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Determination of Lead and Evaluation of Microbial Contamination in Eye Shadows and Lipsticks Samples

Determinação de Chumbo e Avaliação de Contaminação Microbiana em Amostras de Sombras para Olhos e Batons

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Abstract

This work evaluated the quality of new, used, expired and valid samples of eye shadows and lipsticks donated by consumers and purchased in Brazil. Microbiological tests were performed to evaluate the presence of the pathogenic microorganisms *Pseudomonas aeruginosa, Staphylococcus aureus* and *Escherichia coli* and the counts of total aerobic mesophilic microorganisms. In order to quantify lead, samples were submitted to the acid digestion in a block digester. The samples were analyzed under optimized and validated conditions by GF AAS. A 2^3 Factorial Design and CCD were employed to method optimization. Considering the 25 analyzed samples, 75% can be considered reproved according to the microbial limits established by Brazilian sanitary law. Among reproved eye shadows, all were used, 4 were out of their expiry dates and 3 were had no expiry date indicated on their primary packaging. Among reproved lipsticks that were used, 5 were out of their expiry dates, 4 had no expiry date indicated on their primary packaging and 1 was not expired; one of the reproved lipsticks was new and non-expired. Our data show the importance of using and storing this type of products under appropriate conditions. Lead concentrations found in lipsticks varied from 0.45 ± 0.08 mg kg^{-1} to 6.8 ± 0.2 mg kg^{-1}, and in eye shadows from <LOD to 6.2 ± 0.4 mg kg^{-1}, being all samples below to the maximum level. In general, eye shadows samples presented higher lead concentrations than to lipsticks and that child use products presented less concentration of this metal.

Keywords: Lipsticks. Eye Shadows. Lead. Microbial Evaluation.

Resumo

Este trabalho avaliou a qualidade de sombras e batons novos, usados, vencidos e válidos, doados por consumidores e adquiridos no Brasil. Testes microbiológicos foram realizados para avaliar a presença dos microrganismos patógenos Pseudomonas aeruginosa, Staphylococcus aureus e Escherichia coli e a contagem de mesófilos aeróbios totais. Para quantificar o chumbo, as amostras foram submetidas à digestão ácida. Após o preparo, as amostras foram analisadas em condições otimizadas e validadas por GF AAS. Os planejamentos experimentais empregados para otimização do método foram: Planejamento Fatorial Completo 2³ e CCD. Das 25 amostras analisadas, 18 (75%) podem ser consideradas reprovadas segundo os limites microbianos estabelecidos pela legislação sanitária brasileira. Dentre as amostras de sombras reprovadas, todas eram usadas, quatro estavam com prazos de validade expirados, três não apresentavam validade na embalagem primária; dentre os batons reprovados que eram usados, cinco estavam com prazos de validade expirados, quatro não apresentavam validade na embalagem primária e um era válido; um batom reprovado era novo e válido. Nossos dados mostram a importância do consumidor utilizar e armazenar os produtos em condições adequadas, para evitar a contaminação microbiana. As concentrações de chumbo obtidas nos batons variaram de 0,45 ± 0,08 mg kg⁻¹ a 6,8 ± 0,2 mg kg⁻¹, e nas sombras de <LOD a 6,2 ± 0,4 mg kg⁻¹, sendo que todas abaixo do nível máximo para Pb. Observou-se que, em geral, as amostras de sombras apresentaram maiores concentrações de chumbo do que os batons e que os produtos de uso infantil apresentaram menor concentração desse metal.

Palavras-chave: *Batons. Sombra para Olhos. Chumbo. Avaliação Microbiana.*

1 Introduction

Cosmetics, according to the Federal Food and Drug Cosmetic Act criteria (2015), are defined as articles intended to be rubbed, poured, sprinkled, or sprayed on, introduced into, or otherwise applied to the human body or any part thereof for cleansing, beautifying, promoting attractiveness, or altering the appearance and articles intended for use as a component of any such articles, except that such term shall not include soap (U.S. FDA, 2015). Beauty products should be easy to use, effective and safe (DIMRI *et al.*, 2016; DAO *et al.*, 2018; MICHALEK *et al.*, 2019). Eye shadows and lipsticks markets are the largest contributors to cosmetics sales in Brazil, amounting to almost 45% of the beauty sector (BENVENUTTI *et al.*, 2016). In the last twenty years, the international beauty market has grown at rates around 3% to 5.5% per year (ISMAIL, 2018; GUPTA *et al.*, 2019). However, make-up products, especially those that come in contact with mucous membranes, including lipsticks and eye shadows, may cause a number of allergic and infectious reactions (ONURDAG *et al.*, 2010; SAWANT; KELKAR-MANE, 2015; AKIN *et al.*, 2020). The main adverse effects include contact dermatitis by irritation and photosensitivity, and allergic contact dermatitis,

which may start an inflammatory reaction (CASTANEDO-TARDAN; ZUG, 2009). These effects are generally related to the presence of chemical and/or microbiological contaminants in the finished products.

Microbial contamination is a matter of great importance to the cosmetic industry and it can become a major cause of both product and economic losses. Make-up products can act as an ideal nutrient media for microbial growth and, therefore, microbial contamination may result into spoilage or chemical changes on them (FATIMA, 2014; DAO *et al.*, 2018; MICHALEK *et al.*, 2019). The contamination caused by microorganisms in cosmetic products can take place in two stages: either during its production, through raw materials, ingredients or filling; or even through its repeated use by the consumers. In the first case, it is the producer's responsibility to ensure proper microbial preservation of the cosmetic. It is carried to ensure the safety of consumers and to maintain the quality of the product at the level provided in its specification. Then, during the use of the product, the consumer is responsible for its safety, for example, by providing adequate storage conditions. To date, few studies have investigated microbiological contamination of cosmetics. Michalek *et al.* (2019) evaluated microbial contamination in different make-up cosmetics products in Europe, from 2005 to 2018. In this study, considering the 104 reported cosmetics, there was a microbiological risk associated with the presence of 118 microorganisms species. In more than half of the cases (52.54%), the microbial hazard was related to the presence of Gram-negative bacteria. Gram-positive bacteria and fungi were observed in 5.93% and 1.69% of the reported products, respectively. In the group of Gram-negative bacteria, aerobic and facultatively anaerobic bacteria were found most frequently, mainly *Pseudomonas aeruginosa* and *Enterobacter* spp. Among Gram-positive bacteria, *Staphylococcus aureus* was identified most often. In the case of fungi, the most common reported species was *Candida albicans*.

Many companies add antimicrobial preservatives in a required dosage to cosmetics, which protects the product from microbial contamination, also ensuring stability (SMART; SPOONER, 1972). In addition, every cosmetic product should be manufactured in accordance with the Good Manufacturing Practices (GMP) and contain an appropriate preservative system that will last for a given period indicated by expiry dates (EL-BAZZA *et al.*, 2009).

The inorganic pigments present in the formulation of lipsticks and eye shadows may contain some toxic metals, such as lead. Reports in the literature point to the presence of this metal in make-up products (FARIA *et al.,* 2018; LARA-TORRES *et al.,* 2021). Lead is a toxic and cumulative metal, which can cause some deleterious effects to human, affecting central nervous system, kidneys and liver. Lead can deposit itself in bones and, in addition, it causes damage to metabolism mainly by blocking enzymes (KALICANIN; VELIMIROVIC, 2016; KUMAR *et al.*, 2020; NAVARRO-TAPIA *et al.*, 2021). Its introduction into the human body can occur by ingestion, cutaneous absorption or inhalation (KALICANIN; VELIMIROVIC, 2016; KUMAR *et al.*, 2020; LARA-TORRES *et al.*, 2021; NAVARRO-TAPIA *et al.*, 2021). Children can absorb more high levels of Pb rather than adults. It is estimated that adults may absorb 3–10% of an oral dose of water-soluble Pb, whereas for children, it may be as high as 40–50% (KUMAR *et al.*, 2020; NAVARRO-TAPIA *et al.*, 2021). Furthermore, mothers may transfer Pb to the fetus and also to infants during the period of breastfeeding (KUMAR *et al.*, 2020). Pb toxicity principally targets the human central nervous system. Neurobehavioral effects undermine academic performance even after blood levels return to normal (KALICANIN; VELIMIROVIC, 2016; KUMAR *et al.*, 2020; NAVARRO-TAPIA *et al.*, 2021). To perform the quantification of metals in concentrations in the order of mg kg^{-1} (ppm) and µg kg^{-1} (ppb), the Graphite Furnace Atomic Absorption Spectroscopy (GF AAS) is an attractive technique due to its high sensitivity, simplicity of instrumentation and mainly by the absence of nebulization or vaporization systems, which simplifies the introduction of the sample with 100% efficiency (SOARES; NASCENTES, 2013; BATISTA *et al.*, 2015; MACHADO *et al.*, 2020). Experimental design, optimization and validation of the method are extremely important to reach the conditions that provide the best responses. It is also necessary to demonstrate that the method, under the practiced conditions, has all the characteristics to obtain results with the required quality: sensitivy, selectivity, accuracy and precision (INMETRO, 2016; FERREIRA *et al.*, 2018).

Eye shadows and lipsticks do not need to be sterile. According to the Brazilian sanitary law, the microbiological limit for these products is 5×10^2 CFU g^{-1} for total aerobic mesophilic counts. It is also required the absence of the pathogenic microorganisms *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa* (BRAZIL, MS, ANVISA, 1999). Thus, a cosmetic preparation contaminated with any of the above microbial species or containing microbial load higher than the prescribed limits can be considered as a potential health risk to the consumer. Still considering the Brazilian sanitary law, the maximum permitted lead impurity for artificial dyes is 20 mg kg^{-1} expressed as Pb for cosmetics, such as lipsticks and eye shadows (BRAZIL, ANVISA, 2015).

In this context, the aim of this study was to evaluate the microbial contamination of used and non-used, expired and non-expired and donated by consumers and purchased eye shadows and lipsticks samples, from different manufacturers marketed in Brazil. This investigation was also undertaken to determine the concentration of lead in these samples, considering the limits recommended by the Brazilian sanitary law.

2 Material and Methods

2.1 Samples

Thirteen samples of lipsticks and twelve of eye shadows were collected and categorized according to their usage, brands, color and manufacturer (Table 3). For the microbiological tests, the surface of the samples were collected in order to access the product layer that would come in contact with the user. Samples for lead determination, in contrast, were collected avoiding the surface layers for these assays. Twenty-three of the selected samples were donated by consumers and two of them (products for children) were purchased locally.

2.2 Microbiological analysis

Product samples were kept at room temperature after being collected. Surfaces of samples containers were disinfected with aqueous mixture of 70% ethanol (v v^{-1}) before opening and removing contents in a laminar flow cabinet. 1.000 g sample was aseptically weighed and dispersed in 1.00 mL Tween-80 and mixed with a vortex mixer. The total volume was adjusted to 10.00 mL with Leethen Broth (LB). For the lipsticks, because they are fatty products of difficult homogenization, samples were previously melted in a water bath at 40 °C, using 1/3 of the volume of the diluent, and then completed with the total volume of LB at room temperature. The obtained suspension was the 10^{-1} dilution, which was diluted decimally in LB to obtain 10^{-1} - 10^{-3} dilution series.

2.2.1 Total aerobic mesophilic counts

For bacterial counts, the *pour plate* technique was used. 1.00 mL was taken from each suitable dilution and mixed with nutrient agar in sterile duplicate plates. The contents were allowed to solidify. The inverted plates were incubated at 36 ± 2 °C and examined daily up to 48 hours. Then, suitable dilutions were counted. For fungal counts, the *spread plate* technique was used. 0.10 mL from each suitable dilution was spread in duplicate in sterile plates containing solidified potato dextrose agar using a presterilized bent glass rod for each dilution. The medium was let to absorb the inoculum. Then, plates were incubated at 28 ± 2 °C and examined daily up to 7 days. Suitable dilutions were counted. For bacteria and fungi, plates containing 30-300 colonies were counted and the results were recorded per dilution counted. Average colony counts were multiplied by the dilution factor. Results were reported as CFU g^{-1}. All tests were performed in triplicate.

2.2.2 Isolation and identification of pathogens

Samples of the products diluted in Letheen Broth (enrichment broth) were initially incubated at 36 ± 0.5 ºC for 24-48 hours. For the detection of *E. coli*, *S. aureus* and *P. aeruginosa*, samples were subcultured on Mac Conkey Agar, Mannitol Salt Agar and Cetrimide Agar, respectively, and then incubated at 36 ± 0.5 ºC for 48 hours. Plates were observed for the presence of typical and atypical colonies in each medium. Further confirmation for all the isolates was done by Gram staining and biochemical tests, as described by the Microbiology Guide of the Brazilian Association of Personal Hygiene, Perfumery and Cosmetics Industry (ABIHPEC) (ABDI, ABIHPEC, SEBRAE, 2015). All tests were performed in triplicate.

2.3 Pb determination

2.3.1 Instrumentation

A Varian/AA240Z atomic absorption spectrometer equipped with a graphite furnace was used in all measurements of Pb integrated absorbance. Zeeman Effect Background correction was employed. A Varian lead hollow cathode lamp was used at a wavelength of 283.3 nm and a current of 5.0 mA, in accordance with the manufacturer's recommended conditions (AGILENT, 2012).

2.3.2 Reagents and materials

Ultrapure water was obtained using a Milli-Q Water Purification System (resistivity of 18.2 MΩ.cm^{-1}, Millipore Direct-Q 3, Molsheim, France) and purified nitric acid was generated by distillation (Sub-boiling Milestone DuoPur, Italy) immediately before its use in the preparation of solutions and samples. Samples were prepared using purified nitric acid and hydrogen peroxide (Lab Synth). Chemical modifiers were prepared using zirconium, rhodium and palladium standard solutions (SpecSol). Pb bulk solution was obtained from lead standard solution (SpecSol).

2.3.3 Optimization

Before starting the analysis for the method optimization, screening tests were performed to identify the two chemical modifiers that would provide the higher absorbance and the lower background signal. The experiments were conducted to choose the appropriate permanent modifier for the determination of lead content in the samples. The integrated absorbance measurement and the background signal for the samples were obtained using separate graphite tubes treated with permanent modifiers (zirconium, rhodium and palladium) and palladium as a chemical modifier (co-injection of 1 µL of a 1000 mg L^{-1} solution). A tube without a permanent modifier was evaluated as well. Screening tests were done in accordance with the manufacturer's recommended heating program for Pb determination (AGILENT, 2012).

A 2^3 Factorial Design was employed to establish the optimal pyrolysis, atomization temperatures and the best chemical modifier for the determination of lead in lipsticks and eye shadows samples (Table 1).

Table 1 - Factors and levels in 2^3 factorial design for the optimization of lead determination in lipsticks and eye shadows using GF-AAS

Factor	Level		
	Low (-1)	Center (0)	High (+1)
Chemical modifier	Zr	-	Pd
Pyrolysis temperature (ºC)	300	700	1100
Atomization temperature (ºC)	1400	1850	2300

Source: Research data.

The pyrolysis and atomization temperature ranges and chemical modifiers that provided the highest absorbance in the 2^3 factorial design were then used in the Central Composite Design (CCD) to obtain a response surface (Table 2).

Table 2 - Factors and levels in CCD for the optimization of lead determination in lipsticks and eye shadows using GF-AAS

Factor	Level				
	(-1.41)	(-1)	(0)	(+1)	(+1.41)
Pyrolysis temperature (ºC)	500	529	600	571	700
Atomization temperature (ºC)	1500	1529	1600	1671	1700

Source: Research data.

2.3.4 Validation

The validation parameters linearity, selectivity, sensitivity, limit of detection, limit of quantification, trueness and precision were evaluated as established by INMETRO (INMETRO, 2016). Linearity was obtained through external standardization and, to evaluate linearity deviations, the residuals between the measured values and the values calculated from the linear regression were verified. Thus, an aqueous calibration curve was made. The GF AAS equipment diluted a lead solution of 100 µg L^{-1} to construct a ten-point calibration curve. The sensitivity was expressed by the slope of the linear regression calibration curve.

Four calibration curves were made for the selectivity test. For the standard addition method, two curves were obtained, one for eye shadows and another for lipsticks. For the calibration curve with reagent, a 50 µg L^{-1} solution of lead was prepared. The GF AAS equipment diluted this solution to construct a five-point curve using a 50% v/v solution of purified nitric acid. Finally, an aqueous calibration curve was prepared for the calibration curve with reagent, diluting the 50 µg L^{-1} solution in ultrapure water. Then, the slopes of the four calibration curves were statistically compared.

To determine the limit of detection (LOD) and the limit of quantification (LOQ), seven independent blank solutions were analyzed. The limit of detection value was obtained through the equation "LOD= x + t.s", where "x" is the mean of the sample blank values, "s" is the standard deviation of the blank sample, and "t" is obtained from Student's t distribution with (7-1) degrees of freedom at 95% confidence level. The limit of quantification was expressed by the equation "LOQ = x + 10.s, where "x" is the mean of the sample blank values and "s" is the deviation from the blank readings described above. The working range begins just after the limit of quantification and extends to the last linear point of the curve.

The recovery method was used to evaluate accuracy and trueness, where the absorbance of a fortified sample was read and compared to the non-fortified sample and the standard lead solution absorbance. To evaluate precision, tests were performed for repeatability and intermediate precision. The repeatability test consisted of the analysis of seven independent replicates of standard lead solutions at concentrations of 10 µg L^{-1}, 30 µg L^{-1} and 50 µg L^{-1}. These solutions were read in the GF AAS equipment by the same analyst, using the same equipment under the same conditions, at the same place within a short period of time. In the intermediate precision test, the same solutions, prepared as described above, were analyzed under similar conditions in different days.

All analysis followed the GF AAS manufacturer's recommended conditions and the optimized heating program and the best chemical modifier (AGILENT, 2012).

2.3.5 Sample analysis

Approximately 50.0 mg of the lipstick and eye shadow samples were accurately weighed, in triplicate, directly into the digestion tubes, followed by the addition of 3.00 mL of purified nitric acid and 2.00 mL of hydrogen peroxide. The tubes were closed and taken to the block digester (Quimis, Brazil) for one hour at 110º C. Then, the tubes were cooled to room temperature. 3.00 mL of purified nitric acid and 1.00 mL of hydrogen peroxide were added, and the vials were taken again to the block digester under the same conditions. The samples were cooled to room temperature and transferred to graduated polyethylene vials, and the final volume was adjusted to 10.0 mL with ultrapure water. The samples were analyzed by GF AAS using the optimized conditions.

3 Results and Discussion

Table 3 shows data about the lipsticks and eye shadows samples, as well as the obtained results for microbiological tests and lead determination.

Table 3 - Lipsticks and eye shadows sample data, microbiological tests and lead determination results

Type of product	Sample number	Manufacturer	Period of use	Shelf-life	Color	Made in	Total aerobic mesophilic counts (10^2 CFU/g)	*P. aeruginosa*	*S. Aureus*	*E. coli*	Pb concentration (ppm)
Lipsticks	1	I	5 years	**	Red	**	7.65	-	+	-	1.2 ± 0.1
	2	II	5 years	Aug/2014	Peach	Brazil	> 3000	-	+	-	2.1 ± 0.1
	3	II	*	**	Dark red	Brazil	< 0.1	-	-	-	1.39 ± 0.09
	4	II	*	Mar/2017	Golden	Brazil	12.8	-	+	-	0.8 ± 0.5
	5	III	6 months	Expired	Peach	Brazil	6.35	-	-	-	0.84 ± 0.08
	6	III	2 years	Expired	Pink	Brazil	> 3000	-	+	-	1.4 ± 0.6
	7	IV	*	May/2020	Red	Brazil	> 3000	-	+	-	0.61 ± 0.08
	8	V	8 years	**	Red/brown	Canada	< 0.1	-	-	-	1.4 ± 0.4
	9	VI	7 years	**	Dark pink	Italy	18.5	-	+	-	0.79 ± 0.03
	10	VII	3 years	Mar/2018	Pink	USA	8.95	-	+	-	0.7 ± 0.2
	11	VIII	5 years	**	Dark red	**	19.3	-	-	-	1.2 ± 0.2
	12	IX	3 years	**	Orange	Faked	8.50	-	-	-	6.8 ± 0.2
	13	X	New	May/2020	Red	Brazil	6.50	-	-	-	0.45 ± 0.08
Eye Shadows	14	II	*	Aug/2018	Brown	Brazil	< 0.1	-	+	-	1.9 ± 0.2
	15	II	*	Aug/2018	Light pink	Brazil	< 0.1	-	-	-	0.8 ± 0.1
	16	II	*	**	Black	Brazil	< 0.1	-	+	-	2.1 ± 0.4
	17	V	*	**	Silver	Canada	< 0.1	-	+	-	2.9 ± 0.3
	18	V	*	**	Golden	Canada	< 0.1	-	-	-	3.36 ± 0.06
	19	XI	4 years	**	Dark blue	Canada	< 0.1	-	-	-	6.2 ± 0.4
	20	XI	4 years	**	Light blue	Canada	< 0.1	-	+	-	3.5 ± 0.5
	21	X	4 years	Aug/2015	Purple	China	< 0.1	-	+	-	3.0 ± 0.2
	22	X	4 years	Aug/2015	Green	China	< 0.1	-	+	-	3.7 ± 0.1
	23	X	4 years	Aug/2015	Orange	China	< 0.1	-	-	-	2.8 ± 0.2
	24	XII	5 years	12 months	Dark red	China	< 0.1	-	+	-	2.5 ± 0.1
	25	XIII	New	May/2020	Blue	Brazil	< 0.1	-	-	-	0.18 ± 0.07 (<LOD)

*Information not provided by the product user. **Information not provided by the manufacturer on the primary packaging of the product. (+): presence of the microorganism. (-): absence of the microorganism

Source: Research data.

3.1 Microbiological analysis

In order to evaluate the microbiological quality of cosmetics, it is necessary to demonstrate the absence of pathogenic microorganisms that may offer a risk to the consumer and also to determine the number of viable microorganisms in the products. Cosmetics are not marketed as sterile products, and, in our study, all samples of eye shadows presented counts of aerobic mesophilic microorganisms in accordance with the Brazilian sanitary limits (< 5.0 x 10^2 CFU/g), while 11 (84.6%) samples of lipsticks presented counts above the allowable limits (Table III). Microbial contamination of cosmetics has been reported by many authors (BENVENUTTI *et al.*, 2016; DASHEN *et al.*, 2011; SKOWRON *et al.*, 2017; TRAN; HITTCHINS, 1994) and may occur due to the environmental conditions in which the products are manufactured, packed and used by the consumer.

The type of ingredients of a formulation may also favor or inhibit microbial growth. In this study, none of the tested eye shadows presented counts of aerobic mesophilic microorganisms above the limits. This kind of product has a low water activity, and this may explain our data, as water is essential for the microbial growth and metabolism.

Samples 2, 4, 5, 6 and 10 were out of their expiry dates and were still in use by the consumers. Some of these lipsticks have no the expiry dates indicated on their primary packaging. All of them presented counts of viable microorganisms above the sanitary limits, which may contribute to the product deterioration and offer a risk to the consumer´s health. Akin *et al*. (2020) had carried out a study to investigate microbial quality and control of lipsticks, and they found that of 81 samples, 42% yield aerobic plate count and 23% were found to be consisting of mold and yeasts. As in the present work, Skowron *et al.* (2017) showed that expired lipstick samples had a viable microorganism count higher than the samples that were in the validity period (SKOWRON *et al.*, 2017). Even though most cosmetics have a preservative system, it is only capable of protecting the product from microbial contamination during its shelf-life. Many authors agree that,

throughout the years, such preservatives may lose their activity. In addition, lipsticks are products that are often ingested by the consumer and come in contact with the oral mucosa, being able to cause infections depending on the immunity of the host and the type and number of microorganisms present in the product (BENVENUTTI *et al.*, 2016; DAO *et al.*, 2018; MICHALEK *et al.*, 2019).Considering the pathogenic microorganisms, *S. aureus* was found in 7 (54%) of the tested lipsticks and in 7 (58%) of the eye shadows samples. None of the analyzed samples presented the pathogens *E. coli* and *P. aeruginosa* (Table 3). Dao *et al*. (2018) fount that most reports on cosmetics contamination concerned the presence of Gram-negative bacteria. Primarily, two genera of rod-shaped bacteria, aerobic *Pseudomonas* and facultatively anaerobic *Enterobacter* were detected. While *Pseudomonas* species are widely distributed in the natural environment, *Enterobacter* species are found in faeces of humans and other animals, sewage, soil, water and dairy products. The most commonly identified species among all cosmetic products reported by these authors was *P. aeruginosa*. In the present study, these pathogens were not found in the analyzed samples, and only *S. aureus* was detected.

According to the parameters established by RDC nº 481/1999 (ANVISA), these products can be considered reproved due to the presence of this pathogen (BRAZIL, MS, ANVISA 1999). Our results suggest that the preservative system used in these products may not have been efficient during their use, or they could be produced without meeting the Good Manufacturing Practices. Moreover, they also may have been contaminated by the use, since *S. aureus* is a pathogen commonly found in the human skin microbiota.

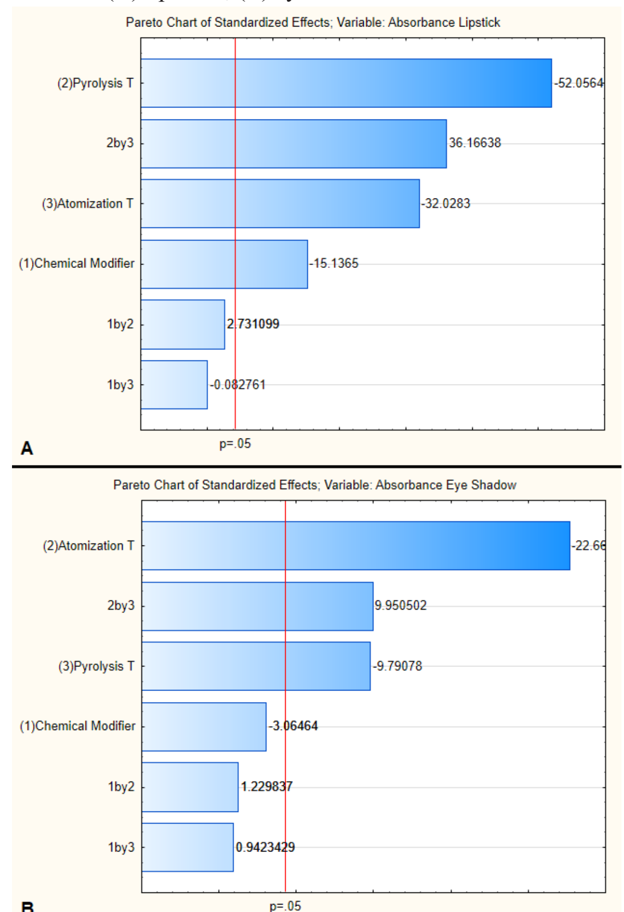
The contamination of cosmetic products by the consumer occurs mostly on their surface through contact with the skin, mucous membranes and applicators such as brushes and sponges (DASHEN *et al.*, 2011). Brushes and sponges can accumulate dead cells, oils from the skin and residues from other make-up, which may generate an appropriate environment to microbial growth (DAWSON; REINHARDT, 1981). *S. aureus* is commonly associated with skin infections, involving edema formation and inflammation. It also produces toxins and can cause diseases in humans such as pneumonias, folliculitis and meningitis, among other infections. As in our investigation, Saeed and Asif (2011) found *S. aureus* in used lipsticks samples, and Benvenutti *et al.* (2016) found this pathogen associated to different used make-up products, such as eye shadows, lipsticks and facial powders (BENVENUTTI *et al.* 2016; SAEED, ASIF, 2011). Another study on branded cosmetic powders conducted by Dashen *et al.* (2011) showed the presence of *S. aureus* as the most prevalent microorganism in the samples analyzed, which is in accordance with our findings (DASHEN *et al.*, 2011). Finallly, in comparative investigations conducted by Siya *et al.* (2019) on various cosmetic products such as eye care products and face care products, it was found that *Staphylococcus* species were the ones that had been regularly detached.

3.2 Lead Determination

3.2.1 Optimization

Considering the chemical modifiers used in the screening tests, the permanent zirconium generated the lowest background signals, while the permanent palladium generated the highest absorbance signals. Due to the obtained results, these two modifiers were tested again in a 2^3 factorial design, along with the pyrolysis temperature and atomization temperature factors, to be statistically compared. Then, using the values obtained in the tests, the Pareto Graphs for lipsticks and eye shadows (Figure 1) was generated. The graph for lipsticks indicates that the pyrolysis temperature has a significant negative effect, which means that this factor generates better results at low levels, close to 300º C. Likewise, the atomization temperature has a significant negative effect, generating better results at low levels, close to the temperature of 1400º C. The chemical modifier factor has also a significant negative effect, which indicates that, for lipsticks, zirconium modifier generates better results. The interaction of the modifier with other factors is not significant and does not affect the obtained results.

Figure 1 - Pareto chart obtained using 2^3 factorial design for optimization of pyrolysis, atomization temperature and chemical modifier: (A) lipsticks, (B) eye shadows



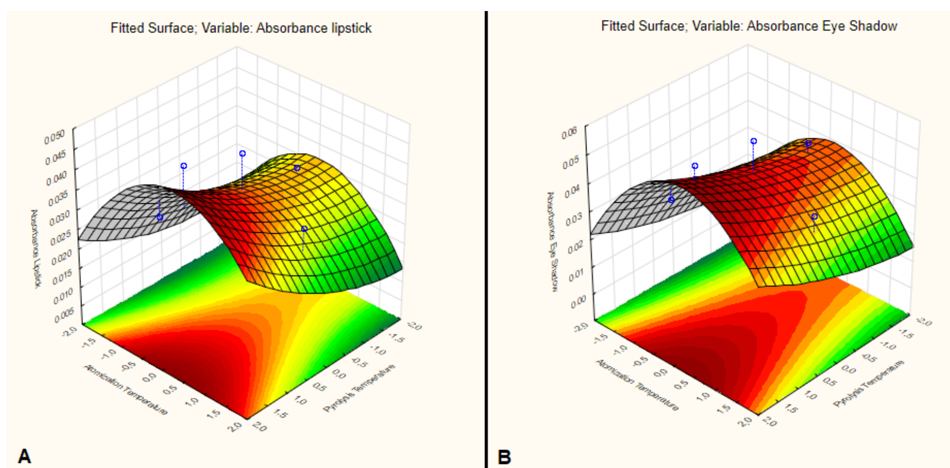
Source: This graph was generated by Statistica® 10.0 from data in Table S1.

Considering the pyrolysis and atomization temperature for eye shadows, the same analysis of the results can be done for the lipsticks samples. The chemical modifier has no significant effect, indicating that for eye shadows the two analyzed modifiers would generate similar statistical results. Therefore, the permanent chemical modifier zirconium was chosen to be used in eye shadows and lipsticks analysis.

Considering the 2^3 factorial design and Pareto Chart interpretation, it was performed a first CCD with pyrolysis (318 ºC, 400 ºC, 600 ºC, 800 ºC, 882 ºC) and atomization (1318 ºC, 1400 ºC, 1600 ºC, 1800 ºC, 1882 ºC) temperature ranges lower than the central point of 2^3 factorial design. However, the response surface generated a saddle point, indicating two regions of increased absorbance. A second CCD design was then performed to obtain a better response surface based on the higher absorbance regions observed in the first attempt to adjust the pyrolysis and atomization temperature levels, as described in Table 2.

From the second CCD results, the response surfaces (Figure 2) were generated for lipsticks and eye shadows samples. For the lipsticks, the response surface showed an atomization temperature critical point of 1,603º C. The critical temperature for the pyrolysis presented a value outside the worked range. However, as shown in Figure 2 (A), we noticed that the temperature that generates the best responses is near the highest level of the experiment, i.e. 700° C.

Figure 2 - Response surface plots for sample absorbance as a function of pyrolysis and atomization temperature: (A) Lipstick, (B) Eye Shadow



Source: This graph was generated by Statistica® 10.0 from data in Table S2.

For eye shadows, the response surface generated a critical point for the atomization temperature of 1,607º C. The pyrolysis critical temperature also presented a value outside the worked range. However, as in the obtained results for lipsticks, the temperature that generates the best responses is also close to 700° C.

From the results above, the optimum temperatures of 700° C and 1,600° C, for pyrolysis and atomization, respectively, were set for the GF AAS analysis of lipsticks and eye shadows samples. Table 4 shows the optimized graphite furnace heating program. Batista, Augusto and Pereira Filho (2015) have also analyzed lead in eye shadows samples after acid digestion in a microwave furnace by GF AAS. These authors employed the pyrolysis and atomization temperatures, 800° C and 1,200° C respectively, recommended by the GF AAS equipment manufacturer (iCE 3000 Series, Thermo Scientific, Waltham, MA, USA) (BATISTA *et al.*, 2015). Soares and Nascentes (2013), when analyzing lead in lipsticks samples using the surface response method, optimized the pyrolysis and atomization temperature, and found optimum temperatures of 900º C and 1,800º C, respectively (SOARES; NASCENTES, 2013).

Table 4 - Optimized graphite furnace heating program for Pb determination in lipsticks and eye shadows samples

Step	Temperature (ºC)	Time (s)	Flow (L/min)	Read	Signal Storage
1	85	5.0	0.3	No	No
2	95	40.0	0.3	No	No
3	120	10.0	0.3	No	No
4	700	5.0	0.3	No	No
5	700	6.7	0.3	No	No
6	700	6.7	0.0	No	Yes
7	1600	1.0	0.0	Yes	Yes
8	1600	2.6	0.0	Yes	Yes
9	1600	2.6	0.3	No	Yes
10	2600	2.0	0.3	No	Yes

Source: Research data.

3.2.2 Validation

To evaluate the linearity deviations, the residuals between the measured values and the values calculated from the linear regression were used. From these residues, the value of "*t*" was calculated by equation "$t_{calculated}$ = residuals/($s_r\sqrt{n}$)", where "s_r" is the standard deviation of the residues and "n" is the number of points in the curve. The unilateral t value for the T-test at 95% confidence is 2.23. As *t* calculated for all points

on the curve were less than unilateral *t*, we can consider that all points belong to the curve and that the whole range analyzed in this test is linear.

The selectivity test was performed to verify the matrix effect in the analysis. The F and T tests were used to compare the calibration curves slopes, since the readings were performed in triplicate for all three methods. For the lipsticks, the F-test indicated that the matrix had no significant effect on the precision of the method at this concentration range, at 95% confidence. The T-test for lipsticks indicated that there is no statistical difference between the inclinations of the curves, confirming that the lipstick matrix does not affect the reading of the sample. For eye shadows, the F-test indicated that the matrix has a significant effect on the accuracy of the method at this concentration range and at 95% confidence. The T-test for the eye shadows indicated a statistical difference between the slopes of the standard addition method curve when compared to the aqueous calibration curves and to the calibration curve with addition of reagent.

Due to the obtained results for the selectivity test, lipsticks samples were analyzed on an aqueous calibration curve and the eye shadows samples were analyzed on a standard addition curve. Table 5 shows the obtained results for the other validation tests.

Table 5 - Figures of merit for method validation for lead determination in eye shadows and lipsticks samples

Figures of merit	Sample type	Results
Equation	Lipstick	y = 0.0014x + 0.0059
	Eye Shadow	y = 0.0013x + 0.0087
R^2	Lipstick	0.995
	Eye Shadow	0.993
Sensitivity	Lipstick	0.0014
	Eye Shadow	0.0013
LOD	-	0.2 mg kg^{-1} (1.1 µg L^{-1})
LOQ	-	0.9 mg kg^{-1} (4.4 µg L^{-1})
Working range	-	1.1 - 50.0 µg L^{-1}
Recovery	Lipstick	85%
	Eye Shadow	103%
Intermediate Precision (n = 14)	10 ppb	11.8%
	30 ppb	2.8%
	50 ppb	4.9%
Repeatability (n = 7)	10 ppb	6.0%
	30 ppb	1.8%
	50 ppb	5.3%

Source: Research data.

3.2.3 Concentrations of lead in the samples

None of the analyzed samples showed lead concentrations above the limits established by the Brazilian sanitary law. An X-ray fluorescence spectrometry analysis was performed on the remaining solid of the digestion to confirm that it was efficient for lead. According to the obtained results, this solid was a mixture of silicon and titanium dioxides, and aluminum and magnesium oxides, corroborating that there was no analyte loss.

Samples 13 and 25, of lipsticks and eye shadows, respectively, had the lowest lead concentration. These samples were intended for child use, which may have contributed for the obtained results. In general, eye shadows samples presented higher lead concentrations when compared to lipsticks, except sample 12. This result may be related to the fact that different types of pigment and other raw materials can be used in lipsticks and eye shadows manufacturing. However, it is worth noting that eye shadows were the products that had the highest lead concentrations, and this contaminant may present cutaneous penetration. Moreover, these make-up products may come into contact with the eyes and, for this reason, lead penetration may be easier to occur due to the characteristics of the eyeball tissue.

Sample 12 that, according to the user, was a counterfeit product, presented a very different lead concentration when compared to the other lipsticks samples. This fact draws attention to the risks of acquiring this type of product. Zhao et al. (2016), when analyzing the lead concentration in 75 samples of lipsticks and 18 samples of lip glosses, found that orange lipsticks had a higher lead concentration when compared to the other colors, and that Pb chromate had been added to the products of this color (ZHAO *et al.*, 2016).

From the eye shadows, sample 19 had the highest lead concentration, being almost the double of sample 22, which presented the second highest lead concentration in this work. Soares (2012), when analyzing samples of eye shadows, found similar concentrations to sample 19 in blue, green and graphite shades from China. Batista, Augusto and Pereira Filho (2015) found lead concentrations of 44 ± 2 mg kg^{-1} and 34 ± 4 mg kg^{-1}, for an orange and blue eye shadows samples, respectively, both from China.

3.3 Principal Component Analysis

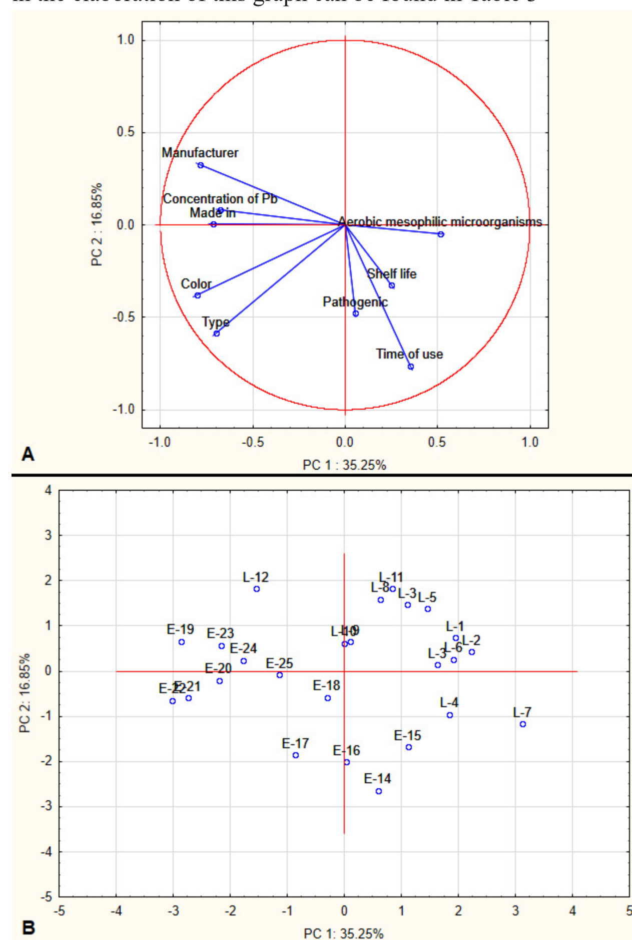
In order to verify correlations between the obtained data and the characteristics of the analyzed samples of lipsticks and eye shadows, a principal component analysis was performed (Figure 3). In this analysis, the type of sample (lipstick or eye shadow), color, country of origin, manufacturer, expiry date and time of use were compared to lead concentration, presence of pathogens and counts of total aerobic mesophilic microorganisms. Figure 3 demonstrates that expiry date and time of use information are in the same quadrant as the data of pathogens and counts of total mesophilic microorganisms, indicating that these factors are directly affected by the expiry date and time of use of the products. This information is consistent with the expected results, since products that have been expired or used for an extended time are more likely to develop contaminating microorganisms.

Figure 3 (A) shows that lead concentration is next to manufacturer and country of origin information, which indicates that these factors are closely related. Color and type of product factors appears in the same quadrant as lead

concentration information (Figure 3A), which indicates that they are more related to the presence of lead in the samples than to the microbiological contamination, due to the distance they appear on the chart. This relation was also expected since lead concentration is related to the pigments used in the products formulation, and manufacturers that are in the same country or region tend to use similar raw materials. Moreover, manufacturers of the same country of origin may be subjected to the same sanitary laws and inspection policies to release their products on the market.

Samples 12 and 19 (Figure 3B) were positioned in the same quadrant, and these were the samples that presented the highest lead concentrations. Next to them are most of the analyzed eye shadows that, as previously described, generally showed a higher lead concentration when compared to lipsticks, which explains the sample type information being closer to the lead concentration in Figure 3 (A).

Figure 3 - Principal component analysis: (A) Variables projection in the factor plane, (B) Samples projection in the factor plane. (E): Eye shadow. (L): Lipstick. The quantitative parameters used in the elaboration of this graph can be found in Table 3



Source: Research data.

4 Conclusion

Our data reveal the importance of microbiological quality control and the need to evaluate the efficiency of the preservatives used in cosmetics products due to the high risk of contamination by opportunistic pathogens. In addition, Good Manufacturing Practices should be followed during the production, storage and transportation stages of a make-up cosmetic, as well as expiry dates should be indicated on their primary packaging. Moreover, it is extremely important that the final consumer use and store the product under appropriate conditions, in order to avoid microbial contamination.

In our work, it was also possible to perform the optimization and validation of the method for lead determination by graphite furnace atomic absorption spectrometry for the analysis of lipsticks and eye shadows samples. All samples had lead concentrations below the limits established by the Brazilian sanitary law. Eye shadows samples showed, in general, higher lead contents when compared to lipsticks, probably related to the difference of raw materials used in each type of product. The products destinated to child use presented less concentration of this metal.

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