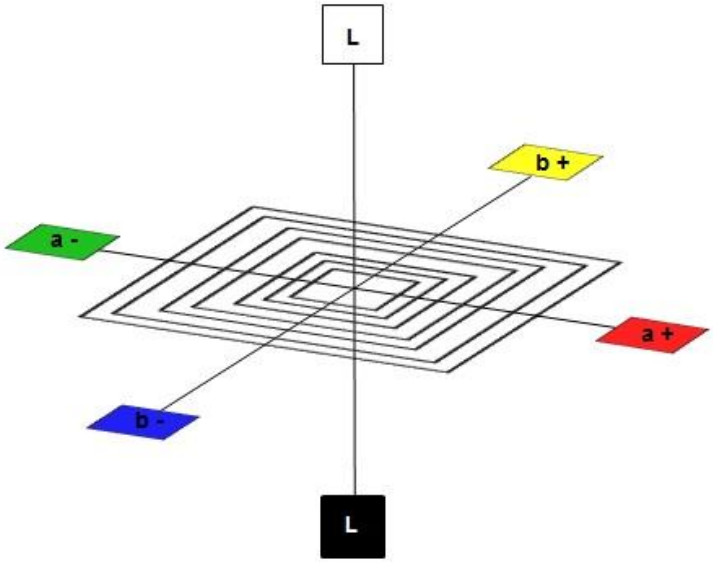

Fig.1. Three dimensional representation of Munsell colour system diagram.

2.6. X-ray diffraction analysis

X-ray diffraction analysis (XRD) was conducted for WMTA powder, WMTA set form with glycerine (SR Gel, Ivoclar, Vivadent AG, Schaan, Liechtenstein) and WMTA set form with glycerine and light irradiated.

For the set form, a WMTA sample was prepared according to the manufacturer's instructions and was placed into a circular metallic mold. The upper surface of the sample was swept with a spatula and left 7 days at 37ºC and 100% humidity in the incubator.

Both powder and set material of WMTA (ProRoot ® MTA, Dentsply Tulsa Dental, Johnson City, TN) were then subjected to (XRD) analysis. An X-ray diffractometer (Panalytical X'Pert Diffractometer, Philips) (**Figure 2**) with general Bragg Brentano scans and

Cu, radiation running at 40 kV voltage and 35 mA current was used in the present study. The scan range was set at 20-60º2Ɵ and with a scan speed of 0,025º2Ɵ per second.

Each phase (crystalline substance) of a compound has a characteristic diffraction pattern consisting of several X-ray peaks. The peaks at the specified intensity representing the diffraction patterns of the tested materials were matched with the standard data documented in the Powder Diffraction Files (PDF) found in the International Centre for Diffraction Data (ICDD) database.

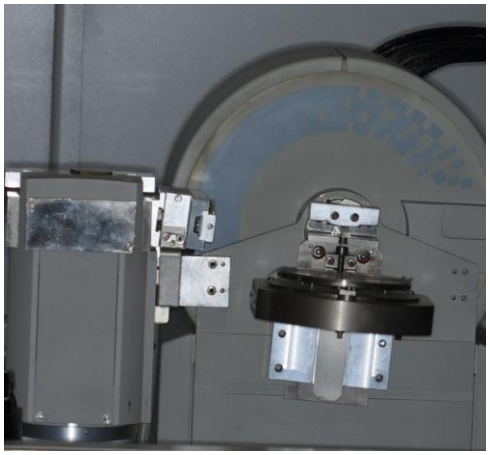

Fig. 2. X-ray diffractometer (Panalytical X'Pert Diffractometer, Philips)

2.7. Statistical Analysis

Statistical analysis was performed using IBM® SPSS® Statistics, version 20. Reliability analysis between the two shade assessment methods, colour macro photographs and spectrophotometer measurements was obtained through intraclass correlation coefficient (ICC) using two-way mixed model effects accessing for consistency between measurements for a confidence level of 95%. Descriptive statistics were obtained for chromatic coordinates L*, a* and b*. Kruskal-Wallis H test was used for group comparison in each period of evaluation (T_1, T_2 and T_3) and post-hoc pairwise comparisons were performed with the Mann-Whitney U test. Group evolution over time was verified with the Friedman test for related samples. Post-hoc analysis was performed with paired samples t-test to determine between which periods colour changes were statistically significant. Mixed ANOVA procedures were used to verify coordinate L* evolution, considering time (T_1, T_2 and T_3) as the within subjects effects and considering groups (1, 2, 3 and 4) as between subjects effects. In order to analyse colour changes for the 4 groups over time, three different colour intervals (ΔE) were calculated: ΔE (T_2-T_1), ΔE (T_3-T_2) and ΔE (T_3-T_1), using the expression:

$$\Delta E = [(L_2^* - L_1^*)^2 + (a_2^* - a_1^*)^2 + (b_2^* - b_1^*)^2]^{\frac{1}{2}}.$$

For these time intervals, in each group, a unilateral t-Student test was realized considering the cut point value of 2,3 as the minimum threshold for perceptible changes in colour difference. Colour differences (ΔE) between T_2 and T_1, T_3 and T_2, T_3 and T_1, were calculated and compared for the 4 groups, using a paired sample test. One-way ANOVA and multiple comparisons were applied to verify the significant statistical differences between the groups (1, 2, 3 and 4), regarding colour variation. The significance level was set at $\alpha = 0.05$.

3. RESULTS

Reliability analysis, between the two shade assessment methods, colour macro photographs (Canon EOS 5D Digital; Canon Inc., Tokyo, Japan) and spectrophotometer (VITA Easyshade® Advance 4.0, Vident$_{TM}$, California, USA) measurements, was obtained through intraclass correlation coefficient (ICC) using two-way mixed model effects accessing for consistency between measurements for a confidence level of 95%. Results can be analysed in **table III**. Consistency between colour measuring methods was not observed.

Table III. Intraclass correlation coefficient (ICC) between measuring methods.

Coordinates	Time	ICC	95% CI [lower bound; upper bound]	*p*
L*	**48 hours**	0,333	[-0,072; 0,644]	0,052
	7 days	0,275	[-0,136; 0,605]	0,092
a*	**48 hours**	-0,188	[-0,544; 0,225]	0,816
	7 days	0,151	[-0,261; 0,516]	0,236
b*	**48 hours**	0,444	[0,058; 0,714]	0,013*
	7 days	0,406	[0,011; 0,691]	0,022*
ΔE	**T_3-T_2**	-0,010	[-0,404; 0,388]	0,518

*– results with statistically significant differences
CI – confidence interval
p – statistical significance

As observed in **Figure 2** the colour variation gap of ΔE [-2,3 ;2,3] in both methods were statistically significant over time since colour difference is not perceived to the human eye in this gap.

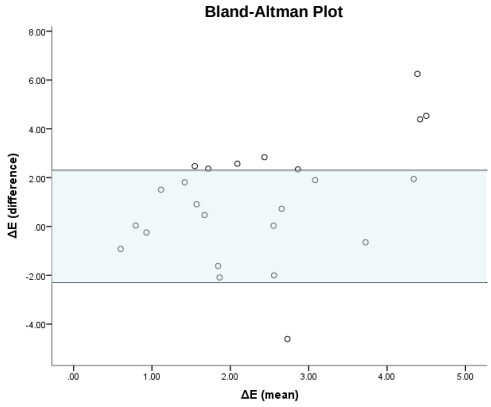

Fig. 3. Bland-Altman Plot for colour variation (ΔE) concerning colour differences between the two different measuring methods.

Descriptive statistics were obtained for the chromatic coordinates L*, a* and b*. **Table IV** represents the different groups measured over time (T_1, T_2 and T_3), as well as, the results for non-parametric Kruskal-Wallis H and Friedman tests.

Figure 4 represents a random sample of each of the 4 groups, at T_1, T_2 and T_3. It is possible to observe the different chromatic shades from all groups and their evolution over time. Group 3 had the most darkening of the samples in T_1, while Group 1 and 2 remained visually stable over time.

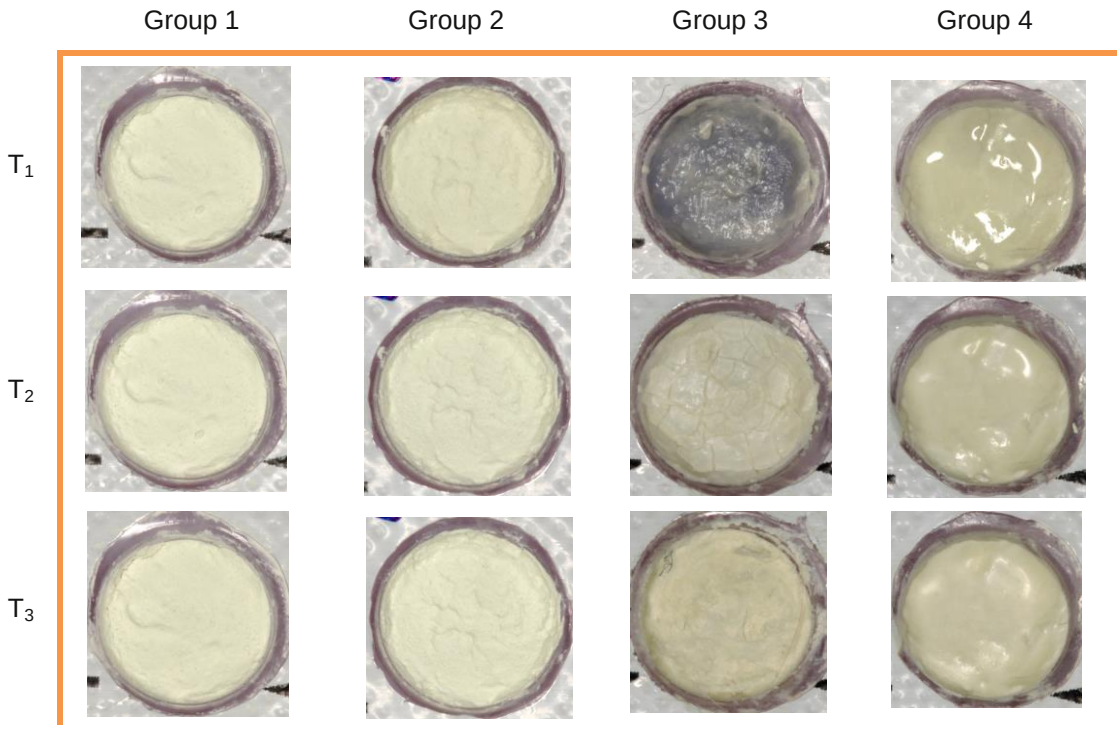

Fig. 4. Random sample of each of the 4 groups, after light irradiation. Group 1 and 2: WMTA; Group 3: WMTA and SR Gel; Group 4: WMTA and Clearfil™ SE Bond.

Table IV. Chromatic coordinates for the 4 groups in T_1, T_2 and T_3

Measuring method	Group	L*				a*				b*			
		T_1	T_2	T_3	*P*	T_1	T_2	T_3	*p*	T_1	T_2	T_3	*p*
Photograph	1	47,589 (1,3998)	49,404 (1,159)	48,395 (0,736)	**0,030***	-1,659 (0,105)	-1,453 (0,167)	- 1,455 (0,156)	**0,042***	7,979 (0,514)	6,712 (0,748)	6,967 (0,782)	**0,006***
	2	47,033 (2,0916)	49,382 (1,451)	48,158 (0,989)	0,115	-1,633 (0,0484)	-1,407 (0,173)	-1,375 (0,135)	**0,001***	8,330 (0,415)	6,540 (0,444)	6,793 (0,527)	**0,006***
	3	34,133 (6,222)	40,219 (1,592)	42,012 (1,203)	**0,002***	-1,100 (0,388)	-1,965 (0,212)	-1,485 (0,117)	**0,002***	5,835 (2,553)	9,232 (0,737)	9,200 (0,632)	**0,009***
	4	37,192 (2,286)	41,953 (1,026)	44,235 (0,892)	**0,006***	-1,878 (0,214)	-1,235 (1,598)	-1,678 (0,119)	0,135	8,743 (0,734)	8,743 (0,734)	8,698 (0,721)	0,311
	p	**<0,01***	**<0,01***	**<0,01***		**0,001***	**0,009***	**0,012***		**0,002***	**0,001***	**0,001***	
Spectrophotometer	1		47,917 (3,965)	46,083 (5,032)	0,102		-1,767 (0,197)	-1,650 (0,226)	0,102		8,267 (0,413)	8,200 (0,261)	0,102
	2		43,683 (6,459)	41,567 (8,677)	0,180		-1,750 (0,327)	-1,533 (0,393)	**0,025***		8,100 (0,379)	7,967 (0,582)	0,180
	3		33,633 (4,526)	31,750 (7,054)	0,414		-0,983 (0,449)	-0,400 (0,395)	**0,014***		10,333 (1,109)	10,167 (1,124)	0,414
	4		46,833 (9,696)	44,550 (8,566)	**0,014***		-2,083 (0,630)	-1,933 (0,622)	0,655		14,317 (3,533)	14,500 (3,349)	1,000
	p		**0,006***	**0,018***			**0,006***	**0,003***			**<0,01***	**<0,01***	

* – values with statistically significant differences

p – statistical significance

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3.1 Digital photographic analysis

- **Coordinate L***

Figure 5 shows the charts regarding colour coordinate L* variation, over time (T_1, T_2, T_3) for each group (1, 2, 3 and 4). It can be observed that L* values for group 1 increased from T_1 to T_2 and suffered a decrease from T_2 to T_3. For group 2 the same evolution was verified. On the other hand, in groups 3 and 4 an increase in L* value was observed over-time.

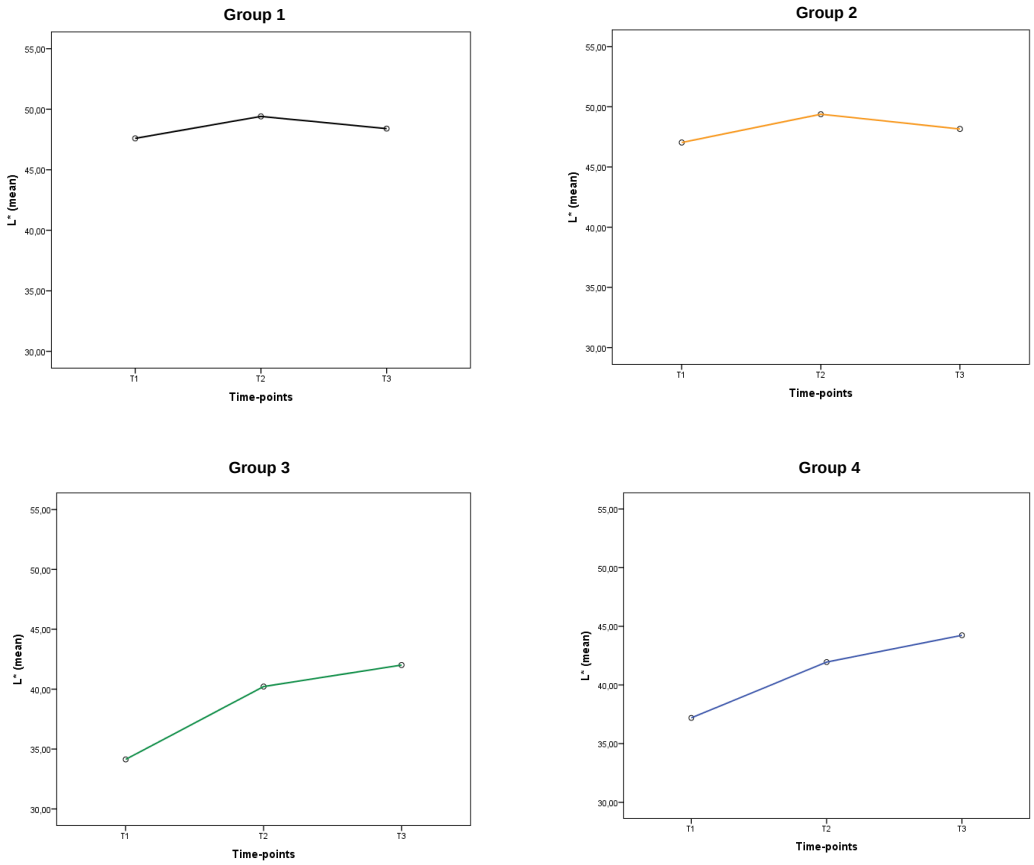

Fig. 5. Coordinate L* variation, over time (T_1, T_2, T_3), for each one of the groups.

Coordinate L* variation was also verified, between time intervals (T_2-T_1), (T_3-T_2) and (T_3-T_1) for each group, with a paired samples t-test. In **table V**, results of post-hoc analysis and coordinate L* variation means are represented.

Statistically significant coordinate L* variation was observed for group 1 at all-time intervals, except between T_1 and T_3, p=0,260. For group 2 only for time interval (T_2-T_1), p=0,012 was the difference significant and group 3 at time intervals (T_3-T_2) and (T_3-T_1), p=0,028 and p=0,034, respectively. Group 4 showed statistically significant variation for all time intervals, p (T_2-T_1) = 0,004, p (T_3-T_2) = 0,010 and p (T_3-T_1) < 0,01.

Table V. Coordinate L* variation between time intervals (T_2-T_1), (T_3-T_2) and (T_3-T_1), for each group (Paired samples t-test.)

Group		Time intervals	Mean difference	*Standard deviation*	*95% CI*	*P*
Photograh	1	**(T_2-T_1)**	1,82	1,30	[0,45;3,18]	0,019*
		(T_3-T_2)	-1,01	0,82	[-1,87;-0,15]	0,003*
		(T_3-T_1)	0,81	1,55	[-0,82;2,44]	0,260
	2	**(T_2-T_1)**	2,35	1,48	[0,79;3,90]	0,012*
		(T_3-T_2)	-1,22	1,53	[-2,82;0,38]	0,106
		(T_3-T_1)	1,12	1,42	[-0,36;2,61]	0,110
	3	**(T_2-T_1)**	6,09	7,30	[-1,58;13,75]	0,097
		(T_3-T_2)	1,79	1,44	[0,28;3,30]	0,028*
		(T_3-T_1)	7,88	6,68	[0,87;14,89]	0,034*
	4	**(T_2-T_1)**	4,76	2,33	[2,31;7,21]	0,004*
		(T_3-T_2)	2,28	1,38	[0,83;3,74]	0,010*
		(T_3-T_1)	7,04	1,55	[5,41;8,67]	<0,01*

* – values with statistically significant differences
CI – confidence interval
p – statistical significance

Regarding colour evolution at each time point (T_1, T_2 and T_3) a Mann-Whitney U test was run to determine if there were differences in time scores between groups. Results showed that statistically significant differences were present between groups 1 and 3, U = 0,00; z = -2,882; p = 0,004 and group 4, U = 0,00; z = -2,882, p=0,004 at all time points. The same applied between groups 2 and 3, U = 0,00; z = -2,887; p = 0,004 for T_1, and U=0,00; z = -2,882; p = 0,004 for T_2 and T_3 respectively. Between groups 2 and 4 there were statistical significant differences for T_1 and T_2, U = 0,00; z = -2,887; p = 0,004 and T_3, U = 0,00; z = -2,882; p=0,004. Groups 3 and 4 had statistically significant differences for T_3, U = 2,00; z = -2,562; p = 0,010.

Figure 6 represents coordinate L* evolution over time (T_1, T_2, T_3), for all groups. Mixed ANOVA tests detected an interaction between group and time on colour variation of colour coordinate L*, F(3,5;23,4)= 4,29; p = 0,012, considering the Greenhouse Geiser Effect. The test indicated that groups have different behaviours regarding L* values evolution over time.

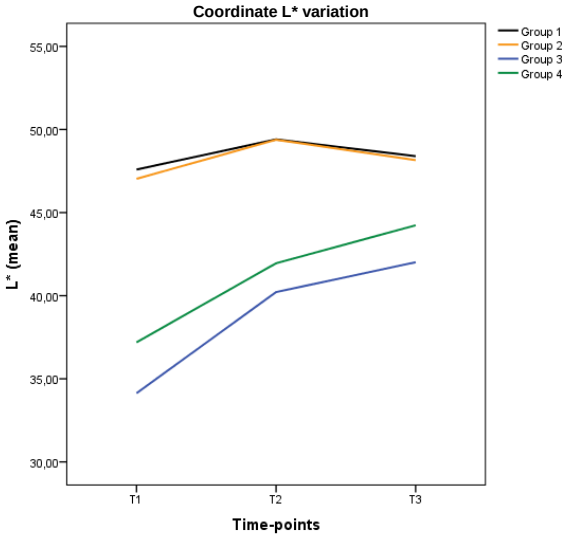

Fig. 6. Coordinate L* variation, over time (T_1, T_2, T_3), for all groups

Figure 7 represents a box-plot chart depicting coordinate L* for each group at different time points (T_1, T_2 and T_3). It is evident that the biggest value variation occurred in group 4 at T_1. There is one outlier in the data for group 3 at time point T_1, as assessed by inspection of the boxplot.

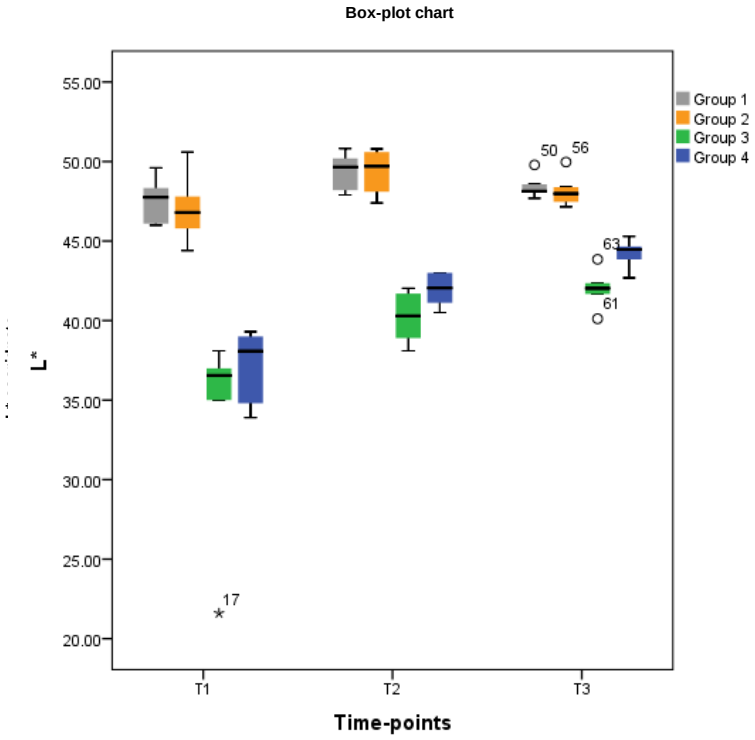

Fig. 7. Box-plot graph representing coordinate L* values dispersion for each group at three time points (T_1, T_2 and T_3).

- **Coordinate a***

Figure 8 shows the chart regarding colour coordinate a* variation, over time (T_1, T_2 and T_3), for each group. It is possible to observe that for group 3, coordinate a* variation behaved differently from all other groups. From time interval T_1 to T_2 there was a significant decrease in a* value, whereas from T_2 to T_3 coordinate a* values increased.

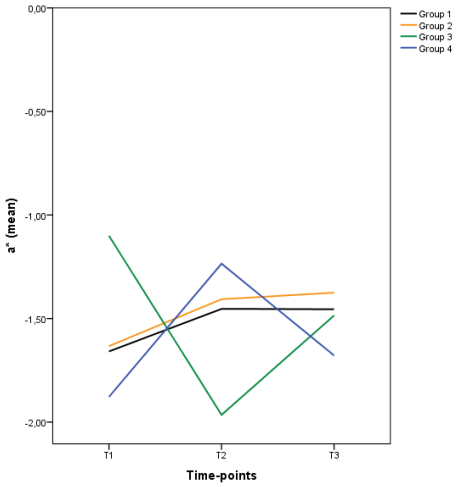

Fig. 8. Coordinate a* variation, over time (T_1, T_2, T_3), for all groups

In **table VI** are represented coordinate a* mean differences between all time-points. Group 3 showed that from T_1 to T_3 a* values decreased, while in all other groups there was an increase regarding these values, being representative of an approach towards redness.

Table VI. Coordinate a* colour differences (Δa*) between T_2-T_1,T_3-T_2 and T_3-T_1 for all groups

Measuring method	Group	Δa*		
		T_2-T_1	T_3-T_2	T_3-T_1
Photograph	1	0,21	-0,002	0,20
	2	0,23	0,03	0,26
	3	-0,86	0,48	-0,38
	4	0,64	-0,44	0,2

- **Coordinate b***

Figure 9 shows the graph regarding coordinate b* variation, over time (T_1, T_2 and T_3), for all groups. Group 3 was the only group with coordinate b* value increase from T_1 to T_2, becoming then stable from T_2 to T_3. Group 4 values were constant over time, while groups 1 and 2 have a value b* decrease from T_1 to T_2.

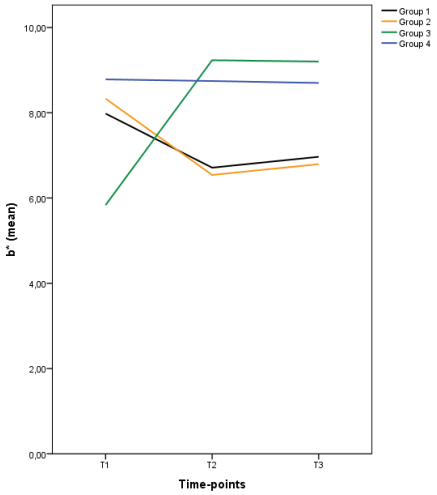

Fig. 9. Coordinate b* variation, over time (T_1, T_2, T_3), for all groups

In **table IV** coordinate b* mean values can be analysed. All groups except for group 3 had a decrease of b* values from the first measuring time-point (T_1) to the last (T_3).

Table VII. Coordinate b* colour differences (Δa*) between T_2-T_1, T_3-T_2 and T_3-T_1 for all groups

Measuring method	Group	Δa*		
		T_2-T_1	T_3-T_2	T_3-T_1
Photograph	1	-1,27	0,26	-1,01
	2	-1,79	0,25	-1,54
	3	3,40	-0,03	3,37
	4	-0,04	-0,05	-0,09

- **Colour variation (ΔE)**

Table VIII represents the mean difference for colour difference intervals (ΔE) between (T_2-T_1), (T_3-T_2) and (T_3-T_1), for all groups.

Table VIII. Mean difference for colour variation (ΔE)

Measuring method	Group	ΔE		
		T_2-T_1	T_3-T_2	T_3-T_1
Photograph	1	2,350	1,172	1,913
	2	3,157	1,701	2,287
	3	7,248	1,927	8,664
	4	5,028	2,689	7,077

Regarding ΔE (T_2-T_1), perceptible threshold of chromatic alterations for the human eye (ΔE ≥ 2,3) were observed for all group at time difference T_2-T_1. Regarding ΔE (T_3-T_2), perceptible threshold of chromatic alterations for the human eye (ΔE ≥ 2,3), is not observed for any of the groups. Analysing ΔE (T_3-T_1) values, perceptible threshold of chromatic alterations for the human eye (ΔE≥2.3) is only observed for group 3 and 4.

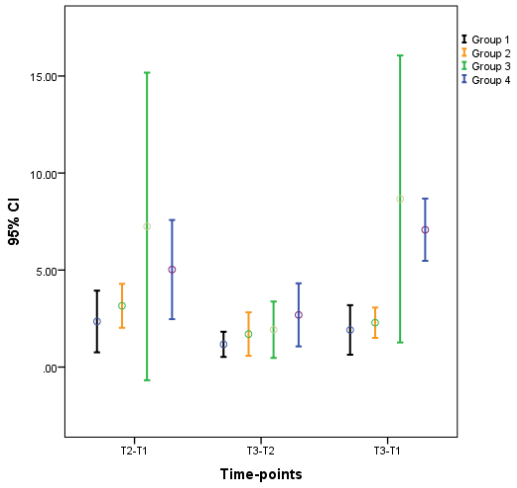

Fig. 10. Error-bar relative to colour differences (ΔE) for each group: ΔE (T_2-T_1), ΔE (T_3-T_2), ΔE (T_3-T_1).

Figure 10 represents an error bar relative to colour differences between (T_2-T_1), (T_3-T_2) and (T_3-T_1), for each group. Between T_2 and T_1 the higher degree of colour alteration (ΔE) in descending order, was for groups 3,4 1 and 2; between T_3 and T_2,

groups 4, 3, 2 and 1.Considering colour differences from T_1 to T_3, the group with the most evident change was group 3, as can be observed in the corresponding chart.

A one-way ANOVA test was conducted to compare the four groups at each time interval. No statistical differences were observed, except for (T_3-T_1), F (3) = 5,127, *p*=0,009.

Tabel Ix. Bonferroni correction - Multiple comparisons

Dependent variable		Groups		Mean difference	95% CI	*Std* error	*p*
Photograph	ΔE (T_2-T_1)	1	2	-0,807	[-7,70;6,08]	2,353	>0,05
			3	-4,900	[-11,79;1,99]		0,303
			4	-2,678	[-9,57;4,21]		>0,05
		2	3	-4,091	[-10,98;2,80]		0,585
			4	-1,871	[-8,76;5,02]		>0,05
		3	4	2,220	[-4,67;9,11]		>0,05
	ΔE (T_3-T_2)	1	2	-0,529	[-2,57;1,51]	0,698	>0,05
			3	-0,755	[-2,80;1,29]		>0,05
			4	-1,516	[-3,56;0,53]		0,252
		2	3	-0,226	[-2,27;1,82]		>0,05
			4	-0,988	[-3,03;1,06]		>0,05
		3	4	-0,762	[-2,80;1,28]		>0,05
	ΔE (T_3-T_1)	1	2	-0,374	[-6,59;5,84]	2,122	>0,05
			3	-6,751	[-12,96;0,54]		0,028*
			4	-5,164	[-11,38;1,05]		0,147
		2	3	-6,377	[-12,59;-0,17]		0,042*
			4	-4,790	[-11,00;1,42]		0,212
		3	4	1,587	[-4,62;7,80}		>0,05

*- values with statistically significant differences
CI – confidence interval
Std error – standard error
p – statistical significance

Multiple comparisons are described in **table IX** and showed that group 3 presented values significantly higher than all the other groups. Analysing the above table, it is possible to see that group 3, showed significant differences when compared with groups 1 and 2 between the last (T_3) colour measurements and the first (T_1) time point. On the other hand, there were no statistically significant differences found in the colour alteration (ΔE) analysis, between group 3 and 4, for all time intervals.

3.2 Spectrophotometric measurements

- **Coordinate L***

Figure 11 shows the graphs relative to colour coordinate L* variation, over time (T_2, T_3) for each group (1, 2, 3 and 4). It can be observed that all L* values suffered a decrease from T_2 to T_3 over time. It is also evident that group 3 had lower L* mean values.

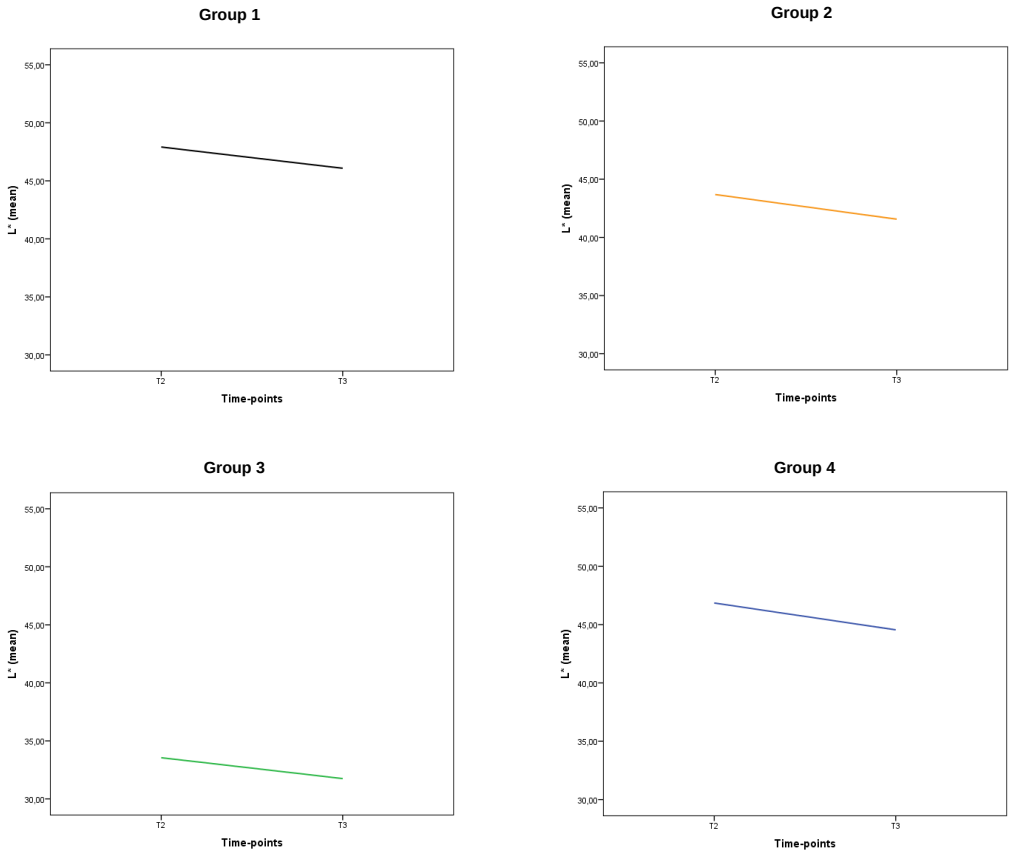

Fig. 11.Coordinate L* variation, over time (T_2, T_3), for each one of the groups.

Coordinate L* variation was also verified, between time interval (T_3-T_2) for each group, with a paired samples t-test. In **table X**, results of post-hoc analysis and coordinate L* variation means are represented.

We observed no statistically significant differences for coordinate L* variation from time point 48 hours to 7 days.

Table X. Coordinate L* variation between time intervals (T_3-T_2), for each group (Paired samples t-test)

Group		Time intervals	Mean difference	*Standard deviation*	*95% CI*	*p*
Spectrophotometer	1	**(T_3-T_2)**	-1,83	±3,66	[-5,67;2,00]	0,274
	2	**(T_3-T_2)**	-2,12	±3,04	[5,31;1,08]	0,149
	3	**(T_3-T_2)**	-1,80	±3,25	[-5,21;1,61]	0,233
	4	**(T_3-T_2)**	-2,30	±2,31	[-4,77;0,13]	0,059

CI – confidence interval
p – statistical significance

Regarding colour evolution at each time point (T_2 and T_3) a Mann-Whitney U test was run to determine if there were differences in time scores between groups. Results showed that statistically significant differences were present between groups 1 and 3, *U*=1,00; *z*=-2,72; *p*=0,006 for time point T_2 and in group 4 no statistically significant differences were found. Between groups 2 and 3, *U*=5,00; *z*=-2,082; *p*=0,037 for T_2 were found statistical significant differences. Between groups 2 and 4 there were no statistically significant differences for T_2 and T_3. Groups 3 and 4 had statistically significant differences for T_3, *U* =-2,326; *z* = -2,562; *p*=0,020.

Figure 12 represents coordinate L* evolution over time (T_2, T_3), for all groups. Regarding the Greenhouse Geiser Effect, a mixed ANOVA tests detected no interaction between group and time on colour variation for colour coordinate L*, F(3;20)=0,035; *p*=0,991. There was a significant decrease for all groups throughout time evaluation. Within-subjects effects detected a significant decrease of L* from T_2 to $T_{3.:}$ F(1;20)=10,07, *p*=0,005. Tests of between-subjects effects detected statistically significant differences between groups: F(3;20)=5,39, *p*=0,007.

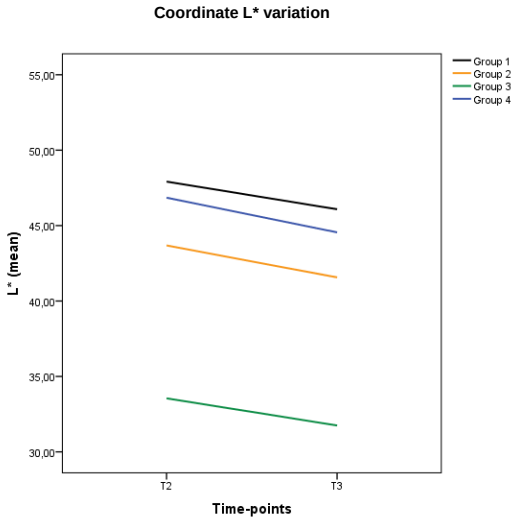

Fig. 12. Coordinate L* variation, over time (T_1, T_2, T_3), for all groups

Figure 13 depicts coordinate L* for each group at different time points (T_2 and T_3). It is evident that the biggest value variation occurred for in groups 3 and 4 at T_3. There is one outlier in the data for group 3 at time point 2, as assessed by inspection of the box-plot.

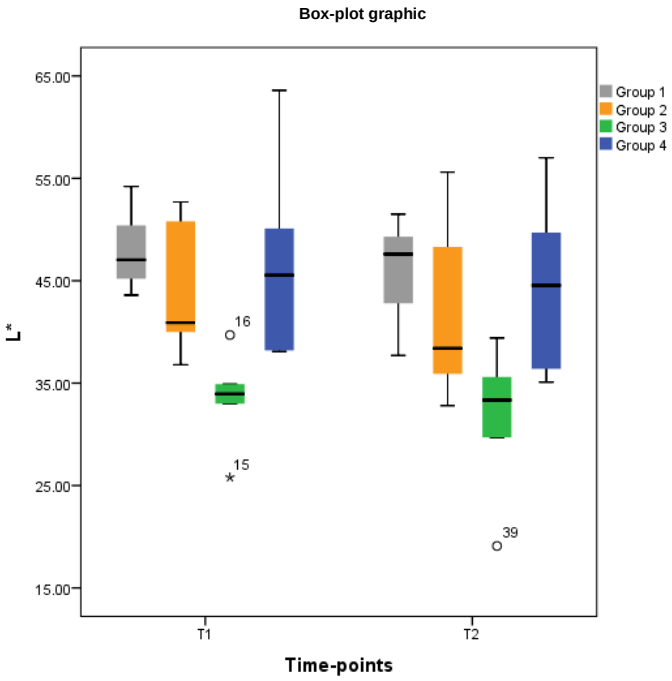

Fig. 13. Box-plot representing Coordinate L* values dispersion at three time points

- **Coordinate a***

Figure 14 shows the graph regarding the colour coordinate a* variation, over time (T_2 and T_3), for each group. It is possible to observe that for group 3, coordinate a* variation had higher values than group 1, 2 and 4 as well as the greatest variation.

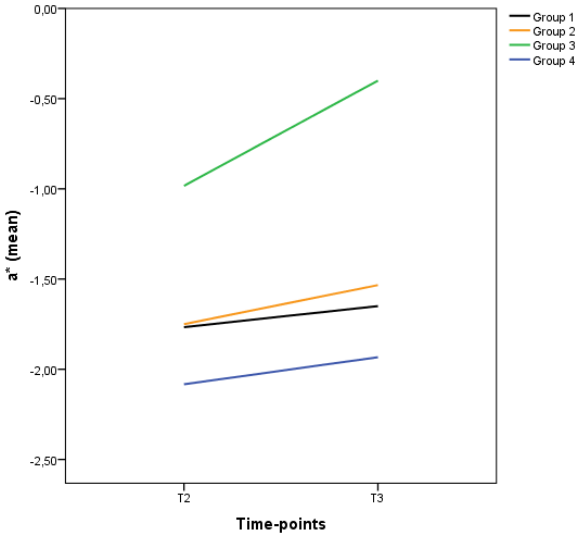

Fig. 14. Coordinate a* variation, over time (T_2, T_3), for all groups

In table XII colour differences for coordinate a* are represented for time interval T_3-T_2. Group 3 had the highest value, being Δa^*=0,58 towards redness.

Table XI. Coordinate a* colour differences (Δa^*) between T_3-T_2 for all groups

Measuring method	Group	Δa*		
		T_2-T_1	T_3-T_2	T_3-T_1
Spectrophotometer	1		0,17	
	2		0,22	
	3		0,58	
	4		0,15	

- **Coordinate b***

Figure 15 shows the graph regarding coordinate b* variation, over time (T_2 and T_3), for all groups. Group 3 has the highest b* coordinate values, while group 1 and 2 have similar values.

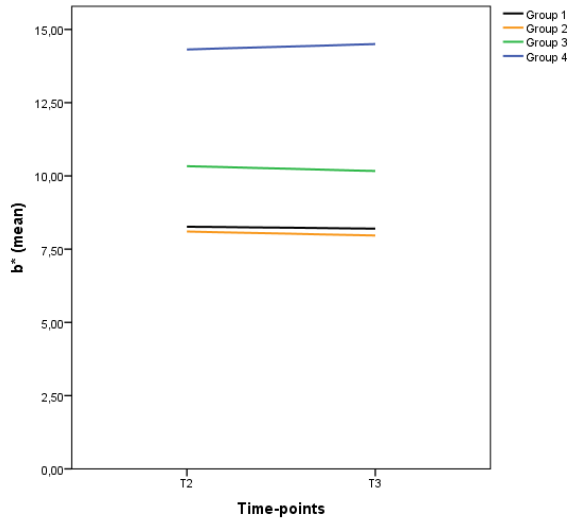

Fig. 15. Coordinate b* variation, over time (T_2, T_3), for all groups

In table XII mean values for coordinate b* variation for each group are shown for time variation T_3-T_2.

Table XII. Coordinate b* colour differences (Δa*) between T_3-T_2 for all groups

Measuring method	Group	Δb*		
		T_2-T_1	T_3-T_2	T_3-T_1
Spectrophotometer	1		9,97	
	2		9,72	
	3		11,15	
	4		16,58	

- **Colour variation (ΔE)**

Table XIII represents the mean difference for colour difference intervals (ΔE) between T_3-T_2 for all groups.

Table XIII. Colour differences (ΔE) between T_3-T_2 for all groups.

Measuring method	Group	ΔE		
		T_2-T_1	T_3-T_2	T_3-T_1
Spectrophotometer	1		3,118	
	2		3,134	
	3		2,915	
	4		2,419	

Regarding ΔE (T_3-T_2), perceptible threshold of chromatic alterations for the human eye (ΔE ≥ 2,3) were observed for group 1 (negative control) and 2. However, for groups 3 and 4, colour alteration was not statistically superior to ΔE=2,3.

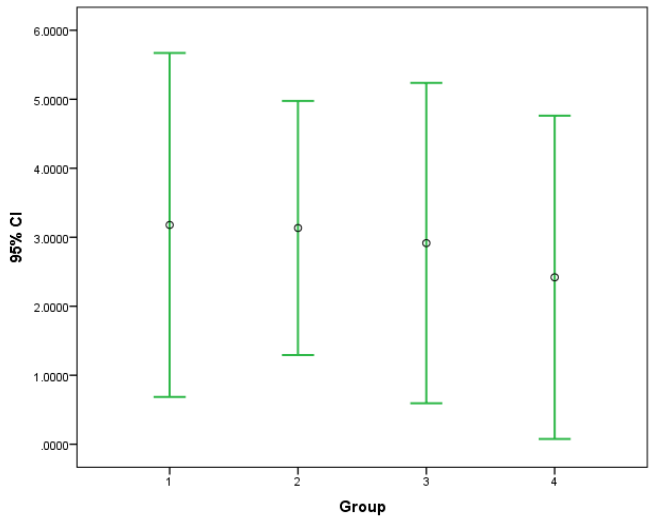

Fig. 16. Error-bar relative to colour differences (ΔE) for each group: ΔE (T_3-T_2)

Figure 16 represents an error bar relative to colour differences between (T_3-T_2) for each group. The groups with the higher degree of colour alteration (ΔE) in descending order, were: groups 1, 4, 3, and 2.

One-way variance analysis (ANOVA) was conducted and detected statistically significant differences between groups. No statistically differences were observed, for (T_3-T_2), F (3) = 0,156, *p*=0,924.

Table XIV. Bonferroni correction - Multiple comparisons

Dependent variable		Groups		Mean difference	*95% CI*	*Std* error	*p*
Spectrophotometer	ΔE (T_3-T_2)	1	2	0,044	[-3,60;3,69]	1,245	>0,05
			3	0,263	[-3,38;3,91]		>0,05
			4	0,760	[-2,89;4,40]		>0,05
		2	3	0,219	[-3,43;3,86]		>0,05
			4	0,715	[-2,93;4,36]		>0,05
		3	4	0,496	[-3,15;4,14]		>0,05

CI – confidence interval
Std error – standard error
p – statistical significance

Multiple comparisons are detailed in **table XIV** and revealed that there were not statistically significant differences in colour alteration (ΔE), among the groups , for all time intervals (T_3-T_2).

3.3 X-ray diffraction analysis

X-ray diffraction (XRD) analysis of WMTA powder showed that the material was crystalline, with specific peaks attributable to specific phases.

A slight change in intensity was observed on the phase of bismuth oxide in the sample with WMTA and glycerine light irradiated, which can indicate the decomposition of bismuth oxide into bismuth, although only a residual amount would be present. The highest peak for bismuth is overlapped with that of bismuth oxide's peak at approximately 31,7-31,9, verified by ICDD. The XRD results are shown in **figure 17** and **18**. The colour lines that are represented in **figure 17** do not always represent existing peaks.

WMTA powder showed strong peaks of bismuth oxide and calcium silicate.

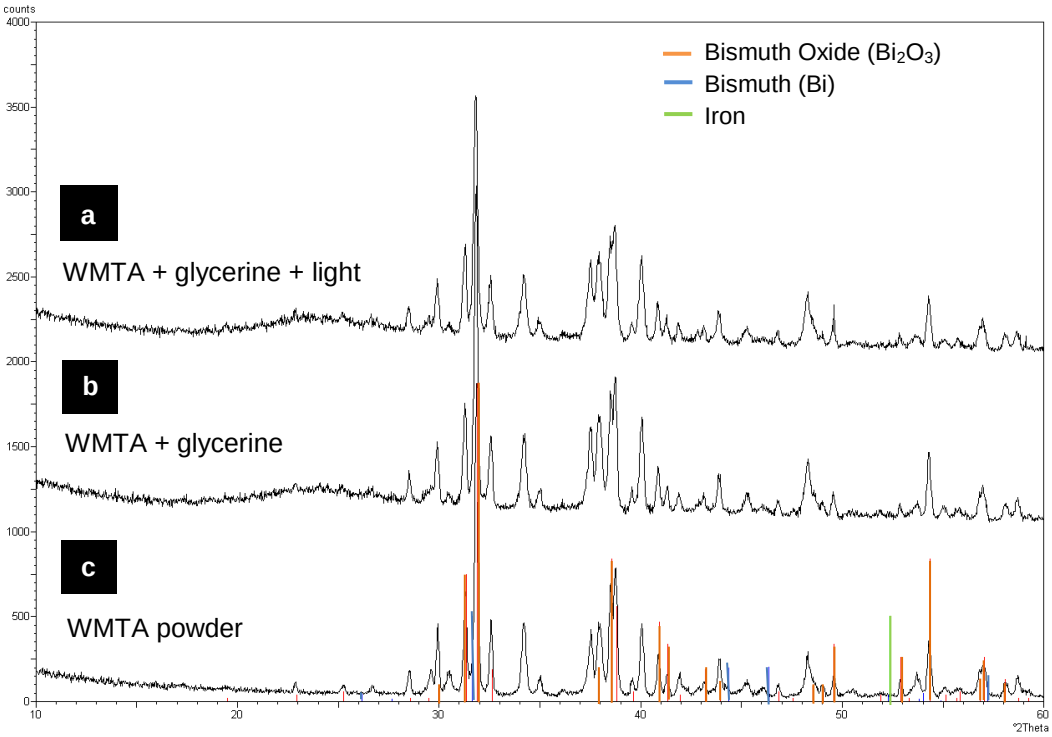

Fig. 17. X-ray diffraction patterns of WMTA in the set form with glycerine and lightirradiated **(a)**, only with glycerine **(b)** and powder form **(c)**.

It is possible to see in **Figure 18**, that the X-ray diffraction patterns of WMTA with glycerine and of WMTA with glycerine and light have an overlapped pattern, verifying that there is probably no chemical alteration.

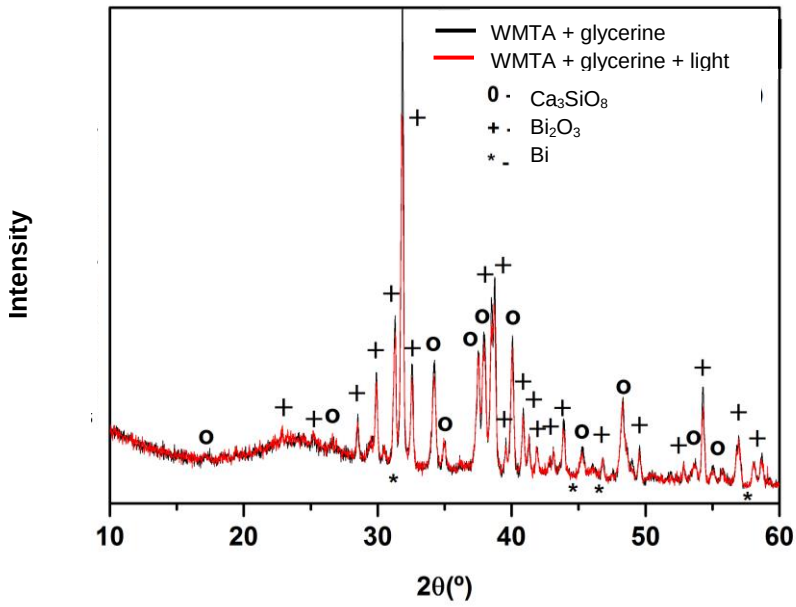

Fig. 18. X-ray diffraction patterns of WMTA in the set form with glycerine and light irradiated and only with glycerine overlapped

According to data relative to coordinate L* variation over time between groups, the null hypothesis should be rejected.

4. DISCUSSION

In recent years, several clinical colour measuring devices have become available[29]. These devices are efficient in quantifying and analysing the natural tooth colour[30, 31] and allow a more uniform and accurate communication between dental technicians and dentists[30]. Some of this technology can be used in colour research, as was done in the present study.

Overall and despite the short period of evaluation, this study showed that there is a tendency for WMTA colour upon contact with glycerine to darken in the first hours and then to fade away possibly due to intrinsic changes in the material. With regard to the alterations in L* coordinate the standard values vary, as indicated from 0 (black) to 100 (white). In this study a significant decrease was measured from the first evaluation period to the second, although until the third evaluation time-point a decrease in coordinate L* value was registered. While for groups 1 and 2 these variations in L* values were not statistically significant.

Mineral trioxide aggregate (MTA) was first described in the dental scientific literature in 1993[32] and was given approval for endodontic use by the U.S. Food and Drug Administration in 1998[33]. MTA was introduced in a grey form, but it had a big potential for discolouration, therefore one of the main reasons for introducing WMTA as a substitute, was to provide a hue match closer to the colour of teeth and thus better aesthetics, as opposed to the contrasting grey colour of GMTA.[34-36]

According to the information in the material safety datasheet, ProRoot® MTA (Dentsply Tulsa Dental, Johnson City, TN) consists of 75% Portland cement, 20% bismuth oxide (Bi_2O_3), and 5% calcium sulphate dehydrate.[37] Portland Cement (PC) differs from MTA by the absence of bismuth ions and the presence of potassium ions.[38] Scanning electron microscopy (SEM) and electron probe microanalysis characterized the differences between GMTA and WMTA and found that the major differences between them were in the concentrations of Al_2O_3, MgO, and FeO.[27, 34] WMTA was found to have 54,9% less Al_2O_3, 56,5% less MgO, and 90,8% less FeO, which leads to the conclusion that the FeO reduction is most likely the cause for the colour change.[27] WMTA was also reported to have an overall smaller particle size than GMTA[39][22] while it was also suggested that the reduction in magnesium could also contribute to the lighter colour of WMTA.[34, 40]

One of the main objectives of our study was to evaluate chromatic alterations of WMTA when submitted to different environmental conditions.

Several studies have reported on the discolouration potential of WMTA. Watts *et al*. (2007) in their experimental study analysed the compressive strength of WMTA

and GMTA (ProRoot ® MTA, Dentsply Tulsa Dental, Johnson City, TN) when mixed with sterile water or local anaesthetic and exposed to an acidic environment. They reported findings of note in their pilot studies and throughout the experiment, yet no scientific measurements were made. It was noted that all WMTA specimens, regardless of mixing agent, time and pH, were grey when removed from the molds. They stated that the top portion of the specimen directly exposed to phosphate buffered saline (PBS) solution remained light in colour. Once removed from the molds and re-placed into the PBS solution, the dark discolouration faded throughout the 28-day trial, though was seen on the internal portion of the specimens upon fracture.[41] The discolouration of WMTA (ProRoot ® MTA, Dentsply Tulsa Dental, Johnson City, TN) was also reported in an ex-vivo study were the main objective was to evaluate the removal efficiency of WMTA used as a root filling material. They reported that one of the findings regardless of the group was the dark discoloration of WMTA in most of the specimens, beyond the surface of the material. They suggested that as this discolouration could compromise the aesthetics of a tooth, further research wass necessary to determine the chemical process leading to colour alteration of this material.[42]

Vallés *et al*. (2013)[25] evaluated the colour stability of WMTA after irradiation with three different curing lights and with a fluorescent lamp in an oxygen-free environment, achieved by the use of a glycerine gel. They prepared thirty samples of WMTA and divided them into four experimental groups. To generate an oxygen-free environment they immersed the samples in pure glycerine for 15 minutes. In the cure light groups the samples were irradiated with a curing light for 20, 60 and 120 seconds, each of the groups with a different device. The fluorescent lamp group samples underwent the same protocol but were not irradiated with a curing light, instead they were left on a laboratory shelf at 1m below an 18W lamp. The negative control group was not immersed in pure glycerine and each sample was only irradiated with one of the curing lights. All samples were kept in the laboratory for 5 days. WMTA discolouration was considered according to light and to time exposure. They found that after 5 days, the samples showed some discolouration except for the negative control group, which remained stable over time. Their pilot study also tested the behaviour of WMTA samples when sealed in test tubes in two different environments, pure nitrogen and pure oxygen. They stated that nitrogen exposed samples darkened after light irradiation while oxygen exposed samples remained stable. Concerning their findings, they hypothesized that the presence or absence of oxygen plays an important part in the discolouration of WMTA.[25]

According to our experimental results samples immersed in pure glycerine and light irradiated suffered immediate darkening although at 48 hours after light exposure the samples were visibly lighter. Still it should be emphasized that the lightening of the samples was progressively noticed before reaching the second colour measuring period. In another of our experimental groups where oxygen was inhibited by an adhesive layer over the sample, dark discolouration wasn't verified after light irradiation. Comparing these two groups, we conclude that WMTA discolouration only occurs temporarily in the absence of oxygen potentiated with glycerine and with a blue light range, which also induces heat. This leads us to ponder whether the discolouration is triggered by the absence of oxygen or if the light application allows the occurrence of a chemical reaction. Furthermore we have also noticed that the WMTA samples placed in contact with the PVC blister where oxygen contact is avoided remained chromatically stable. In order to clarify the role of oxygen in the darkening of WMTA samples, a pilot study was conducted by placing a sample in a vacuum camera and irradiated with the same LED light used in the experiments, for 60 seconds while placed in the camera. No colour alterations were noticed. Therefore no objective correlation can be supported between the darkening of WMTA and the absence of oxygen.

Vallés *et al,*[16] explained the role of oxygen by the presence of compounds that absorb light, such as chromophores. These reach an excited state that can interact with molecular oxygen.[20] This interaction may progress in different ways; one of which is the transfer of energy from the excited chromophore to oxygen. Once the energy transfer ends, the chromophore recovers its initial properties whereas the oxygen dissipates the excess energy as heat. Thus, oxygen might act as a quencher that quickly deactivates the excited state of WMTA, consequently preventing a light-induced decomposition of WMTA that eventually could produce dark or grey by-products. They inferred that the irradiation of oxygen-free samples, coated with glycerine, might create an excited state of WMTA persisting longer, due to the absence of the quenching effect of oxygen, having enough time to decompose and yield dark by-products. It thus concluded that the presence of oxygen could prevent photochemically induced darkening, whereas the absence of oxygen would promote it.

It could be hypothesized that a chemical reaction with glycerine might be present. In our pilot study we substituted the application of glycerine by diesel and a vegetable oil (Fula®, Portugal). It was observed that when the samples where light irradiated for 60 seconds, they darkened, although less than 48 hours later the discolouration had faded. However, none of these materials and glycerine have clinical applicability.

The main problem of WMTA discolouration is in the clinical setting. It has been observed that tooth discolouration occurs, thus leading to the conclusion that perhaps the contact with blood could potentiate the darkening of teeth.

Bismuth oxide (Bi_2O_3) is a yellow substance commonly added to various endodontic materials as a radiopacifier, being part of MTA's composition and it has been suggested to be the main cause of dental discolouration.[43, 44] The reduced black crystals of bismuth atoms are responsible for the darkening of the sample and the presence of these crystals has been identified by X-ray.[45] Increasing the partial pressure of oxygen at a high temperature avoids the formation of metallic bismuth and the sample remains transparent. It is known that Bi_2O3 can be excited by visible and UV light. It has been reported that Bi_2O_3 undergoes a thermal dissociation under high temperature, which yields metallic bismuth and oxygen, the UV-visible diffuse reflectance spectrum for nanocrystallite Bi_2O_3 spans wavelenghts of 300-500 nm, with a maximum of 400 nm.[45] The irradiated Bi_2O_3 behaves in the same way as heated Bi_2O_3, it darkens when irradiated under an oxygen-free environment. Vallés *et al* based their hypothesis in these findings.[16, 25]

The identification of the major constituents present in a material is important as it will contribute to understand the material's physical, chemical and mechanical properties. The use of XRD allows the identification of the major constituents present in a material.[46] The key principle of this technique is based on identifying the diffraction pattern of each crystalline phase characterized by a unique set of peaks (known as Bragg's peaks), with a specific diffracted intensity (y-axis) and diffracted angle at a specific position. Phase identification is accomplished by comparing the data of the tested specimens by using peaks and relative intensities with a very large set of "standard" data provided by the ICDD.[47, 48]

Mineral trioxide aggregate (MTA) contains largely crystalline phases, with the calcium silicate hydrate being the only amorphous phase.[49]

In our XRD analysis the two samples of set WMTA exhibited strong peaks of bismuth oxide. A slight change of intensity on the bismuth oxide phase in the sample light irradiated with WMTA and glycerine was seen, which can be due to the decomposition of bismuth oxide into bismuth, though if so, it would be present in a residual amount.

Despite the standardized experimental set-up, the present model has limitations. The role of oxygen is still unclear, although through our experimental study it was possible to eliminate the role of oxygen as the enhancer of WMTA discolouration. More experimental groups, different measuring methods, chemical and

physical investigation should be used and done in order to clarify the real process of WMTA discolouration.

5. CONCLUSIONS

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Within the limitations of this *in vitro* study it can be concluded that:

- There was no consistency between the two colour measuring methods
- Regarding colour coordinate L* variation, it was possible to confirm that for WMTA plus glycerine and light irradiation there was a clear sample darkening in terms of value throughout time, for both measuring methods.
- Concerning global colour variation (ΔE), the photographic digital analysis method identified significant differences between groups. Group 3 showed differences when compared with the negative control group and the WMTA with the application of light group.
- X-ray diffraction analysis showed that samples of WMTA and WMTA plus glycerine and light irradiation spectra could be overlapped. The presence of bismuth was not identified.
- During the time course of the experience colour variation was noticed. The samples of WMTA with glycerine and light irradiation initially darkened, but discolouration faded away over time.

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