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GERALDO ELIAS MIRANDA

ANALYSIS OF THE FLUORESCENCE OF BODY FLUIDS ON DIFFERENT SURFACES BASED ON THE AGE OF THE SAMPLE

ANÁLISE DA FLUORESCÊNCIA DE FLUIDOS CORPORAIS EM DIFERENTES SUPERFÍCIES DE ACORDO COM A IDADE DA AMOSTRA

PIRACICABA
2014

UNIVERSIDADE ESTADUAL DE CAMPINAS

FACULDADE DE ODONTOLOGIA DE PIRACICABA

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Dissertação apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Mestre em Biologia Buco-Dental, na área de Odontologia Legal e Deontologia.

Dissertation presented to the Piracicaba Dentistry School of the University of Campinas in partial fulfillment of the requirements for the degree of Master in Buco-Dental Biology, in Forensic Dentistry area.

Orientador: Prof. Dr. Eduardo Daruge Junior.

Este exemplar corresponde à versão final da dissertação defendida por Geraldo Elias Miranda e orientada pelo Prof. Dr. Eduardo Daruge Junior.

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Prof. Dr. EDUARDO DARUGE JUNIOR

Prof. Dr. RICARDO HENRIQUE ALVES DA SILVA

Prof. Dr. LUIZ FRANCESQUINI JÚNIOR

ABSTRACT

The use of screening techniques, such as an alternative light source (ALS), is important for finding biological evidence at a crime scene. The objective of this study was to evaluate whether biological fluid (blood, semen, saliva, and urine) deposited on different surfaces changes as a function of the age of the sample. Stains were illuminated with a Megamaxx™ ALS System and photographed with a Canon™ camera. Adobe Photoshop™ was utilized to prepare photographs for analysis, and then ImageJ™ was used to record the brightness values of pixels in the images. Data were submitted to analysis of variance using a generalized linear mixed model with two fixed effects (surface and fluid). Time was treated as a random effect (through repeated measures) with a first-order autoregressive covariance structure. Means of significant effects were compared by the Tukey test. In all tests, a 5% level of significance was established. The fluorescence of the analyzed biological material varied depending on the age of the sample. Fluorescence was lower when the samples were moist. Fluorescence remained constant when the sample was dry, up to the maximum period analyzed (60 days), independent of the substrate on which the fluid was deposited. Therefore, the forensic expert can detect biological fluids at the crime scene using an ALS even several days after a crime has occurred.

Keywords: Forensic Sciences. Fluorescence. Semen. Blood. Urine. Saliva.

RESUMO

A utilização de técnicas de triagem como a *alternate light source* (ALS) é importante para encontrar evidências biológicas em uma cena de crime. O objetivo deste trabalho foi avaliar se a fluorescência do fluido biológico (sangue, sêmen, saliva e urina) depositado em diferentes superfícies sofre variação em função da idade da amostra. A mancha foi iluminada com uma ALS da marca Megamaxx™ System e fotografada com o auxílio do Canon EOS Utility™. A análise das imagens foi feita por meio de uma combinação dos programas Adobe Photoshop™ e ImageJ™. O Adobe Photoshop™ foi utilizado para preparar as fotografias para as análises e o ImageJ™ para registrar o valor do brilho do pixel da imagem. Os dados obtidos foram submetidos na técnica de análise de variância por meio do ajuste de um modelo linear generalizado misto com dois fatores fixos e um terceiro fator, o tempo, analisado como medidas repetidas no formato de efeito aleatório com matriz de covariância do tipo autorregressivo de primeira ordem. Efeitos significativos tiveram suas médias comparadas duas a duas por meio do teste de Tukey. Pode-se concluir que a fluorescência dos fluidos biológicos analisados variaram em função do tempo em que foram expostos. A fluorescência foi menor quando as amostras estavam úmidas e permaneceram constantes quando estavam secas até o tempo máximo analisado (60 dias), independentemente do substrato em que o fluido foi depositado. Portanto, o perito forense pode detectar fluidos biológicos no local do crime usando uma ALS mesmo após vários dias da ocorrência do crime.

Palavras-chave: Ciências Forenses. Fluorescência. Sêmen. Sangue. Urina. Saliva

SUMÁRIO

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Dedico este trabalho a minha mãe **Nilce Rosa Miranda** e ao meu saudoso pai **Paulo Miranda** que me ensinaram os valores da vida.

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"A imaginação é mais importante que a ciência, porque a ciência é limitada, ao passo que a imaginação abrange o mundo inteiro. "

Albert Einstein

INTRODUÇÃO

Amostras biológicas como sangue, sêmen, saliva e urina são importantes evidências encontradas em uma cena de crime. Vários métodos têm sido desenvolvidos para identificar esses fluidos. Um dos mais simples testes presuntivos usados para determinar a maioria das evidências biológicas é a fonte de luz alternativa (ALS) (Virkler e Lednev, 2009; Lee e Koo, 2010).

A ALS é um método efetivo, não-invasivo, não-destrutivo, usado para detectar fluidos biológicos (Lennard e Stoilovi, 2004; Vandenberg e Oorschot, 2006), ferimentos (contusão, equimose, marca de mordida) (Snell e Soltys, 2005; Viner et al., 2014) restos humanos (Gallant, 2013) e uma variedade de outros tipos de evidências. Por isso é recomendado escaniar os locais com essas luzes antes da aplicação de outros reagentes (Seashols, 2013; Vandenberg e Oorschot, 2006).

O princípio atrás da tecnologia ALS é devido às qualidades absortivas e fotoluminescentes do item sob exame (Viner et al., 2014). A fluorescência é definida como a propriedade de absorver luz de um menor comprimento de onda e emitir a luz em um comprimento de onda maior (Lee e Koo, 2010).

Alguns autores (Webb et al., 2006; Lee e Koo, 2010; Gallant, 2013) sugerem que novas pesquisas devem ser realizadas para o aperfeiçoamento da técnica. A maioria dos estudos tem avaliado as manchas somente em tecidos, entretanto, é importante analisar a influência do tempo na detecção da mancha (Snell e Soltys, 2005)

Ainda não foi avaliado o uso das luzes da marca *Megamaxx™,* nem a influência do tempo na detecção de manchas em diferentes superfícies o qual este trabalho se propõe. Portanto essa pesquisa pode contribuir para o aperfeiçoar a técnica e auxiliar o perito forense na coleta de evidências em um local de crime. O objetivo deste trabalho foi avaliar se a fluorescência do fluido biológico depositado em diferentes superfícies varia de acordo com a idade da amostra, quando iluminado com uma ALS.

CAPÍTULO 1: Analysis of the fluorescence of body fluids on different surfaces based on the age of the sample

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ANALYSIS OF THE FLUORESCENCE OF BODY FLUIDS ON DIFFERENT SURFACES BASED ON THE AGE OF THE SAMPLE

ABSTRACT

The use of screening techniques, such as an alternative light source (ALS), is important for finding biological evidence at a crime scene. This objective of this study was to evaluate whether biological fluid (blood, semen, saliva, and urine) deposited on different surfaces changes as a function of the age of the sample. Stains were illuminated with a Megamaxx™ ALS System and photographed with a Canon camera. Adobe Photoshop™ was utilized to prepare photographs for analysis, and then ImageJ™ was used to record the brightness values of pixels in the images. Data were submitted to analysis of variance using a generalized linear mixed model with two fixed effects (surface and fluid). Time was treated as a random effect (through repeated measures) with a first-order autoregressive covariance structure. Means of significant effects were compared by the Tukey test. In all tests, a 5% level of significance was established. The fluorescence of the analyzed biological material varied depending on the age of the sample. Fluorescence was lower when the samples were moist. Fluorescence remained constant when the sample was dry, up to the maximum period analyzed (60 days), independent of the substrate on which the fluid was deposited.

Therefore, the forensic expert can detect biological fluids at the crime scene using an ALS even several days after a crime has occurred.

Keywords: Forensic Sciences, Fluorescence, Semen, Blood, Urine, Saliva

INTRODUCTION

Biological samples, such as blood, semen, saliva, and urine, are important pieces of evidence that can be found at a crime scene. Forensics has developed various ways to identify these fluids. One of the simplest tests that is used to detect most biological evidence is the alternative light source (ALS) [1,2].

The ALS is a non-invasive, non-destructive method that is used to detect biological fluids [3,4], wounds (contusions, ecchymosis, bite marks) [5,6], human remains [7], and a range of other types of evidence. An area should be scanned with an ALS before the application of other reagents [8,4].

The principle behind ALS technology is based on the absorptive and photoluminescent qualities of the item under examination [6]. Fluorescence is the property of absorbing light of a lower wavelength and emitting light of a greater wavelength [2].

Some authors [2,7,9] have suggested that new research should be performed to perfect the ALS technique. However, most studies have evaluated stains only on fabric, and it is important to analyze the influence of time on detecting the stain [5].

There has been no analysis of the use of *Megamaxx*™ brand lights or the influence of time on the detection of stains on different surfaces. Therefore, these factors are the subject of this study. This research can help improve the ALS technique and aid the forensic expert in collecting evidence at the crime scene. The objective of this study was to evaluate whether the fluorescence of a biological fluid deposited on different surfaces changes according to the age of the sample, when illuminated with an ALS.

MATERIALS AND METHODS

Body fluids (blood, semen, saliva, and urine) for the experiments were obtained from a volunteer donor. Samples were utilized shortly after their collection, without using preservatives, except for the intravenous blood that was collected in a tube containing ethylenediamine tetraacetic acid (EDTA). The EDTA was employed to avoid coagulation and does not interfere with blood detection [8]. The Research Ethics Committee of the Dental Faculty of Piracicaba (FOP/UNICAMP) approved the study under case no. 051/2012.

Body fluids were deposited on the following porous and nonporous surfaces: wood, black cotton fabric, white cotton fabric, paper, and white tile. The surface-fluid unit was exposed to an ALS (Megamaxx™ System; Sirchie, Youngsville, NC, USA) at 1 minute, 1 hour, 24 hours, 10 days, 35 days, and 60 days after the fluid was deposited on the surface. These exposure times can be considered as the storage time/age of the stains, which were stored at room temperature.

Stains were illuminated with the ALS at a wavelength of 455 nm (as suggested by the manufacturer) and viewed with orange glasses. The ALS equipment was mounted on a tripod, to maintain a fixed distance between the ALS and the analyzed stain. A diffusor attached to a lamp was used to make the light softer.

Photographs were obtained with a Canon EOS 60D digital camera, using a Canon EF-S 60mm f/2.8 Macro USM lens (Canon Inc., Tokyo, Japan) and an orange-colored lens filter (Tiffen Company, NY, USA). The camera was mounted on a tripod to avoid movement. The camera was controlled via a computer with the Canon EOS Utility™ software (Canon Inc.). The camera's ultraviolet (UV) filter was removed, to guarantee that the camera's sensor would pick up light near the UV range, as practiced by Lee [10]. Photographs were obtained in a completely darkened room.

Photoshop™ (Adobe Systems, San Jose, CA, USA) was utilized to prepare the photographs for analysis, and then ImageJ™ (National Institutes of Health, Bethesda, MD, USA) was employed to record the brightness values of pixels in the images. First, Photoshop™ was used to remove regions of interest (ROIs) measuring 100 × 100 pixels

from the images. These ROIs contained the stain and background (Figure 1). These ROIs were transformed into 8-bit/channel "grayscale" images, so that the color information could be discarded and consistency maintained during the analyses [11]. The new images were saved in .TIFF format. The ROIs were obtained in the same position in all of the tests. The surface on which the fluid was deposited (background) served as the control for the experiment. Next, the ImageJ™ software was used to obtain the average brightness value for the ROI, with the following command: "Analysis" > "Histogram". For each pixel, a numerical value was assigned, ranging from 0 (completely black pixel) to 255 (completely white pixel), which represented its brightness on the grayscale [11].

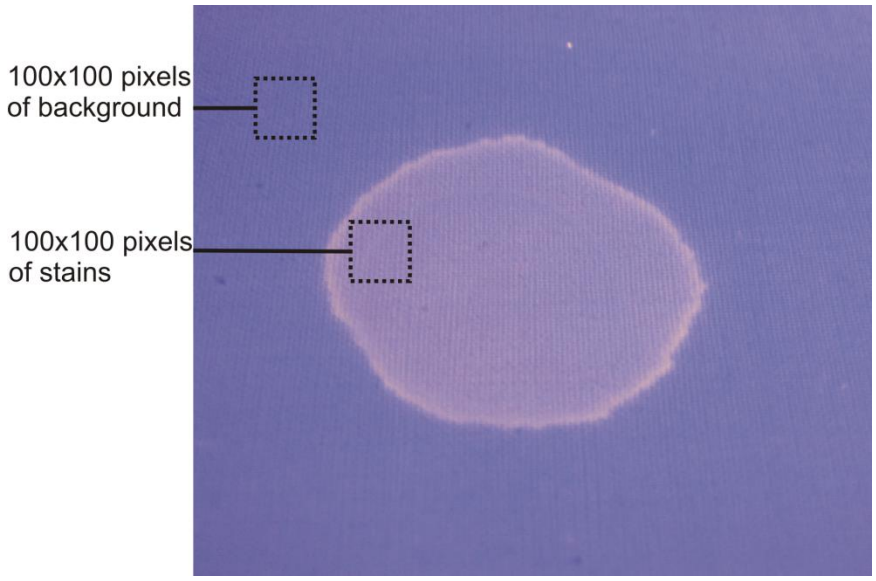


Fig. 1. Regions of interest (ROIs) selected for analysis.

Data were submitted to analysis of variance (ANOVA), using a generalized linear mixed model with two fixed effects (surface and fluid). Time was treated as a random effect (through repeated measures) with a first-order autoregressive covariance structure. The assumption that the error adhered to a Gaussian distribution was analyzed through the asymmetry and kurtosis coefficients and the Shapiro-Wilk test. Means of significant effects were compared by the Tukey test. In all tests, a 5% level of significance was used. Statistical values were calculated by the SAS System (SAS Institute Inc., Cary, NC, USA).

RESULTS

Table 1 shows the results from ANOVA comparing the mean brightness values for different fluids, surfaces, and time periods.

Table 1. ANOVA results for effects studied under an ALS, with the appropriate model for the experiments, using two fixed factors and time (sample age) defined through repeated measurements.

Effect	Degrees of freedom		Analysis of variance	
	Numerator	Denominator	F Statistic	p-value
Surface	4	50	80257.5	0.0001
Fluid	4	50	61791.2	0.0001
Age	5	250	7537.6	0.0001
Fluid*surface	16	50	6836.0	0.0001
Surface*age	20	250	167.49	0.0001
Fluid*age	20	250	995.65	0.0001
Fluid*surface*age	80	250	158.57	0.0001

There were significant differences in the brightness values between at least two levels for all of the effects tested (Table 1). Tables were made for each fluid, to compare each of the surfaces (Tables 2, 3, 4, 5, 6). Figures 2–5 show the stains illuminated with natural light and with an ALS. Means were arranged according to the age of the sample. Letters were assigned to represent differences between the means from Tukey's test, ranging from the highest (letter A) to the lowest average (letter C). There was no significant difference between means with the same letter at that particular time. As expected, differences were not found between the mean brightness values at different times in the control group for each surface (Table 2).

Table 2. Mean brightness values with respect to the "sample age" factor in the control group for each surface.

Surface	Sample age	Mean	SD	Confidence interval (95%)		Tukey group (α = 0.05)
				Upper	Lower	
Tile	1 minute	50.00	0.00	–	–	A
	1 hour	50.00	0.00	–	–	A
	24 hours	50.00	0.00	–	–	A
	10 days	50.33	0.58	51.77	48.90	A
	35 days	50.00	0.00	–	–	A
	60 days	50.33	0.58	51.77	48.90	A
Wood	1 minute	45.00	0.00	–	–	A
	1 hour	44.67	0.58	46.10	43.23	A
	24 hours	45.00	0.00	–	–	A
	10 days	44.67	0.58	46.10	43.23	A
	35 days	44.67	0.58	46.10	43.23	A
	60 days	45.00	0.00	–	–	A
Paper	1 minute	55.00	0.00	–	–	A
	1 hour	55.00	0.00	–	–	A
	24 hours	55.33	0.58	56.77	53.90	A
	10 days	55.00	0.00	–	–	A
	35 days	54.67	0.58	56.10	53.23	A
	60 days	55.00	0.00	–	–	A
White fabric	1 minute	60.00	0.00	–	–	A
	1 hour	59.67	0.58	61.10	58.23	A
	24 hours	60.00	0.00	–	–	A
	10 days	59.67	0.58	61.10	58.23	A
	35 days	60.00	0.00	–	–	A
	60 days	60.00	0.00	–	–	A
Black fabric	1 minute	18.67	0.58	20.10	17.23	A
	1 hour	18.67	0.58	20.10	17.23	A
	24 hours	18.67	0.58	20.10	17.23	A
	10 days	19.00	0.00	–	–	A
	35 days	19.00	0.00	–	–	A
	60 days	18.67	0.58	20.10	17.23	A

Table 3. Mean brightness values with respect to the "sample age" factor for different surfaces treated with semen.

Surface	Sample age	Mean	SD	Confidence interval (95%)		Tukey group (α = 0.05)
				Upper	Lower	
Tile	1 minute	50.00	0.00	–	–	C
	1 hour	70.33	0.58	71.77	68.90	B
	24 hours	101.00	0.00	–	–	A
	10 days	101.00	0.00	–	–	A
	35 days	101.67	1.15	104.54	98.80	A
	60 days	101.33	0.58	102.77	99.90	A
Wood	1 minute	45.00	0.00	–	–	C
	1 hour	55.00	0.00	–	–	B
	24 hours	69.00	0.00	–	–	A
	10 days	69.33	0.58	70.77	67.90	A
	35 days	69.33	0.58	70.77	67.90	A
	60 days	69.00	0.00	–	–	A
Paper	1 minute	55.33	0.58	56.77	53.90	C
	1 hour	69.00	0.00	–	–	B
	24 hours	78.67	0.58	80.10	77.23	A
	10 days	78.00	1.00	80.48	75.52	A
	35 days	77.67	0.58	79.10	76.23	A
	60 days	77.67	0.58	79.10	76.23	A
White fabric	1 minute	62.33	0.58	63.77	60.90	C
	1 hour	70.00	0.00	–	–	B
	24 hours	80.00	0.00	–	–	A
	10 days	80.33	0.58	81.77	78.90	A
	35 days	80.33	0.58	81.77	78.90	A
	60 days	80.00	0.00	–	–	A
Black fabric	1 minute	11.33	0.58	12.77	9.90	C
	1 hour	14.67	0.58	16.10	13.23	B
	24 hours	20.00	0.00	–	–	A
	10 days	20.67	1.15	23.54	17.80	A
	35 days	20.33	1.15	23.20	17.46	A
	60 days	20.33	0.58	21.77	18.90	A

Table 4. Mean brightness values with respect to the "sample age" factor for different surfaces treated with blood.

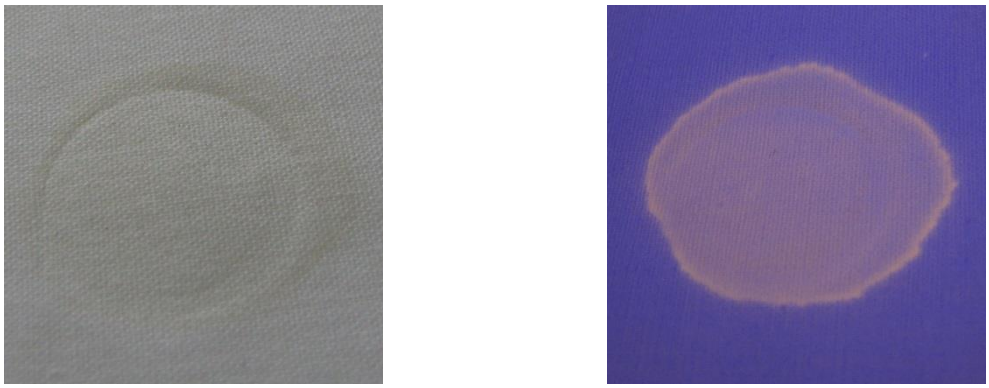
Surface	Sample age	Mean	SD	Confidence interval (95%)		Tukey group (α = 0.05)
				Upper	Lower	
Tile	1 minute	20.00	0.00	–	–	C
	1 hour	22.33	0.58	23.77	20.90	B
	24 hours	27.00	0.00	–	–	A
	10 days	27.33	0.58	28.77	25.90	A
	35 days	27.00	0.00	–	–	A
	60 days	27.33	0.58	28.77	25.90	A
Wood	1 minute	18.33	0.58	19.77	16.90	C
	1 hour	22.00	0.00	–	–	B
	24 hours	29.67	0.58	31.10	28.23	A
	10 days	29.33	0.58	30.77	27.90	A
	35 days	30.33	0.58	31.77	28.90	A
	60 days	30.00	0.00	–	–	A
Paper	1 minute	20.00	0.00	–	–	C
	1 hour	24.00	0.00	–	–	B
	24 hours	30.00	0.00	–	–	A
	10 days	30.33	0.58	31.77	28.90	A
	35 days	30.00	0.00	–	–	A
	60 days	30.00	0.00	–	–	A
White fabric	1 minute	20.33	0.58	21.77	18.90	C
	1 hour	24.00	0.00	–	–	B
	24 hours	27.33	0.58	28.77	25.90	A
	10 days	26.67	0.58	28.10	25.23	A
	35 days	26.33	1.15	29.20	23.46	A
	60 days	26.67	0.58	28.10	25.23	A
Black fabric	1 minute	11.67	1.15	14.54	8.80	C
	1 hour	14.67	0.58	16.10	13.23	B
	24 hours	29.33	0.58	30.77	27.90	A
	10 days	29.33	0.58	30.77	27.90	A
	35 days	29.33	0.58	30.77	27.90	A
	60 days	29.00	0.00	–	–	A

Table 5. Mean brightness values with respect to the "sample age" factor for different surfaces treated with urine.

Surface	Sample age	Mean	SD	Confidence interval (95%)		Tukey group (α = 0.05)
				Upper	Lower	
Tile	1 minute	50.00	0.00	–	–	C
	1 hour	63.00	0.00	–	–	B
	24 hours	85.00	0.00	–	–	A
	10 days	84.67	0.58	86.10	83.23	A
	35 days	85.00	0.00	–	–	A
	60 days	84.67	0.58	86.10	83.23	A
Wood	1 minute	45.00	0.00	–	–	C
	1 hour	50.00	0.00	–	–	B
	24 hours	65.00	0.00	–	–	A
	10 days	64.67	0.58	66.10	63.23	A
	35 days	65.00	0.00	–	–	A
	60 days	65.33	0.58	66.77	63.90	A
Paper	1 minute	55.67	0.58	57.10	54.23	C
	1 hour	65.00	0.00	–	–	B
	24 hours	80.00	0.00	–	–	A
	10 days	80.00	0.00	–	–	A
	35 days	80.33	0.58	81.77	78.90	A
	60 days	80.00	0.00	–	–	A
White fabric	1 minute	60.33	0.58	61.77	58.90	C
	1 hour	65.00	0.00	–	–	B
	24 hours	74.33	1.15	77.20	71.46	A
	10 days	75.00	0.00	–	–	A
	35 days	74.67	0.58	76.10	73.23	A
	60 days	75.00	0.00	–	–	A
Black fabric	1 minute	11.33	0.58	12.77	9.90	C
	1 hour	13.67	0.58	15.10	12.23	B
	24 hours	18.33	0.58	19.77	16.90	A
	10 days	18.67	0.58	20.10	17.23	A
	35 days	18.67	0.58	20.10	17.23	A
	60 days	18.67	0.58	20.10	17.23	A

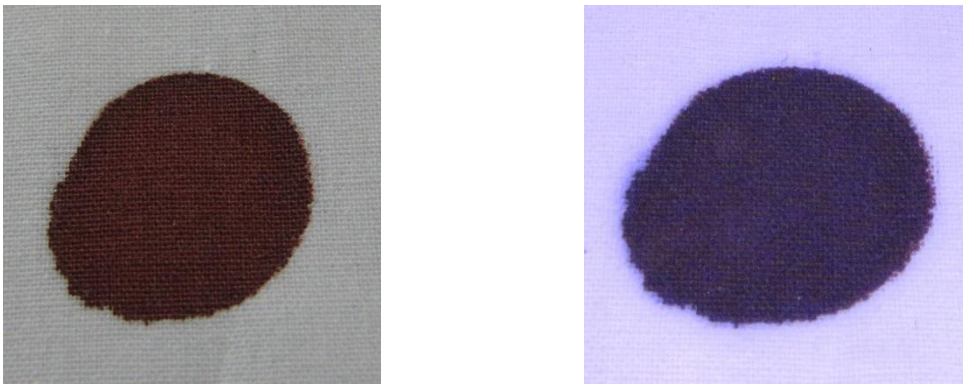
Table 6. Mean brightness values with respect to the "sample age" factor for different surfaces treated with saliva.

Surface	Sample age	Mean	SD	Confidence interval (95%)		Tukey group (α = 0.05)
				Upper	Lower	
Tile	1 minute	50.00	0.00	–	–	A
	1 hour	50.67	0.58	52.10	49.23	A
	24 hours	53.00	0.00	–	–	A
	10 days	53.00	0.00	–	–	A
	35 days	53.33	0.58	54.77	51.90	A
	60 days	53.33	0.58	54.77	51.90	A
Wood	1 minute	40.33	0.58	41.77	38.90	B
	1 hour	42.00	0.00	–	–	AB
	24 hours	45.00	0.00	–	–	A
	10 days	44.67	0.58	46.10	43.23	A
	35 days	45.00	0.00	–	–	A
	60 days	45.00	0.00	–	–	A
Paper	1 minute	55.00	0.00	–	–	A
	1 hour	55.67	0.58	57.10	54.23	A
	24 hours	57.67	0.58	59.10	56.23	A
	10 days	57.67	1.53	61.46	53.87	A
	35 days	57.67	0.58	59.10	56.23	A
	60 days	58.00	0.00	–	–	A
White fabric	1 minute	60.33	0.58	61.77	58.90	B
	1 hour	62.00	0.00	–	–	B
	24 hours	68.00	0.00	–	–	A
	10 days	67.33	1.15	70.20	64.46	A
	35 days	67.33	1.15	70.20	64.46	A
	60 days	68.00	0.00	–	–	A
Black fabric	1 minute	11.33	0.58	12.77	9.90	C
	1 hour	18.00	0.00	–	–	B
	24 hours	25.00	1.00	27.48	22.52	A
	10 days	24.00	1.73	28.30	19.70	A
	35 days	23.33	0.58	24.77	21.90	A
	60 days	25.33	0.58	26.77	23.90	A



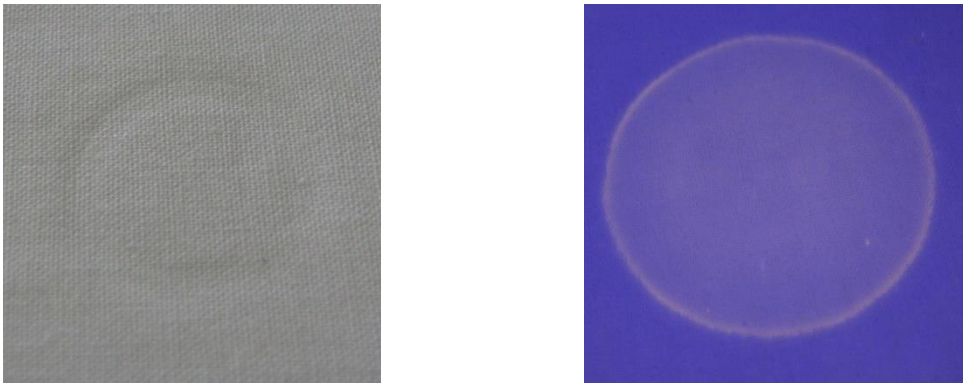
A B

Fig. 2. (A) Semen exposed to natural light. (B) Semen exposed to the ALS.



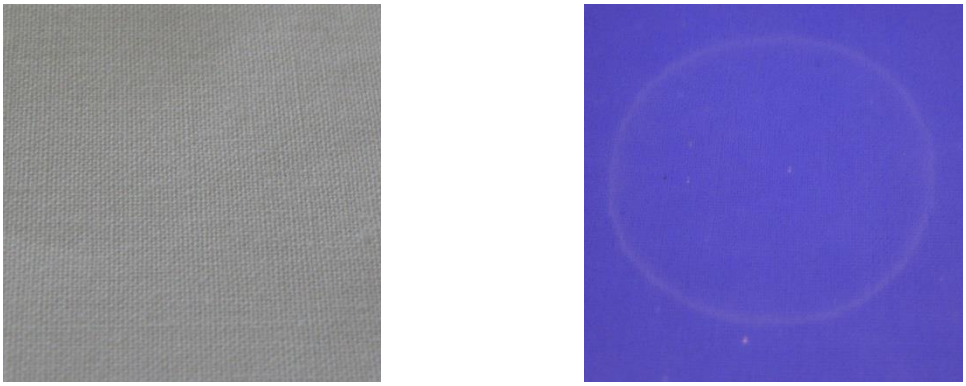
A B

Fig. 3. (A) Blood exposed to natural light. (B) Blood exposed to the ALS.



A B

Fig. 4. (A) Urine exposed to natural light. (B) Urine exposed to the ALS.



A B

Fig. 5. (A) Saliva exposed to natural light. (B) Saliva exposed to the ALS.

DISCUSSION

Comparisons of the mean brightness values of semen (Table 3), blood (Table 4), and urine (Table 5) revealed that on all surfaces, the average brightness was significantly lower at 1 minute than at all other times, followed by the average brightness at 1 hour. From 24 hours to 60 days, there was no statistical difference in fluorescence (constant brightness). Similar results were found in a study showing that moisture had a degrading effect on the ability to see a stain by ALS compared to a stain stored at room temperature for a significantly longer period [4]. This result suggests that the forensic expert can return to a crime scene to obtain dry stains days later, if they cannot be seen easily on the first exam because of moisture.

The same behavior occurred with saliva (Table 6), except for the substrates of tile and paper, which did not show significant differences between the different periods. This result probably stems from the fact that saliva is more difficult to detect because of the lack of solid particles [12]. Saliva exhibits low-intensity fluorescence compared to semen, and the fluorescence stays concentrated at the edges of the stain (Fig. 5) [4,13]. Another explanation for this difference with saliva was that the area collected for analysis was adjacent to the edge (Fig. 1), which, as the authors cited above have shown, is less fluorescent. For this reason, the result did not show a significant difference between times for the tile and paper substrates. New studies could explore this aspect in detail, by

measuring the fluorescence at the edges of saliva stains. Despite the low fluorescence, this technique is still useful with saliva because any improvement in the capacity to find an area in which to collect DNA is an advantage, as saliva stains cannot be seen with the naked eye.

The results of this study are similar to those found by other authors [14-15], who did not observe a difference in saliva and semen samples when exposed for 3 to 5 weeks, nor in the type of fabric used [14]. Semen continues to fluoresce with the same intensity months after it is placed on fabric [15]. However, these previous authors used only semen and saliva deposited on fabric. Another study that used blood samples on cotton cloth showed that after 7 weeks, an ALS permitted detection of the blood, revealing no discernable effect on seeing the stain during this period [9].

One factor to consider is the nature of the substrate on which the stain is found (e.g., absorbent or non-absorbent). Absorbent materials, such as wood, fabric, carpeting, and walls, have grooves or slits. Compared to non-absorbent materials, absorbent surfaces are easier to analyze because they retain large quantities of fluid in a relatively non-degraded state. With non-absorbent surfaces, such as tiling, metal, and glass, it is more difficult to retain the stain and avoid its degradation [16].

The results showed that whether the substrate was absorbent or not did not influence the drying time for the different fluids; all of the fluids showed the same trend on all surfaces. The average brightness of the same fluid differed when it was deposited on different surfaces (Tables 3, 4, 5 and 6), indicating that the type of substrate and its color affected detection of the stain. Each substrate had its own brightness, as indicated by the results of the control group (Table 2). Nevertheless, these data were not the focus of this study as various authors have already demonstrated that the type of material, especially its color and absorptiveness, influences the detection of stains [4,8,14,17]. Some materials are dark, highly absorbent, or exhibit fluorescence [17].

The fluorescence of biological materials (semen, blood, urine, and saliva) varied depending on the age of the sample. Fluorescence was lower when the samples were moist. Fluorescence remained constant when the samples were dry, up to the maximum time analyzed (60 days), independent of the substrate (fabric, paper, wood, or tiling) on which

the fluid was deposited. Thus, the forensic expert can detect biological fluids at a crime scene by using an ALS even several days after a crime. New studies applying additional variables are needed to improve the technique.

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CAPÍTULO 2: Use of the alternate light source to detect tooth and bone

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Esta dissertação está baseada na Informação CCPG UNICAMP-002/2013 que regulamenta o formato alternativo para dissertações de Mestrado e teses de Doutorado e permite a inserção de artigos científicos de autoria ou coautoria do candidato.

ABSTRACT: The alternate light source (ALS) is a simple method for biological detection in forensics. The aim of this study was to identify the combination of wavelength and filter that best detects tooth and bone, and to determine which biological materials (enamel, dentin and bone) have highest fluorescence intensity when exposed to ALS. Tooth and bone samples were illuminated with ALS and photographed. Image analysis was done using Adobe Photoshop™ and ImageJ™ software. Data were subjected to analysis of variance. Significant effects were identified by the Tukey test. In all tests, a 5% level of significance was established. It was concluded that the best combination for detecting tooth and bone is an illumination wavelength of 455nm with an orange filter. The fluorescence of dentin is greater than that of enamel, which in turn is greater than that of bone. This knowledge can help in forensic screening to detect fragments of these biological materials.

KEYWORDS: forensic science, fluorescence, tooth, bone, alternate light source, crime scene investigation

ALS is a simple method used to locate most of the biological evidence at a crime scene (1,2). ALS is effective, non-invasive, non-destructive, and can detect biological fluids (3), human remains (4), teeth and bones (5) and various other types of evidence. To

positively identify a victim in many cases, it is essential to accurately locate and recover fragments of bones and teeth. Therefore, the use of an ALS should be considered when searching for human bones, teeth, and other remains (5).

The principle behind ALS technology is based on the absorptive and fluorescent properties of the item under examination (6). Fluorescent materials absorb light at low wavelengths and emit light at wavelengths greater than those absorbed (2). Not all substances show fluorescence, and furthermore, each fluorescent material requires excitation by a specific range of wavelengths. Light whose wavelength is outside this range does not contribute to the excitation of the sample. Therefore, to detect a specific material, it is important to know the best combination of wavelength and filter.

By contributing to the development of technologies for biological detection, the results of this study may assist forensic experts in identifying evidence both at crime scenes and in the lab. This is important because incomplete analysis may result in the loss of crucial evidence such as teeth and small bones. Moreover, some authors (2,4) have suggested that new research should be performed to perfect the ALS technique. The objective of this study was to identify the combination of wavelength and filter that best detects tooth and bone, and to verify which biological material (enamel, dentin and bone) has greater fluorescence when exposed to ALS.

MATERIALS AND METHODS

The experiments were performed using samples of tooth and bone (biological material) that were mixed with styrofoam balls (inert material). The Research Ethics Committee of the Piracicaba Dental School (FOP/UNICAMP) approved the study under case no. 051/2012.

The samples were then exposed to a Megamaxx™ Alternative Light Source (ALS) System (Sirchie, Youngsville NC, USA) that comprises light sources operated at individual wavelengths of 455, 470, 505, 530, 590, and 625 nm. The ALS equipment was mounted on a tripod, to maintain a fixed distance between the ALS and the analyzed sample. A diffusor attached to a lamp was used to make the light softer and more evenly distributed.

Photographs were obtained with a Canon EOS 60D digital camera, using a Canon EF-S 60mm f/2.8 Macro USM lens (Canon Inc., Tokyo, Japan), and yellow, orange or red filters (Tiffen Company, NY, USA). The camera was mounted on a tripod to avoid movement. The camera was controlled via a computer with the Canon EOS Utility™ software (Canon Inc.). The camera's ultraviolet (UV) filter was removed, to guarantee that the camera's sensor would pick up light near the UV range, as practiced by Lee (7). Photographs were obtained in a completely darkened room.

Photoshop™ (Adobe Systems, San Jose, CA, USA) was utilized to prepare the photographs for analysis, and then ImageJ™ (National Institutes of Health, Bethesda, MD, USA) was employed to record the brightness values of pixels in the images. First, Photoshop™ was used to select regions of interest (ROIs), measuring 100 × 100 pixels, from images of enamel, dentin and bones (Figures 1 and 2). These ROIs were transformed into 8-bit/channel "grayscale" images, so that the color information could be discarded, to maintain consistency during the analyses (11). The new images were saved in .TIFF format. The ROIs were obtained in the same position in all of the tests.

FIG. 01 – ROIs exposed to natural light.

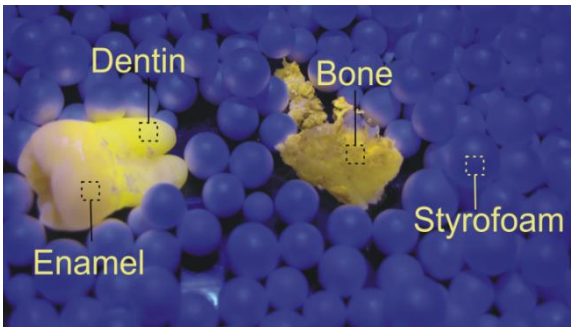


FIG. 02 – ROIs exposed to the ALS.

Next, ImageJ™ software was used to obtain the average brightness value within the ROI. Each pixel was assigned a numerical value which represented its brightness on the grayscale (8), ranging from 0 (completely black pixel) to 255 (completely white pixel).

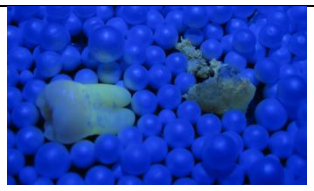
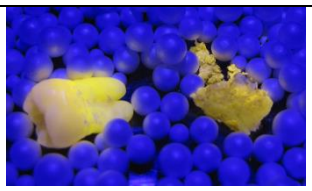
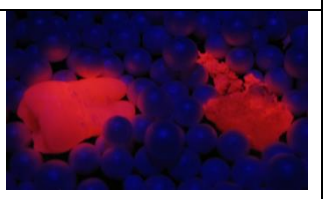
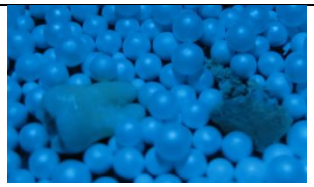
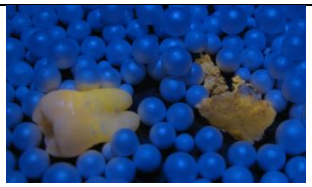
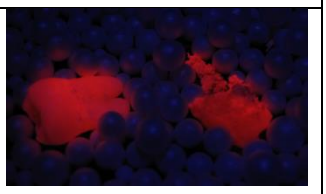
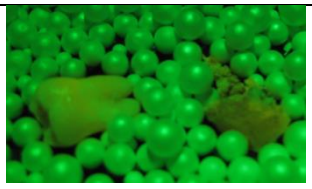
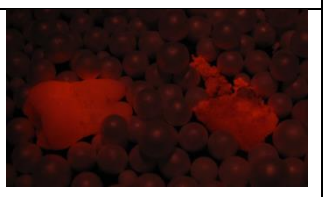
Data were submitted to analysis of variance (ANOVA), using a generalized linear mixed model with three fixed effects. The assumption that the error adhered to a Gaussian distribution was analyzed through the asymmetry and kurtosis coefficients and the Shapiro-Wilk test. The mean values of significant effects were compared by the Tukey test. In all

tests, a 5% level of significance was used. Statistical values were calculated by the SAS system (SAS Institute Inc., Cary, NC, USA).

RESULTS

Table 1 shows photographs of the biological and inert material upon varying the incident light wavelength and emission filter. Table 2 shows the results of ANOVA, applied to the factors affecting mean brightness: materials, filters, wavelengths, and their interactions up to third order.

Table1 - Photographs of biological (tooth and bone samples) and inert (styrofoam balls) materials under different combinations of illumination wavelength (nm) and filter.

Filter / **nm**	**YELLOW**	**ORANGE**	**RED**
455			
470			
505			

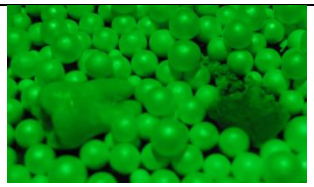
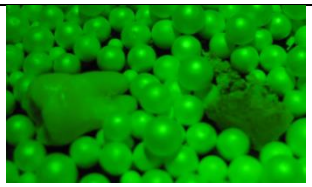
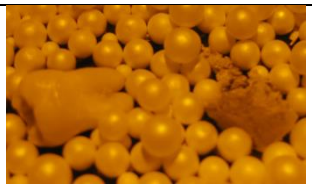
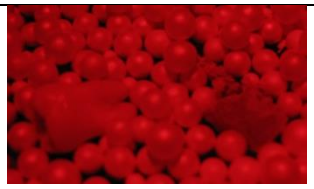
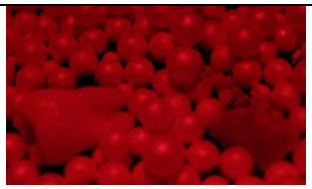
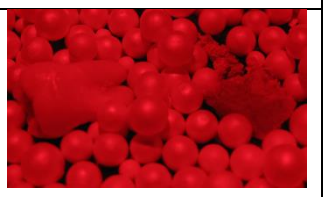
530			
590			
625			

Table 2 - ANOVA results for factors that influenced the brightness, with an appropriate model for randomized experiments with factorial (6 × 3 × 4).

Effect	Degrees of freedom		Analysis of variance	
	Numerator	Denominator	F Statistic	p-value
Wavelength	5	142	13,706.50	<0.0001
Filter	2	142	41,386.60	<0.0001
Material	3	142	24,462.40	<0.0001
Wavelength *Filter	10	142	9,125.12	<0.0001
Wavelength *Material	15	142	6,963.00	<0.0001
Filter*Material	6	142	3,732.00	<0.0001
Wavelength *Filter*Material	30	142	1,315.40	<0.0001

Figure 3 shows the results of the Tukey-Kramer test for comparison of mean brightness due to the interaction of the triple wavelength of 455nm and the orange filter. Means labeled with the same letter in Figure 3 do not differ at a significance level of 5%.

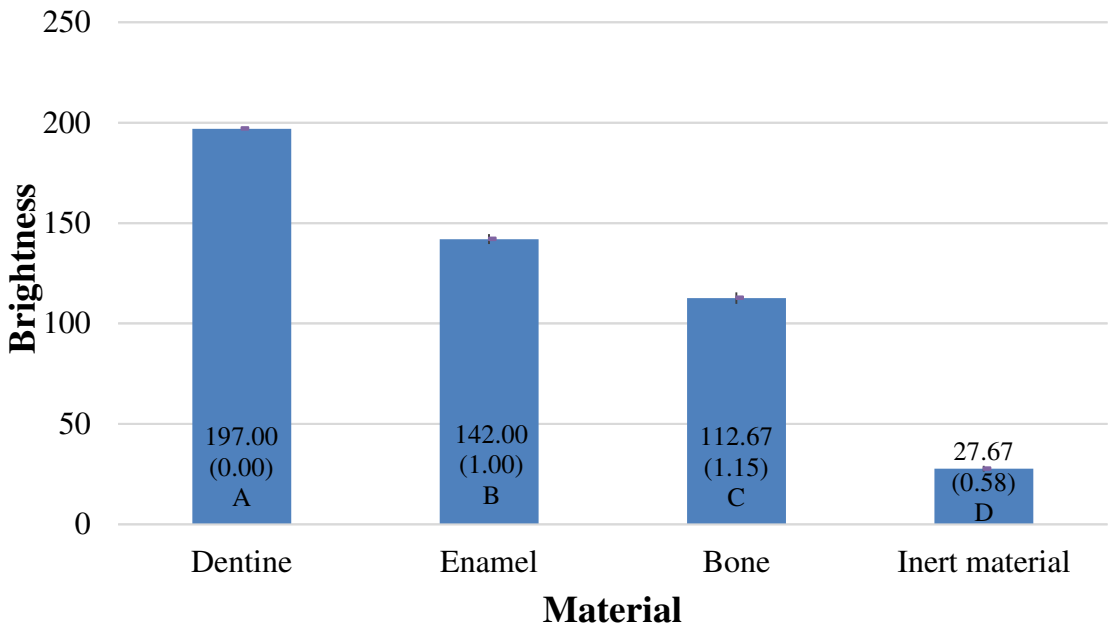


FIG 3 - Mean (standard deviation), limits of the confidence interval (95%) and Tukey's test for comparison of mean brightness of different materials used when the wavelength of 455nm and orange filter.

The difference between the brightness of biological and inert material was calculated to check the visibility of the bone, dentin and enamel. The result is shown in Figure 4. The same ANOVA model was used as in Table 3, however, in this case the inert material was not examined because it was taken as the reference brightness. This results in a factor (6 × 3 × 3) as shown in Table 3.

Table 3 - ANOVA results for factors affecting the brightness of the biological material studied in relation to inert material. The model for randomized experiments with factorial arrangement was applied.

Effect	Degrees of freedom		Analysis of variance	
	Numerator	Denominator		Numerator
Wavelength	5	108	48,792.50	<0.0001
Filter	2	108	17,959.30	<0.0001
Material	2	108	23,043.00	<0.0001
Wavelength *Filter	10	108	8,249.61	<0.0001
Wavelength *Material	10	108	851.46	<0.0001
Filter*Material	4	108	1,483.97	<0.0001
Wavelength *Filter*Material	20	108	281.51	<0.0001

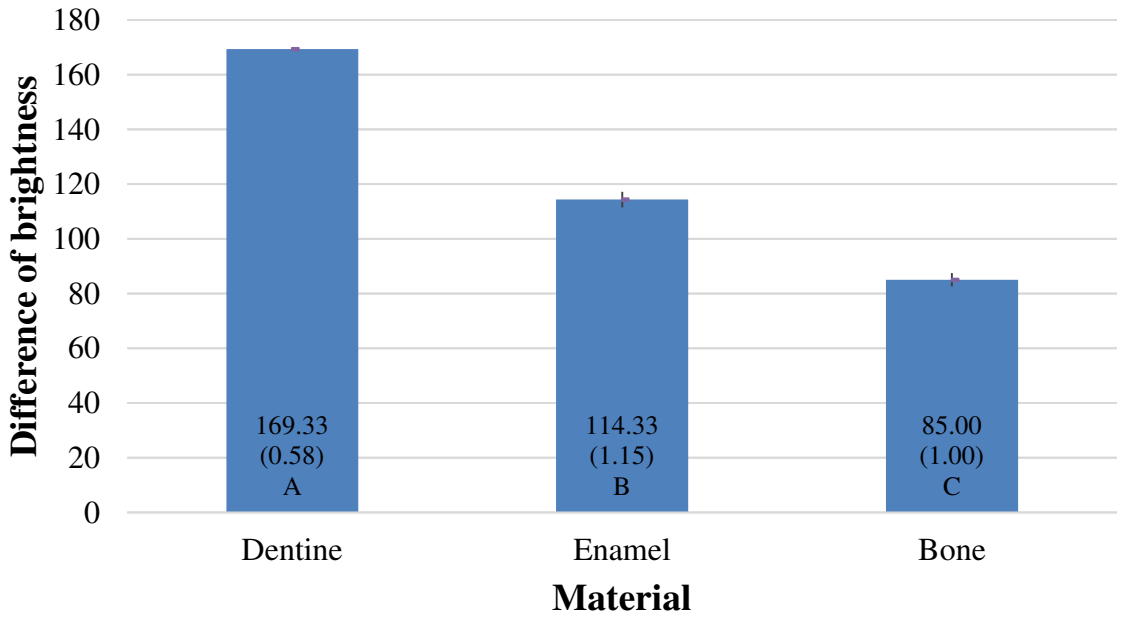


FIG 4 - Mean (standard deviation), limits of the confidence interval (95%) and Tukey's test for comparing the mean difference in the brightness of different materials in relation to inert material, using 455 nm illumination and an orange filter.

DISCUSSION

The best combination to detect bone and tooth via ALS is 455 nm illumination with an orange filter on the camera (Figures 3 and 4). Under these conditions, the biological material had markedly higher fluorescence than the inert material. A similar result has been reported in a previous study (5), but the authors of the previous study did not evaluate the difference in brightness between the biological material and the background on which it was deposited. This difference in brightness is important because it represents how the biological material (dentin, enamel and bone) will appear against inert material at a crime scene, and thus how easy it would be for the forensic expert to visually detect. Because the present study has identified that the orange filter and 455 nm illumination produce the greatest difference in brightness between biological and inert material (Figure 4), this combination should be added to the toolbox of the forensic expert for finding such biological evidence at a crime scene or in the laboratory.

A previous study had mixed samples of tooth and bone with various kinds of stone, and found that the fluorescence of the tooth and bone allowed them to be easily differentiated from the stone samples (5). However, in contrast to the present study, these authors did not assess which biological material possessed greater fluorescence. In the present study, after the best combination of illumination and filter were identified, the different types of biological material (bone, dentin and enamel) were analyzed to determine which had the higher fluorescence. The results (Figures 3 and 4) showed that the fluorescence of dentin is greater than that of enamel, which in turn is higher than that of bone. This knowledge can help the forensic expert to screen and detect biological materials, for example in situations where teeth and small bones are fragmented and mixed with dirt and other debris, both at the scene and in the laboratory. Even small fragments of bone and enamel emit significant fluorescence, which are best viewed in a dark environment (5). These small fragments of evidence may be useful for identifying the victim.

ALS can also be used in dentistry to locate resin restorations, since light can reveal the contrast between the tooth and resin. The speed with which ALS can be deployed makes this technique well-suited to be an adjunct method in dental examinations. Studies have shown that dentin strongly fluoresces due to its higher amount of organic material.

Enamel is also fluorescent, albeit to a lesser degree (9,10). However, these prior studies did not analyze the fluorescence of bone.

Inspection with light via ALS is rapid and can reveal evidence that would otherwise be difficult to observe (10); its usage can provide additional information to contribute towards positively identifying crime victims. ALS has also been used to identify bone in locations that have been damaged by fire. Its advantages in this case are: it reduces potential contamination, and aids in the recovery of remains, after a perimeter has been established encompassing the area to preserve the evidence. Due to the presence of charcoal residue and other debris, bones are difficult to immediately detect during investigation of a fire scene. Because teeth are more resistant to heat and are usually located in or close to the remains, ALS is especially useful in this case (4). Further research is important to advance the development of this technique.

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CONCLUSÃO

A fluorescência dos fluidos biológicos (sêmen, sangue, urina, saliva) variou em função do tempo em que foram expostos. A fluorescência foi menor quando as amostras estavam úmidas e permaneceu constante quando estavam secas até o tempo máximo analisado (60 dias), independentemente do substrato (tecido, papel, madeira, azulejo) em que o fluido foi depositado. Portanto, o perito forense pode detectar fluidos biológicos no local do crime usando uma ALS mesmo após vários dias da ocorrência do crime.

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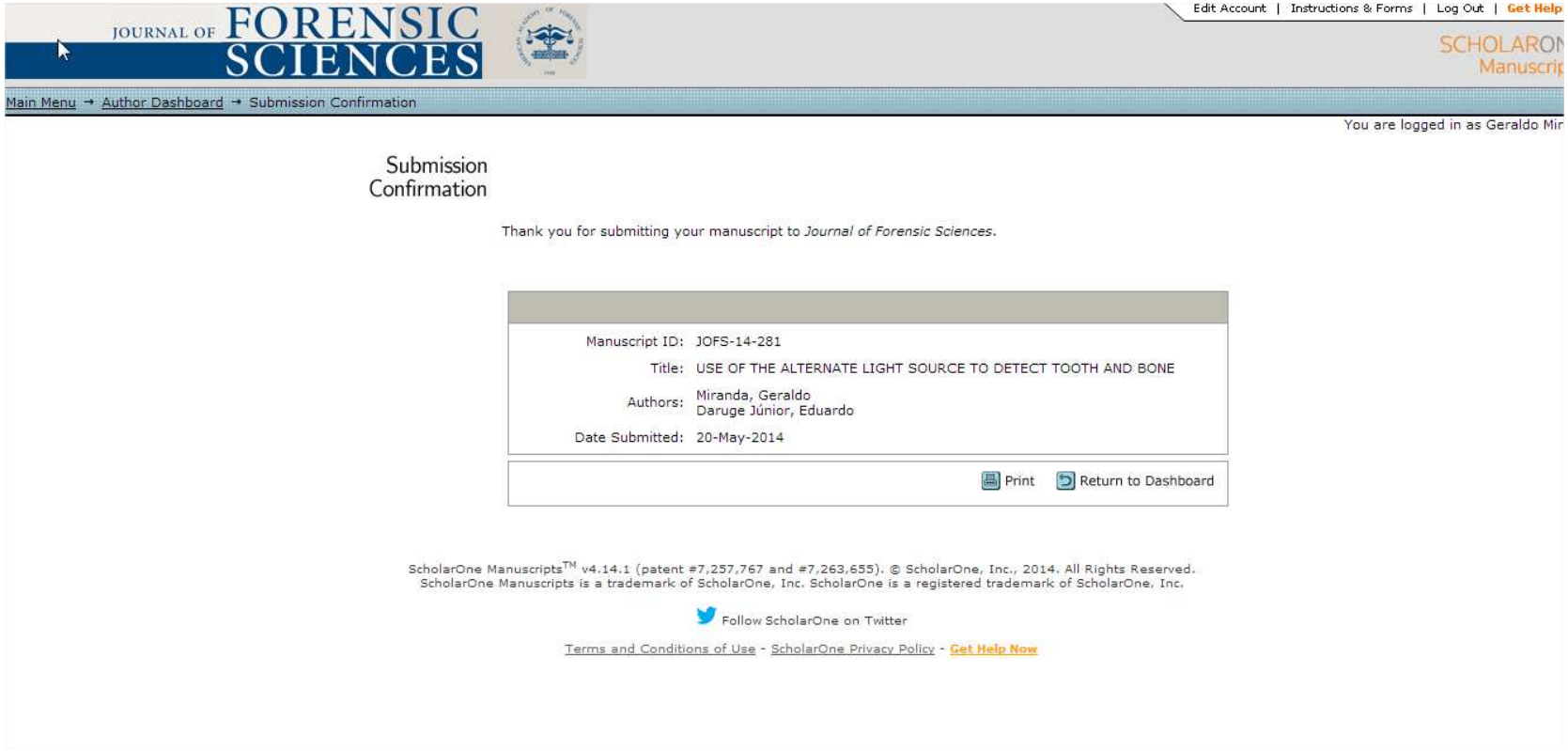

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ANEXO 4 – CERTIFICADO DO COMITÊ DE ÉTICA EM PESQUISA DA FOP-UNICAMP

19/07/12 Comitê de Ética em Pesquisa - Certificado

COMITÊ DE ÉTICA EM PESQUISA
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CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa **"Evidenciação de material biológico com o uso do Megamaxx Alternative Light Source (ALS) System"**, protocolo nº 051/2012, dos pesquisadores Geraldo Elias Miranda, Eduardo Daruge Júnior e Felippe Bevilacqua Prado, satisfaz as exigências do Conselho Nacional de Saúde - Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 07/07/2012.

The Ethics Committee in Research of the School of Dentistry of Piracicaba - State University of Campinas, certify that the project **"Detection of biological material with the use of the Megamaxx Alternative Light Source (ALS) System"**, register number 051/2012, of Geraldo Elias Miranda, Eduardo Daruge Júnior and Felippe Bevilacqua Prado, comply with the recommendations of the National Health Council - Ministry of Health of Brazil for research in human subjects and therefore was approved by this committee at 07/07/2012.

Profa. Dra. Lívia Maria Andaló Tenuta
Secretária
CEP/FOP/UNICAMP

Prof. Dr. Jacks Jorge Junior
Coordenador
CEP/FOP/UNICAMP

Nota: O título do protocolo aparece como fornecido pelos pesquisadores, sem qualquer edição.
Notice: The title of the project appears as provided by the authors, without editing.

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