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"Assessing Hair's Internal Porosity Using Scanning Electron Microscopy"

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1. Introduction

Human hair is highly reticulated queratin material that is organized in a complex porous structure [1]. This structure can present porous in different levels, from the microstructure to the macrostructure due to the organization of the keratin filaments and the amount of crosslinks [2,3], then, hair porosity is significantly impacted by the integrity of hair fibers, influencing moisture retention, mechanical properties, and thermal resistance [4-6], thus the investigation of hair porosity and the effect of cosmetic product over this parameter Is key on the research and development of cosmetic products. Historically, porosity has been broadly studied and several methodologies has been developed for their evaluation, but, few of them have been used on the cosmetic industry to evaluate the porosity of hair fibers and claims validation. relied on indirect measurements due to the high cost and complexity of direct methods. Kaushik et al. studied hair porosity using the Brunauer, Emmett and Teller method known as BET [7]; in this method, the hair is placed in a high vacuum and then nitrogen gas is introduced by pressure into the hair pores; if the pressure required to fill the pores is high, this indicates that the pores are smaller. This method provides results on the porosity of the hair as a whole, but its use assumes that in the water vaporization stage the internal structure of the hair is not altered and due to the high vacuum and sample preparation the method becomes expensive, making it difficult to use the methodology at a commercial level. Scanavez et al. used X-ray microtomography to evaluate cortex porosity; this technique uses reconstructed 3D images from which a direct assessment of the number of pores is made [8]. Other options that have been proposed for evaluating hair fiber porosity include the use of SAXS (Small-Angle X-ray Scattering) [9] and XRD (X-ray Diffraction); these techniques use synchrotron light sources to study the molecular organization of the hair fiber, and porosity is calculated based on the intensity of the scattered or diffracted light—the greater the light intensity, the higher the porosity [10]. Despite the good results obtained with these techniques, like BET, they have the disadvantage of being costly and not easily accessible, which makes their use challenging in the cosmetics industry for claim validation. Some authors obtain hair fiber porosity through indirect measurements, that is, they measure other properties that can be correlated with porosity. For example, Sedik et al. placed hair strands in a container of water and observed

whether the strands floated on the surface or sank. Based on this observation, they classified the strands as: Low porosity for those that floated; Normal porosity for those in which part of the strand floated and part sank; High porosity for those in which all strands sank [1]. This type of indirect measurement is used by consumers because it is easy to perform and does not require expensive measuring instruments, however it does not provide numerical measurements of porosity or information about the shape of the pores, that are important data in the study of the interaction between the hair strand and cosmetic products. However, advancements in image analysis and SEM (Scanning Electron Microscopy) now offer promising alternatives for directly evaluating hair porosity. Scanning Electron Microscopy (SEM) is a technique that uses ionizing radiation to generate images of surfaces. Although the technique requires a high vacuum and coating of the hair sample, it is much more cost-effective than X-ray microtomography, SAXS, or BET. SEM is a widely used technique, and many laboratories are equipped with the necessary instruments and offer analyses at an affordable price. With this technique, it is possible to obtain high-resolution, highly magnified images, which allows for direct visualization of hair pores when the strand is cut transversely. Some microscopes even come with software that enables direct pore measurement from the image; however, this software is sold separately and is usually expensive, often not justifying the investment. The only drawback of using SEM to measure porosity is that it assumes the distribution of pores is the same throughout the hair strand, which is not always true [11]. However, this limitation can be reduced by performing evaluations at multiple points along the same strand. On this context, this study aimed to develop a reliable, cost-effective methodology to measure hair porosity by quantifying the number and size of internal pores using SEM cross-sectional images. The approach involved two phases. In the first phase, we optimized the preparation of hair cross-sections by testing combinations of embedding media and cutting tools to preserve internal hair morphology. Three embedding media and three cutting tools were evaluated. In the second phase, the methodology was validated for reproducibility and sensitivity to cosmetic treatments. Four groups of Caucasian hair swatches were used: one untreated control and three double-bleached groups treated with different products. Cross-sections of these samples were analyzed via SEM, and images were processed using a plugin developed during phase one. This plugin quantified the number and area of internal pores.

2. Materials and Methods

The experiment was divided in two fases. The first phase was the selection of the best inclusion medium and cutting tool for obtain transversal cuts of hair fiber that preserves the internal extructure, permiting the observation of the internal porosity. For this, were used Caucasian natural curly type II hairs whitout treatments. The hairs were separated in small swatches with 0,5cm of length are were placed in a mold for histological cuts in the perpendicular position. The mold were then fill with the resin.

The resins used for the inclusion were: Historesin embedding kit Leica, Polyster Resin Arazyn 25100, Epoxi Resin 2001 and O.C.T compound tissue-tek. All the resin were prepared following the fabricant instructions. The cuts of the hard resins were performed on a microtome with tungsten or glass knife and for the O.C.T compound the cuts were performed on a cryostat Leica CM1860 at -30°C. All the cuts were performed at 10µm, 8µm and 6µm.

After performed the cuts, those were visualized with an optical microscope Olympus BX5, accopled with a digital camera DP74 using the 20X lens. The program used for obtained the images was the CellSens Standard. The best cuts were then taken to SEM microscopy and

the images were used for developing the evaluation method of thew pores. The software ImageJ [12] was used for the measurement with the plugins Labkit and MorphoLibJ.

On a second fase, after selected the inclusion media and the development of the method for pore evaluation, it was verified if the method was reprodutible and have good differentiation of cosmetic products. For this, Natural Indian Hair culy type I was used and divided in 4 groups of 3 swatches each. 3 of the groups were double bleached using oxidant 30 vol (hydrogen peroxide) and a comertial bleaching powder in a proportion 1:3 (w/w); 10g of the mixture were applied per each grame of hair and it was let it to rest 35min before rising. Then the hair were treated with cosmetic products as showed on Table 1.

Table 1. Treatments applied on hairs watches

Group Number	Chemical Treatment	Cosmetic treatment
Group 1	Natural	SLES 10%
Group 2	Double Bleached	SLES 10%
Group 3	Double Bleached	Hair Mask
Group 4	Double Bleached	Leave-in

The experiment was repeated on two different days. After treatment, 10 fibers from each group were randomly selected and analyzed using a SEM microscope. The images obtained were analyzed with ImageJ software, following the same method developed in Phase 1 of this study. The results were then graphed, and statistical comparisons were performed using GraphPad Prism, version 8.3.4, both between the treatment groups on each day and across the same groups on different days to verify the reproducibility and the diferentiation.

3. Results

3.1. Investigation of the best combination of Inclusion medium and cutting tool.

The hairs included on the Epoxy resin were cutted on the microtome with glass knife and with tungsten knife, for both cases performing it was impossible to perform the cuts in thickneses below 10µm, as the material roll in it self. Only the cuts of 10µm were collected; it was perceived that this cuts presented low attachment with the glass slide making difficult the collection of the cuts(Figure 1a); also after taking them to the optical microscope, there were found some hols on the resin (Figure 1b), were the hairs were deatached, then this resin wasent selected for the process.

(**a**) (**b**)

Figure 1. Histological cuts of hairs included on Epoxy resin. (a)Image of the 10µm cuts on the glass slide ; (b) Image of the cuts on the optical microscope using the 20X objetive.

The experien with the polyester resin was almost the same as with the Epoxy resin, the this resine was also descarted.

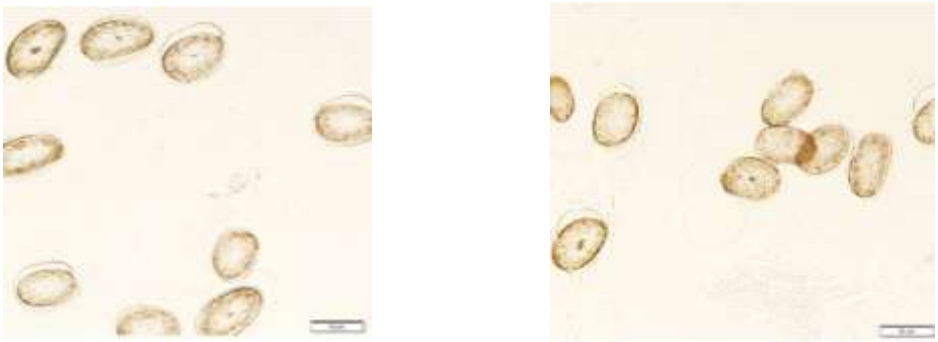
A commertial acrylic historesins was also used, here the inclusion process was simple and the cuts were possible on all the thicknesses (6µm, 8µm and 1 µm) and with both knifes, with good adhesion to the glass slide (Figure 2a), the cuts were then taken to the optical microscope.

(**a**) (**b**)

Figure 2. Histological cuts of hairs included on a comertial acrylic historesin. (a)Image of the 10µm cuts on the glass slide ; (b) Image of the cuts on the optical microscope using the 20X objetive

The visualization on the microscope, show that the hair fibers presented some cracks on the surface (Figure 2b) that seems not be due to the regular damage of the hair, then a second acrylic hystoresin from another brand was used and the same type of crack were found. The cuts with glass knife presented lower amount of cracks than the cuts performed with tungsten kniff but as the objective of the project was evaluation of the interior porosity of the hair, the images obtained wasn't satisfactory and this combination of inclusion media and knife were not selected.

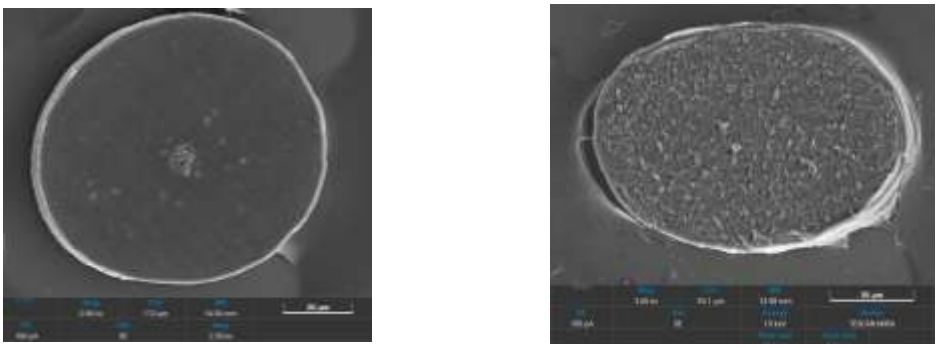
Then, it was decided to perform the cuts at -30°C using the cryostat, here the cuts were performed with tungsten knife and with stainless steel knife, as those were the only available for the equipment. For includind the hair, it was used O.C.T compound, that is higly used as inclusion media for biological soft tissues, but normally is not used for hair inclusion. Here with both knifes the cuts obtained presented conserved morphology when observed at the optical microscope (Figure 3), then both were taken to SEM microscopy.



(**a**) (**b**)

Figure 3. Histological cuts of hairs included on O.C.T compound 8 µm using the 20X objective. (a) Cutt with stainless steel knife; (b) Cutt with tungsten Kinfe;

The images obtained with SEM showed big differences between the cuts, where the surface was more conserved on the cuts performed with stainless steel (Figure 4). Then this combination was selected to the second phase of the project. The obtained SEM images were also used for investigation of the model that will be used for the measurement of the internal porosity.



(**a**) (**b**)

Figure 4. Histological cuts of hairs included on O.C.T observed at the SEM. (a) Cutt with stainless steel knife; (b) Cutt with tungsten Kinfe;

For the model the open-source program Fiji ImageJ was used together with the plug-in Labkit and the plug-in MorpholibJ. This plug-in uses machine learning algorithms that permits to train the model in order to identify the porous in an easy way, increasing the precision of the measurement. At the beginning, a manual selection of porous was made on the image, in order to teach the algorithm, which can be considered as a porous region, this process was performed several times until the algorithm perform the identification automatically. For the quantification, the image was binarized and using macros already developed by our lab the area and number of pours was calculated.

3.2. Verification of the reproducibility and the differentiation of the method.

For the validation, the experimental design presented on the Table 1 was used. Here the hairs were included on the O.C.T. compound and cut on the cryostat with stainless steel knife. The sections were taken to the SEM where 5 pictures were taken from each group. The images were evaluated with the program Fiji ImageJ as presented on the item 3.1 of this document and the results of porous area were tabulated (Table 2) and graphed (Figure 5). For verification of the differentiation, this is the capability of the method to distinguish treatments, the groups on day 1 were compared between each other using Anova with Dunnett post-test using a confidence interval of 95%. The groups were considered statistically significant different when the p value was lower than 0,05. Also, the reproducibility of the method was verified, for this the groups of the day 1 were compared with the groups of the day 2 that suffer the same treatment. The comparison was performed using a non- pared T student test.

Table 2. Results obtained of porous area for each treatment group on each day

Day 1			
Group 1	**Group 2**	**Group 3**	**Group 4**
Natural	Double Bleached	Hair Mask	Leave-in
1,943	3,281	2,218	2,454
0,001	2,832	2,123	2,349
2,051	2,762	2,139	2,536

2,133	5,525	1,606	2,678
0,067	2,258	1,756	2,467
		Day 2	
Group 1	**Group 2**	**Group 3**	**Group 4**
Natural	Double Bleached	Hair Mask	Leave-in
2,046	2,973	2,939	2,973
0,768	2,506	2,708	1,970
0,520	3,140	1,439	3,121
2,497	2,290	2,136	3,101
0,567	2,987	1,711	2,529

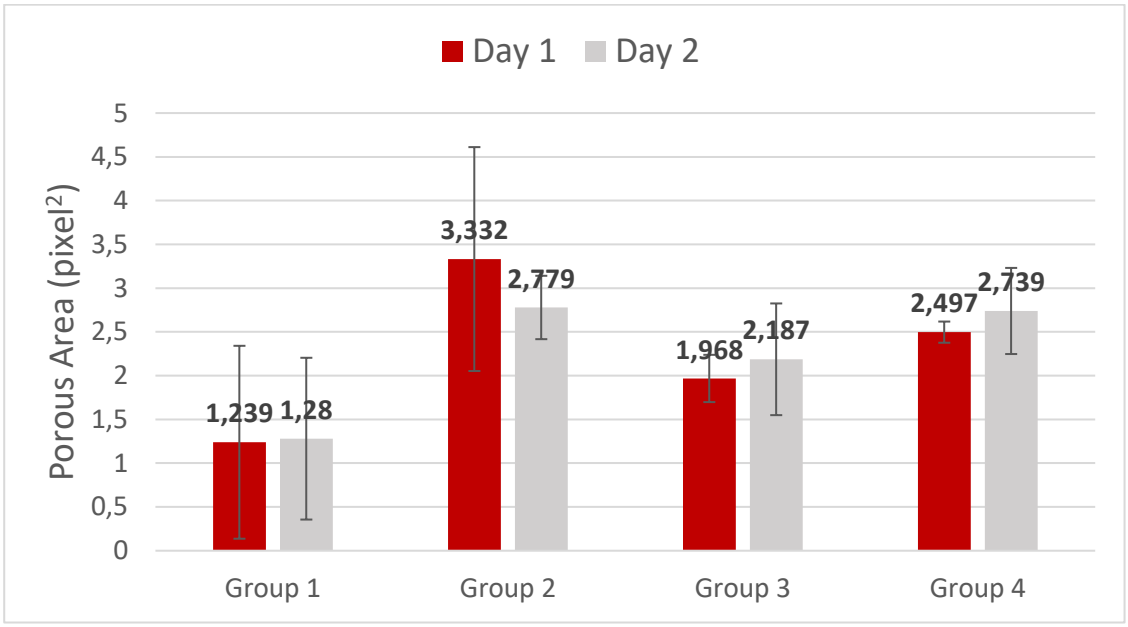


Figure 5. Results of average porous area (pixels^2) obtained for the fibers treated with the different treatments and on the different days.

The statistical comparison shown that there were no statistically significant differences between the results obtained on day 1 and the results obtained on day 2 for each group. Also, the natural hair (Group 1) presented lower porosity than the double bleached hair (group 2), but presented no statistically significant differences with the treated groups (Groups 3 and 4). Also, the Group 3, presents lower porosity than the Group 4 and Group 2.

4. Discussion

The investigation of the best combination of inclusion media, knife and cutting tool was crucial for the obtention of images of transversal cuts with preserved morphology of the interior of the hair fiber. The results showed that some of the hair fibers included on the commercial acrylic historesin presented small fissures on the surface for both knifes (glass and tungsten); the type of fissures make us believe that the historesin is reacting with the hair keratin, cracking the hair fibers; for some analysis this type of cracks do not interfere on the evaluation, but on this case, the morphology of the interior was crucial, for this reason, this combination was excluded, even when this was the standard method used for characterization of hair fibers. The use of cryotome appear as the best option for performing the cuts, as it was a fast and simple method

increasing the productivity on our lab and a simple view on the optical microscope, the morphology was well preserved. Looking more closer (SEM), the cuts performed with stainless steel knife presented a more preserved structure than the ones made with tungsten knife, showing the importance of the cutting tool used during the experiment.

Finally, after evaluation of the internal porosity of the hair fibers, the average value of hair fibers of some groups presented a high standard deviation, due to the natural differences between one fiber to other, even if the hairs are from the same batch. In order to reduce the standard deviation, the suggestion for future experiments is increase the samples from 5 to 12, having higher sample representativity and reducing the deviation.

5. Conclusion

Results demonstrated that the methodology reliably differentiated between treated and untreated samples, showing statistical differences in pore number and area. The technique was reproducible across multiple testing days. These findings highlight the potential of SEM-based analysis as a practical alternative to expensive techniques like SAXS or BET for assessing the impact of cosmetic products on hair porosity.

In conclusion, this method provides a direct, accessible way to evaluate internal hair porosity, enabling cosmetic companies to substantiate claims about product efficacy in improving hair structure.

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Conflict of Interest Statement.

NONE.

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