**Universidade do Minho**
Escola de Ciências

Ana Luísa Sá Fonseca

# Evaluation of chemical and physical alterations in hair fibres with different techniques

July, 2020

Ana Luísa Sá Fonseca **Evaluation of chemical and physical alterations in hair fibres with different techniques**

UMinho | 2020

**Universidade do Minho**
Escola de Ciências

Ana Luísa Sá Fonseca

# Evaluation of chemical and physical alterations in hair fibres with different techniques

Master's Dissertation
Master in Chemical Analysis and Characterisation Techniques

Under the supervision of
**Dr. Marta de Oliveira Ferreira**
and
**Prof. Dr. Susana Costa**

July, 2020

*“Pour chaque fin il y a toujours un nouveau départ.”*

(Antoine de Saint-Exupéry)

# ACKNOWLEDGEMENTS

Este projeto recebeu fundos do programa de investigação e inovação Horizon 2020 da União Europeia sob o acordo de concessão n.º 691128.

Neste espaço, quero expressar o maior e mais sincero agradecimento, na minha língua materna, a todos os que me apoiaram e ajudaram direta e indiretamente durante todo o processo de realização deste projeto, sem os quais não seria possível concretizar esta etapa académica e profissional.

Primeiramente, gostaria de agradecer à Dra. Marta Ferreira, Diretora Geral da inovapotek, pela sua orientação, disponibilidade e recomendações para a concretização deste projeto, assim como por todas as oportunidades que me proporcionou ao longo destes anos, essenciais para o meu crescimento e enriquecimento académico, profissional e pessoal.

Agradeço também à Professora Susana Costa pela orientação neste estágio, pela sua prontidão e disponibilidade e por nunca ter desistido de mim.

Ao Jatin, ao David, ao Long e ao Matt, meus colegas e orientadores na Universidade de Nottingham, agradeço toda a ajuda e apoio técnico na execução das técnicas utilizadas, ajuda essencial para o sucesso deste projeto. À Rachael, minha colega em Nottingham, agradeço o apoio, a companhia e o ombro amigo durante a minha estadia em Nottingham.

A todas as minhas colegas da inovapotek, todas as que passaram pela empresa desde o início desta minha aventura, o meu grande obrigada por me terem integrado tão prontamente, pelo auxílio e apoio e por todos os ótimos momentos. É um orgulho poder integrar uma equipa tão alegre e unida. Um agradecimento especial à Rita Matias e à Bárbara Tavares, duas amigas para a vida e dois pilares essenciais neste processo e em todo o meu percurso profissional na empresa, a quem irei ser eternamente grata.

Agradeço aos meus amigos e à minha família, todos sem exceção, que são um poço de motivação e animação. À Filipa e à Joana, um agradecimento especial, amigas de todas as horas, que me fazem acreditar que sou capaz de tudo, independentemente dos percalços no meio do caminho. À Bia, agradeço toda a ajuda e apoio nesta fase final e a linda amizade.

Um obrigada cheio de saudades ao meu grande companheiro de quatro patas, Keno, que foi a melhor e maior companhia durante 15 anos e que me animou tantas vezes durante este processo, mesmo sem se aperceber.

Por último, mas o maior agradecimento de todos, aos meus pais, que são os meus maiores exemplos de vida. Obrigada por nunca desistirem de mim, pela paciência e motivação, por tudo o que me proporcionaram ao longo da vida e por todos os valores que me incutiram.

## STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

Universidade do Minho, ____ of _____, 20___

Name: _____

Signature:

_____

**Avaliação de Alterações Químicas e Físicas em Fibras Capilares com Diferentes Técnicas**

# RESUMO

O cabelo humano está constantemente exposto a agressões externas provocadas por fatores ambientais ou tratamentos capilares que danificam as fibras capilares, afetando as suas propriedades físico-químicas, principalmente ao nível da cutícula. Pelo exposto, existe uma grande necessidade de prevenir e reparar o cabelo destes tipos de dano e a indústria cosmética progride nessa direção. Contudo, a maioria das técnicas utilizadas atualmente para a avaliação de possíveis danos no cabelo não são suficientemente sensíveis nem precisas para demonstrar o que acontece na superfície das fibras. Estas permitem apenas identificar danos drásticos que atingem o córtex das fibras ou apresentam somente imagens da superfície das fibras, não fornecendo informação quantitativa.

O objetivo deste estudo foi desenvolver e validar técnicas inovadoras que ainda não tinham sido exploradas para a análise de cabelo, para estudar as alterações químicas e físicas que ocorrem na cutícula do cabelo após um dano químico, após um dano por calor e após um dano químico, por exposição à radiação UV e à poluição.

Quando danificado, a superfície do cabelo torna-se mais irregular, havendo consequentemente um aumento da rugosidade. Assim, a rugosidade da superfície de fibras capilares foi analisada por Profilometria Ótica e sobretudo dois tipos de danos foram analisados: químico e por calor. Após testar e desenhar a metodologia para a avaliação da superfície das fibras capilares através desta técnica, os resultados finais mostraram um aumento da rugosidade da superfície para ambos os tipos de dano quando comparando com cabelo normal. As diferenças obtidas não foram estatisticamente significativas nas condições testadas, não validando a metodologia. Porém, esta técnica tem um grande potencial e um maior número de amostras poderá aumentar a significância dos resultados.

Foi também estudada a avaliação por ToF-SIMS. O protocolo foi desenvolvido de forma a calcular quantitativamente o grau de dano das fibras através do nível de degradação do ácido 18-metil-eicosanóico (18-MEA), sendo que foram analisados os três tipos de dano. Os resultados mostraram uma diminuição do rácio do pico de intensidade 18-MEA/CN- no cabelo danificado comparado com cabelo normal para todos os três tipos de dano. Este protocolo foi validado, apresentando ser uma técnica muito sensível, exata e precisa para a avaliação de mudanças em lípidos na superfície do cabelo induzidas por diferentes danos.

Palavras-chave: cabelo; cuidados de cabelo; ToF-SIMS; Profilometria Ótica.

**Evaluation of Chemical and Physical Alterations in Hair Fibres with Different Techniques**

## ABSTRACT

The human hair is constantly exposed to external aggressions by environmental factors or hair treatments that damage the hair fibres, affecting their physicochemical properties, mainly at the cuticle level. Therefore, there is a great need of preventing and repairing the hair fibres from these type of damages and the cosmetic industry keeps progressing in that direction. However, the majority of the techniques used for the evaluation of the hair damage are not sensitive nor precise enough to show damages on the hair surface, only showing drastic damages that reach the cortex of the hair fibres and giving images of the surface of the hair, not providing quantitative information.

The aim of this study was to develop and validate cutting-edge techniques, not yet explored for hair analysis, to analyse the chemical and physical alterations occurring on the hair cuticle after chemical, heat and chemical + UV + pollution damage.

When damaged, the surface of the hair becomes more irregular, having consequently an increase of the surface roughness. So, the hair surface roughness was analysed by Optical Profilometry and mainly two types of damage were analysed: chemical and heat. After testing and designing the methodology to evaluate the hair fibres surface through this technique, the final results showed an increase of the surface roughness for both types of damage when compared with normal hair. However, the differences were not statistically significant in the test conditions, not validating the methodology; still, it has a great potential and a higher number of samples could probably increase the results' significance.

Hair damage was also evaluated by ToF-SIMS and the protocol was designed to calculate the damage degree of the hair fibres through the methyl eicosanoic acid (18-MEA) level of degradation. The three types of damage prepared were analysed. Results showed a significant decrease of the mean peak intensity ratio 18-MEA/$CN^-$ on the damaged hair compared to normal hair for all three types of damage. The protocol designed for the ToF-SIMS was validated, showing to be a very sensitive, accurate and precise technique to evaluate the lipid changes on the hair surface induced by the different damages, showing significant differences that are not detected with the traditional techniques normally used.

**Keywords:** human hair; hair care; ToF-SIMS; Optical Profilometry.

# TABLE OF CONTENTS

# LIST OF ACRONYMS AND ABBREVIATIONS

**18-MEA** – 18-methyleicosanoic acid

**2D** – Two-dimensional

**3D** – Three-dimensional

**AFM** – Atomic force microscopy

**A.U.** – Arbitrary units

**CA** – California

**CMC** – Cell membrane complex

**CN** – Cyanide

**CRO** – Contract Research Organization

**CV** – Coefficient of variation

**DSC** – Differential scanning calorimetry

**DTA** – Differential thermal analysis

**DTG** – Differential thermogravimetry

**eV** – Electron volt

**GCP** – Good clinical practice

**GLP** – Good laboratory practice

**HC-BU** – High current bunched

**ISAC** – Interface and Surface Analysis Centre

**ISO** – International Organization for Standardization

**IRS** – Infrared spectroscopy

**LMIG** – Liquid metal ion gun

**NPL** – National Physical Laboratory

**OCT** – Optical Coherence Tomography

**pA** - Peak amplitude

**PM** – Particulate matter

**Ra** – Arithmetical mean height of a line

**Sa** – Arithmetical mean height of an area

**SD** – Standard deviation

**SEM** – Scanning electron microscopy

**SIFIDE** – Sistema de Incentivos Fiscais à Investigação e ao Desenvolvimento Empresarial

**SLES** – Sodium laureth sulfate

**SPF** – Sun Protection Factor

**TEM** – Transmission electron microscopy

**TG** – Thermogravimetry

**TMA** – Thermomechanical

**ToF-SIMS** – Time-of-Flight Secondary Ion Mass Spectrometry

**UoN** – University of Nottingham

**U.S.A** – United States of America

**UV** – Ultraviolet radiation

**UK** – United Kingdom

**XPS** – X-ray photoelectron spectroscopy

## LIST OF FIGURES

## LIST OF TABLES

# CHAPTER 1. INTRODUCTION

## 1.1. DISSERTATION ORGANIZATION

This dissertation is divided into 6 chapters, each chapter describing the following:

Chapter 1. Introduction:

In this first chapter, it is framed the subject and the goals of this dissertation, as well as the institutions where the project took place are presented.

Chapter 2. State of Art:

In this chapter, it is explored all the theoretical scientific basics for the understanding of the work developed, based on adequate bibliography. The topics addressed on this chapter are the following: claim substantiation on cosmetics; the structure of the hair; the different types of hair damage; the different types of hair care cosmetic products; examples of different *ex vivo* studies for the evaluation of the efficacy of hair care cosmetic products, focusing on the two techniques explored on this work (optical profilometry and time-of-flight secondary ion mass spectrometry – ToF-SIMS).

Chapter 3. Technical procedures:

In chapter 3 it is described all the practical work developed during the project, presenting the techniques and methods used, including the samples preparation and materials used, the equipment used and the experimental procedures and conditions.

Chapter 4. Results and discussion:

Here, all the results that were obtained during the practical work (including the results from both preliminary tests and validation of the methodologies) are presented and critically discussed.

Chapter 5. Conclusion and future work:

In this chapter, the results presented on the previous chapter are evaluated taking into account the aims of this project and the relevant conclusions of this analysis are stated. Also, it is mentioned the limitations of the validated methodologies and it is also pointed out possible solutions for their improvement and work that can be developed in the future to overcome these limitations.

Chapter 6. References:

In this chapter it is listed the references of the bibliographic research made over the time that this project was developed.

## 1.2. INSTITUTIONS PRESENTATION

This project was developed at inovapotek, Pharmaceutical Research & Development (Porto, Portugal), in partnership with the University of Nottingham (Nottingham, England).

inovapotek is a Contract Research Organization (CRO) founded in 2008 by two researchers from the Pharmaceutical Technology Department of the Faculty of Pharmacy of University of Porto, Portugal, which provides customized R&D, Testing, Regulatory and Consulting services to the Personal Care, Pharmaceutical, Medical Devices and Food Supplements industries. The aim of inovapotek is to help cosmetic companies to develop new and innovative products, ensuring their stability, safety, efficacy and regulatory compliance. This is ensured through customized formulation development services, stability studies and microbiological analysis, safety, efficacy, SPF and consumer testing, sensorial analysis, regulatory affairs' services according to the European cosmetic regulation and consulting and training. The company works with finished cosmetic products brands and also with cosmetic ingredients manufacturers and providers, helping them to solve their problems throughout the entire product development chain.

inovapotek is a Spin-off of University of Porto, certified with ISO 9001 and ISO 27001, approved by the French Ministry of Higher Education and Research (Crédit d'Impôt Recherche) and Approved by the Portuguese Ministries of Economy, Innovation and Development and of Science, Technology and Higher Education (SIFIDE).

inovapotek's main expertise is on safety and efficacy testing of cosmetic products and ingredients, conducting clinical studies at its facilities, but also performing *in vitro* and *ex vivo* studies. All sort of products can be tested, including skin care, body care, hair care, hygiene and sun care products with inovapotek. As inovapotek's mission is "to be in the scientific research vanguard in the cosmetic and pharmaceutical technology fields, promoting the development of innovative products that meet the consumers' demands and needs", there is a constant need of innovation of the present methodologies, exploring, developing and validating new techniques, focusing on the clients' needs and following the progression of the cosmetic and pharmaceutical industries.[1]

The University of Nottingham (UoN) is a public research university officially founded in 1948 which located in the UK city of Nottingham. Today, the university has five faculties: Arts, Medicine and Health Sciences, Science, Engineering and Social Sciences, and it also has other campus on China (Malaysia, Kuala Lumpur and Ningbo).[2], [3]

Interface and Surface Analysis Centre (ISAC) is a University of Nottingham (UoN) centre of excellence in surface and interface analytics setup in partnership with the UK's national metrology institute the National Physical Laboratory (NPL). ISAC offers access to a huge variety of surface analytical facilities, including techniques as Atomic force microscopy (AFM), Raman spectroscopy, contact angle, Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS), X-ray photoelectron spectroscopy (XPS), Optical profilometry, among others. This centre setup, co-ordinate and project manage top quality materials characterisation services and consultancy for and in collaboration with, commercial and research partners. [4]

### 1.3. PROJECT FRAMEWORK

According to the European Regulation on cosmetic products, in order to be attributed a function to a product it is necessary to substantiate the attributed claim through efficacy test, which can be performed *in vivo*, *in vitro* or *ex vivo*. For that matter, inovapotek has the challenge to develop and/or optimize specific solutions that can go hand-in-hand with the evolution of the cosmetic industry.[5]

Nowadays, the consumers have a massive range of cosmetic products available on the market, namely hair care products, with properties that are more specific every day, and expecting to get high quality and performance products. With this, the cosmetic industry has been developing new products which meet the consumers' expectations.

The human hair is constantly exposed to external aggressions by environmental factors or hair treatments and daily styling that damage the hair fibres, affecting their physicochemical properties, mainly at the cuticle level. On the other hand, the desire for products that improve the look and feel of hair has created a huge industry for hair care.

Characterization of the structure and physical and mechanical properties of hair are essential to develop better cosmetic products. Therefore, there is a great need of preventing and repairing the hair fibres from these type of damages and the cosmetic industry keeps progressing in that direction. However, the majority of the techniques used for the evaluation of the hair damage are not sensitive nor precise enough to show damages on the hair surface. The classic mechanical methods only show drastic damages that

reach the cortex of the hair fibres and imaging techniques give images of the surface of the hair, not providing quantitative information.

## 1.4. AIM OF THE PROJECT

The aim of this project was to develop and validate cutting-edge techniques, not yet explored for hair analysis, to analyse the chemical and physical alterations occurring on the hair cuticle after an external damage. This could help to understand and analyse different features of the hair, as well as to properly explore the efficacy of different cosmetic hair products.

After exploring a vast range of possible techniques to be used, two main techniques were tested: Optical Profilometry (a light microscope technique which can take 3D measurements from the sample) and "Time-of-Flight" + Secondary Ion Mass Spectrometry (ToF-SIMS) (which allows chemical surface analysis of the first few nanometres depth of the sample and helps to understand lipid differences on the hair surface).

# CHAPTER 2. STATE OF ART

## 2.1. COSMETICS AND CLAIM SUBSTANTIATION

Aesthetics and health are an increasing concern of the consumers, which expect that cosmetic products to be effective in meeting their needs. Nowadays, it is easy to obtain any kind of information and consumers are more aware and demanding regarding the benefits described in product's label.[6] Taking this into account, it is important for the manufacturers of the cosmetic products to properly substantiate the claims described on their products.

Claims for cosmetic products, including hair products, are required in order to have a successful marketing and promotion of the products. In the light of the European Regulation of the Cosmetic Products, claims need to be substantiated. So that a certain function can be attributed to a cosmetic, it is necessary that a claim can be supported through efficacy tests using appropriated and standard methodologies (valid, reliable and reproducible), which is dependent on the desired claim.[5], [7] Substantiation testing is required when a claim refers to the efficacy of the product or a benefit or improvement in a skin/hair/nail as a result of using that product, respecting the ethical considerations and the main principles of good clinical practice (GCP) as well as good laboratory practice (GLP).[7]

These tests can be performed *in vivo*, *in vitro* or *ex vivo*.[5], [7] *Ex vivo* tests with human hair tresses can provide valuable data to support product claims, being efficient and reliable alternative to *in vivo* and *in vitro* testing. The performance of *ex vivo* tests have advantages when compared with *in vivo* studies: it can provide the necessary data in a shorter period of time; there is not the need to select a panel of subjects with the needed characteristics and availability to participate on the study; it is possible to use of specific hair tresses, with the exact characteristics needed; it is easier to have a standardized assay; are easier to perform when a study is more complex.[6], [8] Normally, *ex vivo* tests are sensitive toward discrimination between products with both high precision and reliability.[7]

## 2.2. HAIR STRUCTURE

A hair is a very organized cylindrical structure divided into two components: root (the part which is inside the follicle) and fibre (the part which protrudes from the skin surface). The hair fibre has three main structures: cuticle (the external layer), cortex (the layer in between) and medulla (the axial central component) (Figure 1).[6], [8], [9]

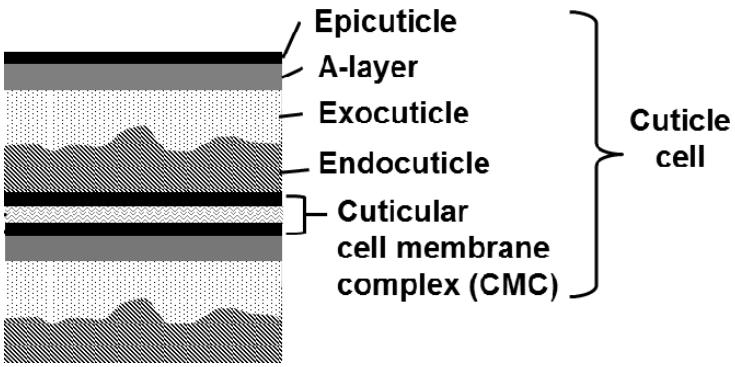



Figure 1. Fine structures of hair cuticle layer. [10]

The cuticle is the external layer of a hair fibre that forms a protective barrier and governs the frictional properties of the hair fibres, being also largely responsible for maintaining the structural integrity of hair.[6], [11] It consists of a high cross-link density proteins and it protects the inner tissues of the hair.[12] It covers the hair fibre from the scalp to the end and it is the most important component of the human hair, since it is the most external layer of the hair fibre and the first to be affected when the hair is damaged. Subsequently, the condition of the cuticle is responsible for the visual and tactile properties of the hair. Each cell of the cuticle has a rectangular shape and they overlap in such way, that only 1/6 of them are exposed, composing a laminar sorted flattened cell layers. These cells contain a thin external membrane, so-called the epicuticle, which is a protein coat covered by a strong lipid structure covalently bounded. The three major layers of the hair cuticle are the A-layer, the exocuticle and endocuticle. The A-layer lies immediately beneath the epicuticle cell membrane and it contains more than 30% of cystine in its composition. The exocuticle corresponds to about 55% of the cuticle and is also rich in cystine. The proteins of the exocuticle and from the A-layer are highly cross-linked by cystine. This highly cross-linked region of the hair fibre provides physical resistance to the hair and also gives to the cuticle resistance to external chemical damages, making the hair fibre rigid. The endocuticle has a low grade of cystine (about 3%), being a much softer layer than the superior ones.[8], [13]–[15]

Between the cuticle and the cortex there is structure composed by a proteinous layer and two lipidic layers, called cell membrane complex (CMC). This basically glues hair cells together, but not much is known about this structure yet.[16]

The cortex is the major component of the hair fibres and it is located just below the cuticle. Such as the cuticle, the cortex cells are filled by crossed-links of cystine. The cells have spindle shape and their distal surface is rough, irregular and they are tie crossly to each other. This structure lends mechanical properties, such as tensile strength and elasticity to the hair fibre.[6], [8]

The medulla is a thin cylindrical layer in the centre of the hair fibre, being rich in lipids and poor in cystine. It function has not been clearly defined yet; however it is known that it has a small effect on most aspects of cosmetic hair treatments.[6], [8]

The hair fibre is formed by inert cells, the majority keratinized. At a molecular level, hair fibres have a very rigid structure, allowing them to have both flexibility and mechanical resistance. Human hair is prominently composed of proteins, following by water and lipids, having also trace elements and pigments. The lipid and protein fractions play a major role in the structure and integrity of the hair fibre, protecting it against external agents. About 80% of the hair composition is keratin, a protein with a high grade of sulphur, from the cystine which is the main amino acid of this protein. Keratin is the protein which gives the hair strength, flexibility, durability, and functionality.[8], [13], [17], [18]

One major component of the outer surface of the cuticle is the lipid 18-methyleicosanoic acid (18-MEA), representing more than 40% of the total covalently bound fatty acids in human hair and about 50% in the hair fibre. [12], [13], [15], [19] 18-MEA is a branched-chain fatty acid covalently bound through thioester linkages to the cuticle surface of hair fibres and it plays an important role in surface hydrophobicity and in inducing hair to feel smooth to the touch. [12], [13], [19], [20] This lipid creates a hydrophobic surface and acts as a boundary lubricant to reduce friction resistance between hair fibres, not allowing the water between the hair fibres to spread. As the hair fibres are not tightly bind together, they can move easily, rearranging themselves. This reduces hair disorder alignment, contributing to a moist feeling of hair.[13], [19]

## 2.3. HAIR DAMAGE

Although human hair does not have a vital function, it has a psychological and social importance, being possible to change it according to fashion trends, culture or social values.[6] However, hair is easily damaged by a variety of mechanisms, like exposure to chemicals, colouring, environment or daily hair drying, leading to a unhealthy and bad looking hair on a macroscopic level detectable by the consumers.[7], [21] In fact, hair damage can be manifested in many ways, including changes along the fibres' surface. Being the cuticle the outer layer of the hair fibres, it is the most exposed to the outer impacts which can lead to its degradation and to the impair of its structural integrity, including the loss of the external lipids and the disorganization of the cells, influencing the sensory perception of hair.[13], [15], [19], [21] In fact, the gradual loss of cuticle layers can eventually lead to complete fibrillation of the fibre.[11]

Hair damage can be divided into physical (like friction from washing, towel drying, grooming, etc.) or chemical (like bleaching, perming, etc.) causes. Photodegradation oh human hair, i.e., exposure to UV radiation, has been of growing interest as it is known that sunlight can lead to dryness, texture alteration, colour degradation and lustre, also increasing stiffness and brittleness. Also hair dryers and straightening plates also cause damage the hair.[9], [14] So, hair is in constant exposure to aggression on our daily basis.

For example, the atmosphere surrounding us is polluted mainly by exhaust gas and particles from cars, industrial emissions and emission from "simple" household chores such as cooking and cleaning.[17] In a chemical point of view, ozone, sulphur dioxide and cigarette smoke represent a great part of the air pollutants.[15] There is a particular focus on particulate matter (PM), which are oxides of nitrogen and ozone, and have been shown to be harmful to the skin. Other pollutants that are on the sight of both consumers and cosmetic industry are the heavy metals and pollen.[22] In fact, when in contact with pollution, hair is subject to these environmental aggressions and some of its characteristics are affected: it loses volume, the colour, natural or artificial, fades, it looks drier and it misses strength and elasticity. Also, hair becomes more porous and less pleasant to the touch. The truth is that hair is quite vulnerable, lacking systems for self-protection, contrary to the skin, that has mechanisms to fight against stress and to regenerate normal metabolism.[17]

The atmosphere pollution also plays an important role in the degradation of certain atmospheric layers, unprotecting us from UV irradiation.[17] It is well known that the UV components of sunlight damage human hair, breaking down the disulfide bonds inside the hair fibre and on the surface of the cuticle, attacking both the melanin pigments and keratin of hair and oxidizing internal lipids. UV radiation can alter the mechanical properties of the hair, by oxidizing the cysteine present in hair's keratin to cysteic acid. It can also cause a decrease in hydration, increased permeability, leading to a loss of colour and shine and an increase in combing resistance.[18]

Hair dryers and flat irons are frequently used to dry and straight the hair and they can also damage the hair fibres, causing for example roughness, dryness and colour loss. Repeated cycles of wetting and blow-drying causes multiple cracks on hair cuticles.[7], [9] The thermal insult of hair from hot flat ironing appliances, for example, causes damage to the hair surface (cuticle including micropore formation and cuticle cell disintegration) and the structural proteins in the cortex. The internal and surface damage resulting from thermal treatment increases hair breakage especially with the additional stress of hair combing.[23]

One of the most affecting chemical damages is bleaching and colouring the hair. This type of alkaline treatment removes the 18-MEA from the hair fibres' surface through oxidative cleavage of the thioester bonds for example, one of the most important lipids of the cuticle, as mentioned above. This results in sulfonate groups that are fixed tightly inside the highly cross-linked protein layer, leading to the decrease of hair's surface hydrophobicity due to their low mobility (Figure 2).[12], [13], [19], [20] Bleaching hair also results in oxidation of cysteine with formation of cysteic acid, similarly to what happens when the hair is exposed to UV radiation.[21] Visually, the scales of the cuticle of the hair fibres begin to lift and partially detach from the surface.[11]

As mentioned above, hair fibres with 18-MEA do not let water to spread between the hair fibres. So, the removal of the lipid 18-MEA has the opposite effect, letting the water to spread easily between the hair fibres, tangling the hair as the hair fibres are binding together tightly (Figure 3). This affects the shape of hair tresses in wet conditions, influencing the hair appearance and sensory perception of hair, has this dries the hair and make it hard to comb.[13], [19]

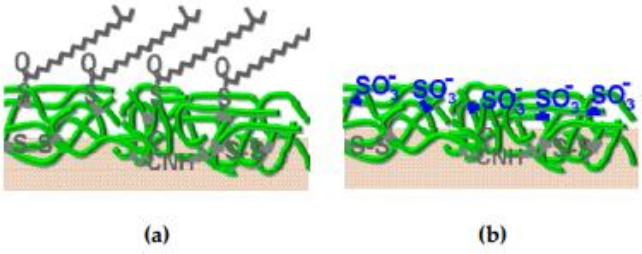



Figure 2. Illustration of epicuticle's degradation: (a) Untreated hair; (b) Chemical damaged hair. [19]

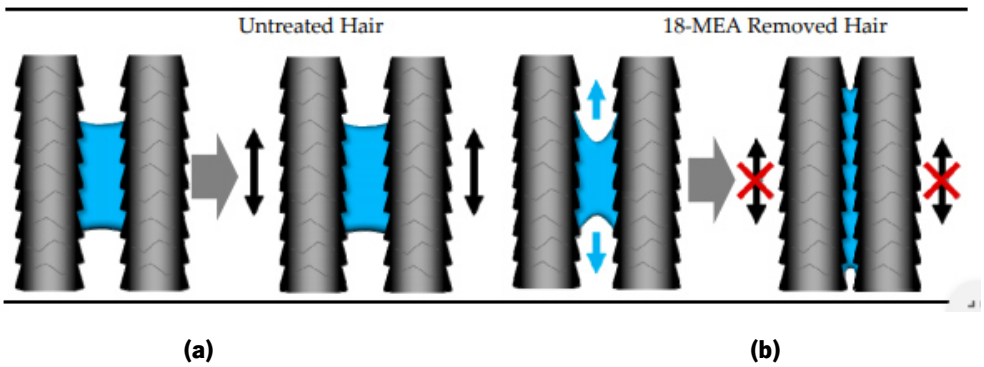



Figure 3. Representation of the interactions between hair fibres in wet environment when undamaged (a) and when damaged (b).

## 2.4. HAIR CARE PRODUCTS

Healthy and good looking hair are looked-for properties for many women with hair damaged by chemical treatments, heat styling and environmental factors, having created a huge industry for hair care, as there is the necessity of being updated with the latest hair fashion styles combined with hair protection and beauty.[7], [8], [15], [19]

It is important to focus in one of the newest and growing interest of the consumers: products to protect the hair of the pollution we are exposed every day and to prevent the hair from the damage caused by it. For example, between 2011 and 2013 there was a 10% rise in the number of beauty and personal care products launched carrying an anti-pollution claim around the world, being 31% of these launches of hair products. Also, the cosmetics industry in particular has been alerting the consumers to the immediate and perceptible effects of the pollution.[24] Hair care industry is continuously committed formulate new, more effective and safe products.[25]

There can be two types of hair care cosmetic products related to hair damage: the ones which protect the hair and prevent it from damage; and the ones which treat already damaged hair. Depending on the formulation of the products, they can function either way.

Antioxidant ingredients are usually used in cosmetic formulations claimed to help reducing damages on hair fibres. They protect against oxidative damage and also help to repair the hair, neutralising free radicals and retard lipid oxidation.[18] For example, it has been shown that treatments with antioxidants improve the mechanical proprieties that are lost after damaging the hair with UV irradiation, preventing protein degradation and lipid peroxidation, which suggests an improved integrity of the fibre. Also, it was showed that antioxidants also improve the cuticle scale, smoothing the scale edge as well as they conserve the colour and shine of dyed hair tresses.[18] Nowadays the cosmetic industry provides a range of products with antioxidants that reduce the negative effects of air pollutants and colouring the hair and detoxify both scalp and hair.[15], [18]

Vitamins are also greatly used on this type of formulations. For instance, vitamin B5 functions as humectant, increasing the water content and improving the elasticity of hair.[18]

Silicones have also been used in hair treatments for damaged hair for many year. The low surface free energy of the silicones provides pleasing aesthetic properties imparted to the hair. The deposition of silicones in the hair surface forms a uniform thin coating on each hair fibre, due to their high coefficients of spreading. This external coating operates as a physical protection mechanism, creating a physical barrier which prevents hair damage. This barrier reduces the combing forces and the triboelectric

charging, giving to the hair a soft feel, a more lustrous appearance and an improved manageability.[21], [26]

## 2.5. EFFICACY OF *EX VIVO* STUDIES OF HAIR CARE PRODUCTS

Evaluating efficacy of hair care products *in vitro* or *ex vivo* involves use of normally highly accurate equipment. Those techniques have advantages over in vivo studies as mentioned above, besides also using specific hair tresses. It is well described and there are evidences that properties and effects of cosmetic products on hair fibres can be assessed through many ex vitro and vitro methods, some of them described below.[6]

A great way to understand the behaviour of the hair fibre surface is using imaging analysis techniques, such as Scanning electron microscopy (SEM), Transmission electron microscopy (TEM), Atomic Force Microscopy (AFM) or Optical Coherence Tomography (OCT).[6], [8]

For example, SEM is a great methodology frequently used on efficacy studies of hair care products. SEM is an electron microscope capable of producing highly amplified and sharp of sample surfaces, so it can help to evaluate the possible changes in the surface morphology of the hair fibres due to the different treatments.[6], [8], [11], [18] A great utility of this image analysis equipment is that it allows to evaluate the sample's surface topography and composition.[6] However, this is not a quantitative methodology, only qualitative and this is not a so simple technique to be used for routine.[11] Also, for certain hair strain properties, structural and morphological changes and particle deposition over the surface, for example, it is necessary a metallization of the surface, i.e., covering the surface with a micrometre film of a conductive material.[6], [8]

AFM is an imaging analysis technique commonly used for the analysis of hair surface as well. This method enables studying material surfaces beyond the scope of electronic and optical microscopy, where a probe has physical contact with the sample, acquiring each point of the topographic component. The advantage is that there is no need to make the sample conductive, as in such techniques such as SEM.[6], [8], [19]

Infrared spectroscopy (IRS), Raman spectroscopy, Photoluminescence Spectroscopy, Electrophoresis, Diffuse Reflectance Spectrophotometry are some of the spectroscopy analysis methods used to evaluate physical, structural and morphological changes on hair.[6]

Protein degradation is one of the conditions which can be quantitatively analyse through spectrophotometric methods in order to understand the damage degree of the hair fibres.[6], [18] The hair fibre, when exposed to physical or chemical damages, may suffer damage to its structure and thus its protein composition is altered. Since hair is primarily a proteinaceous material, methods used to

analyse the amount of protein abraded from hair can perfectly be used to assess the damage degree of hair tresses.[6], [11] However, these are spectroscopy analysis, which take a lot of preparation and do not help to understand specifically what happens on the surface of the hair fibre. [6]

Thermal analysis is defined as a "group of methods by which a physical property of a substance and/or its reaction products is measured as a function of temperature and/or time while the substance is subjected to a controlled temperature program and under a specific atmosphere".[27] Some techniques that are included on this type of analysis are thermogravimetry (TG), differential thermogravimetry (DTG), differential thermal analysis (DTA), differential scanning calorimetry (DSC) and thermomechanical (TMA).[6] These techniques are helpful to understand the efficacy of thermal protective cosmetic products, as well as to demonstrate changes in hair mechanical properties, for example, after being submitted to high-temperatures of curling ironing or straightening plates. For examples, DSC is a well-known technique to study the degradation of hair keratin due to high-temperature exposure and to evaluate the effect of hair care products on keratin structure. [6], [7], [23] However, for a better characterization of the hair and products, it is recommended to use a combination of the mentioned methods.[6]

As breaking stress evaluates fibre integrity, mechanical strength/resistance of the hair is measured many times to evaluate the damage degree of a hair fibre and/or the efficacy of hair care products, using instruments like an Instron Tensile Tester, a Dynamometer or a Texturometer to evaluate the tensile properties of the hair fibres, measuring strength proprieties or elasticity of hair fibre due to tension force or load.[6], [8], [11], [18] The principle of the methodology is the application of a force by the instrument or loading to disrupt the fibres, which is registered by software, converting the information into a load graph of elongation.[6] However, there is a long-standing hypothesis, that the cortex is primarily responsible for the tensile properties of the hair and that the cuticle has little involvement; so, these type of evaluations are not the indicated to analyse the condition of the cuticle, which is the first layer to be affected by external damages. Also, these techniques lacks sensitivity and precision desired to analyse minimal changes on the hair.[11], [18]

It is possible to assess the combing resistance as well, with a Dynamometer or a Texturometer for example, adapting a proper comb on the instrument, which will comb the hair tress with a certain setting and the forces that resist its motion from the point of insertion until it clears the tip end of the swatch are recorded.[6], [7]

A lot of these methodologies can be applied to support a "damage hair reduction" claim, which is the case of protein loss, SEM, AFM, etc. Nevertheless, with the development of new innovative hair care

products in a market in growing competition, there is an increasing need for more polished methods to meet the evolution in these product innovations and consumer concerns and desires.[7]

## 2.6. OPTICAL PROFILOMETRY

Optical profilometers can in principle be used to measure the roughness of surfaces that are not accessible for mechanical profilometers.[28] Optical Profilometry qualifies and quantifies the relative contribution of different characteristics of a samples' surface (topography), such as structure, waviness, roughness, volume, and others, through the production of a detailed three-dimensional surface map of a test surface.[29], [30] So, through this technique, it is not only possible to obtain high-resolution images of the samples' surface, it is also possible to quantify various topographical characteristics.

Optical profilometry is a rapid, non-destructive and non-contact three-dimensional technique based on interference microscopy used to measure surface topography and height variation across a sample.[31], [32] An optical profilometer (Figure 4) is a microscope based on the principle of two-beam optical interferometry, in which light from a lamp is split into two paths by a beam splitter: one path directs the light onto the surface under test, the other path directs the light to optically flat reference. Wave properties of light are used and reflections from the two surface are recombined and projected onto an array detector. The variation in optical path length of light reflected from the two surfaces are calculated, generating a profile of the samples' surface topography. The sample is scanned over a vertical (z axis) and lateral (x and y axes) ranges, vertical (z axis) resolution can be on the order of several angstroms while lateral (x and y axes) resolution depends upon the ranging from microscopic to atomic scales. [30]–[33]

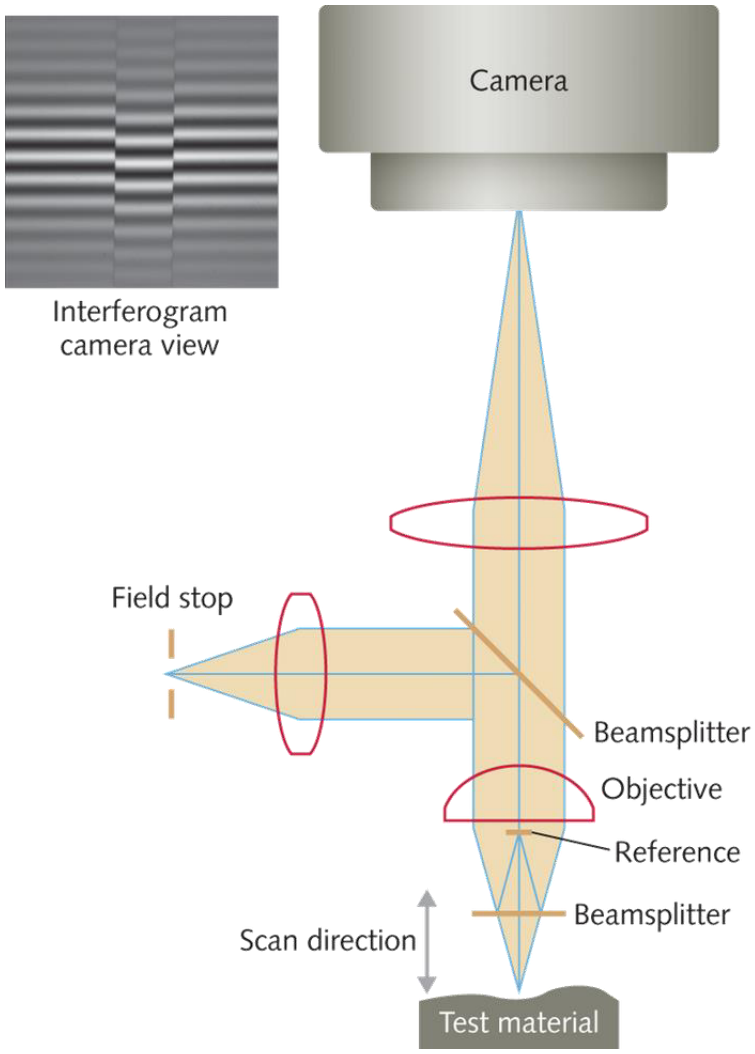



Figure 4. Representation of an Optical Profilometer. [29]

This technique has several advantages: it gives true 2D and 3D high-resolution imaging; provides fast data acquisition over large areas; it is a non-contact and non-destructive technique, being possible to preserve the analysed sample; gives a quantitative aspect through the calculation various roughness parameters, such as Ra (arithmetical mean value of the movement of the profile above and below the centre line of the surface) and Sa (the arithmetic mean deviation); it has a large Z-axis range, from a few nanometres up to feature heights as great as 2 cm; it gives a variable field of view, from 6 mm down to 8 µm; and it delivers critical dimension measurements (X,Y, and Z).[31], [34] This technique enables the samples' surface to be studied more precisely, as the optical light beam that sweeps the surface detects certain tiny microirregularities, which the majority of the mechanical profilometers' stylus are not able to penetrate.[34]

This method has not been well explored when it concerns to the hair surface analysis. However, there is a lot of potential, as it gives us a lot of qualitative and quantitative information with high resolution. Besides

the imaging of the hair cuticle, it is also possible to conclude about the damage degree of the hair fibres through the quantification of the surface's roughness, knowing that, with the damage, the surface has a more irregular profile, having consequently an increase of the surface roughness.

## 2.7. TIME-OF-FLIGHT SECONDARY ION MASS SPECTROMETRY - TOF-SIMS

Time-of-flight secondary ion mass spectrometry (ToF-SIMS) is a surface-highly sensitive analytical method that is able to detect species present at low (z to ppb) concentrations as well as provide the spatial and/or depth distribution of the species, describing the chemical composition and distribution of the samples' surface.[35]–[37] It is used for surface spectroscopy, surface imaging and depth profiling, without destructing the sample in analysis.[36], [37]

A ToF-SIMS instrument includes, a ultrahigh vacuum system, which increases the mean free path of ions liberated in the flight path, a particle gun, a flight path and a mass detector system (Figure 5).[37] ToF-SIMS uses a focused pulsed primary ion beam ($Bi_n^+$, $Cs^+$, $Ar^+$, etc.) to remove molecules from the very outermost surface of a sample, inducing a fragmentation cascade. So, there is the desorption of neutrals, secondary ions (positive or negative) and electrons from the first few monolayers of the sample. The secondary ions are then accelerated into a "flight tube" on their way towards a detector and their mass is determined by measuring the "time-of-flight" of the particles, i.e., the exact time at which they reach the detector, on a scale of nanoseconds.[36], [37] The information obtained from a ToF-SIMS spectrum related with one single analysis is tremendous, as both elemental species and high mass molecular fragments can be detected.[35]

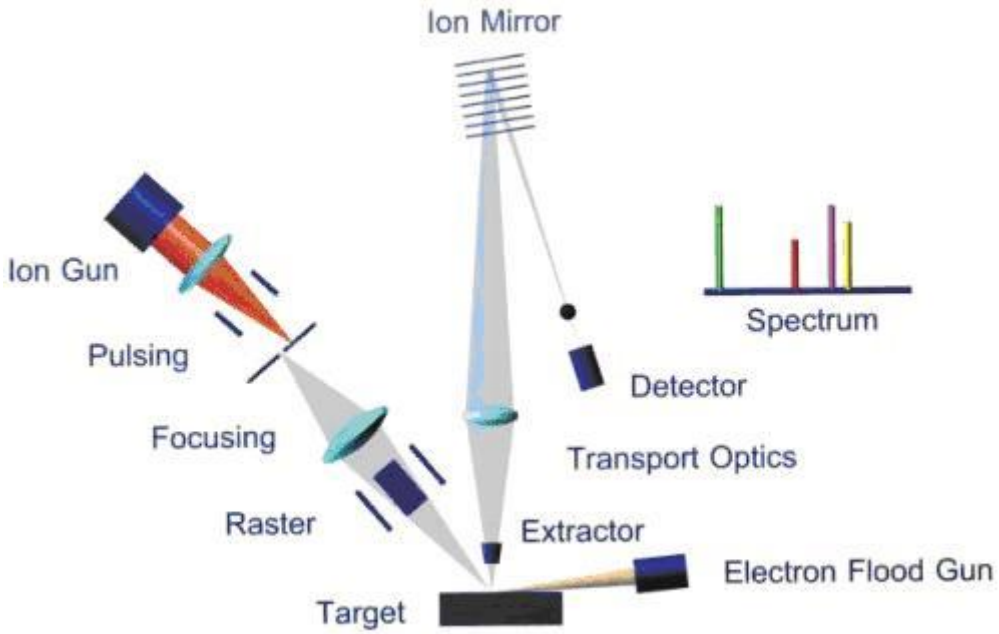



Figure 5. Schematic diagram of an IonTOF ToF-SIMS instrument. [37]

With this technique it is possible to distinguish particles with the same nominal mass, because of its high mass resolution; detect ions, isotopes and molecular compounds; trace elements or compounds in the ppm to ppb range, because of its high sensitivity; obtain sub-micron images to map any mass number of interest; analyse the chemical stratigraphy on material surfaces due to its depth profiling capabilities; and to retrospectively produce maps and interrogate regions of interest for chemical composition by storing information from one sample to another.[37] A single secondary ion mass spectrum can be used to describe the constituents of one specific point of the sample's surface. Alternatively, if the incident beam is rastered across several points of a surface's area, it is possible to build a chemical image map of that selected area.[36]

This technique has been used to see the deposition of various components on the hair surface, like silicones[21], various substances from hair treatments[38]–[40], metals from pollution contamination[41], to characterize elemental distributions on whole and longitudinal sections of hairs[38], to characterize the human hair structure with longitudinal sectioning[40] and another numerous applications. Nevertheless, ToF-SIMS is not so commonly used and it is not so well described for the characterization and analysis of the level of degradation of the hair fibres' surface.

Still, semi-quantitative analysis of 18-MEA through ToF-SIMS can give us information about the state of the hair cuticle. This fatty acid is detected through ToF-SIMS as the negative molecular ion peak at m/z 341, the molecular ion peak of 18-MEA, on the negative spectrum obtained through the equipment. The

oxidative cleavage of thioester bonds can be evaluated by monitoring the change in the peak m/z 341 intensity. The $CN^-$ ion is used for normalization, since the matrix of hair surface is keratinous protein and the $CN^-$ ion is strongly detected in the ToF-SIMS measurement of hair samples. So, the peak intensity ratio of the molecular ion peak of 18-MEA and the $CN^-$ peak (m/z 26) from the matrix protein is used to evaluate the 18-MEA amount on the hair.[12], [19], [20] With this, it is possible to quantify the degree of damage of a hair fibre surface and compare untreated and treated hair fibres, along with comparing hair treatments, in order to verify the efficacy of hair cosmetic products used for damage prevention or treatment of damaged hair.

# CHAPTER 3. TECHNICAL PROCEDURES

In this chapter it is described the several damage procedures performed to mimic the everyday factors which leads to hair damage on different hair tresses. The samples preparation was performed at inovapotek's facilities and the different damage protocols were previously developed by inovapotek's personnel. Some treated samples with products which protected or changed the hair chemical composition and possible physical alterations on the surface of the hair fibres were also prepared.

After the samples' preparation, these had to be properly packaged for transportation, as they had to be sent to the School of Chemistry of University of Nottingham facilities, were they were analysed.

Some techniques were examined before choosing the most promising ones, being necessary to understand the application of each technique in order to identify the most reliable ones. After this identification, it was important to establish the amount of sample needed to perform the analysis, the error of the technique and the number of replicas necessary. Then, it was possible to validate and develop a methodology with the most promising techniques. With these preliminary tests, it was also possible to understand the time needed to perform each analysis and the costs.

Two main methods were used to measure the damage degree of each sample: optical profilometry and "Time-of-Flight" + Secondary Ion Mass Spectrometry (ToF-SIMS). Also on this chapter it is presented the equipment used and the optimization of the used methodologies for the analysis of damaged hair samples.

## 3.1. SAMPLES' PREPARATION

Dark brown straight hair from Caucasian donors was used and several samples were prepared, regarding the different possible type of damages that hair fibres can suffer on the daily basis. The samples prepared are presented on the Table 1.

Table 1. Prepared samples of damaged hair and respective virgin and treated hair

| Condition | Samples |
|---|---|
| Chemical damage – hair discoloration | Virgin hair without treatment |
| | Discoloured hair without treatment |
| | Discoloured hair with shampoo treatment |
| Heat damage – hair stretching with straightening plates (8 heat damage cycles) | Virgin hair fibres without treatment |
| | Damaged hair fibres without treatment |
| | Damaged hair fibres with serum treatment (with an active ingredient) |
| | Damaged hair fibres with serum treatment (placebo) |
| Chemical, UV and pollution damage - hair discoloration, followed by UV radiation exposure and tobacco smoke exposure | Virgin hair without treatment |
| | Chemical, heat, UV and Tabaco damaged hair |

### 3.1.1. VIRGIN HAIR SAMPLES

All respective virgin hair samples (from the same hair donor) of the three type of damage performed were washed with a 12% sodium laureth sulfate (SLES) solution in order to remove any type of possible contamination on the hair surface.

60 μL per gram of hair tress of the SLES solution were applied to each pre-wet hair tress with tap water, with constant water flow of 2420 mL/min, for 10 seconds. The application was performed with a syringe from the top to the bottom of each hair tress. After massaging the hair tress manually during 30 seconds to simulate hair wash, the product was rinsed off with tap water, with constant water flow of 2420 mL/min, for 30 seconds. The excess of water was removed manually and the hair tresses were combed five times with a wide tooth comb. The samples were let dry at room temperature for 20 to 28 hours (24 ± 4 hours).

### 3.1.2. CHEMICAL DAMAGED SAMPLES

Before being submitted to damage, two hair tresses were washed with 12% SLES as described above. For the chemical damage, the hair discoloration was made by mixing in a non-metallic container 1 part

Farmavita® Life Bleaching Powder Professional and 2 parts of oxidant Farmavita® Farmacolor Cream Developer (4 grams of Farmavita® Life Bleaching Powder Professional + 8 mL of oxidant Farmavita® Farmacolor Cream Developer per 4 g hair tress). The mixture was applied to the correspondent hair tresses with the help of a brush, on both sides. The hair tresses were protected with aluminium foil and allowed to stand for 30 minutes. Then, the samples were washed with tap water for 2 minutes, with constant water flow of 2420 mL/min in order to remove the mixture.

After the discoloration, a hair tress without treatment was washed with a 12% SLES on the same way the virgin hair tresses were washed, as explained above.

### 3.1.3. CHEMICAL DAMAGED SAMPLES TREATED WITH SHAMPOO

After being exposed to the chemical damage process, one of the hair tresses was treated with a shampoo containing a cider vinegar/glycerine extract and Mallow flowers[1], used to wash a hair tress after discoloration. 60 μL per gram of hair tress of shampoo were applied with a syringe from the top to the bottom to the pre-wet hair tress (with tap water, with constant water flow of 2420 mL/min, for 10 seconds). After massaging the hair tress manually during 30 seconds, the product was left on for 2 minutes and afterwards rinsed it with tap water, for 30 seconds, with constant water flow of 2420 mL/min in order to remove the treatment shampoo. The hair tress was combed five times with a wide tooth comb and left to dry at room temperature for approximately 24 hours to 72 hours (on weekends). The product was applied ten times, repeating the procedure described.

### 3.1.4. HEAT DAMAGED SAMPLES

In order to properly damage the hair fibres and to ensure that each individual hair fibre directly contacts with the flat iron and the heat is evenly distributed, 60 fibres with 16 cm long were collected from a hair tress and three suitable hair supports were prepared (20 hair fibres per support).

To assemble the hair fibres on the supports, the hair fibres were glued spaced from each other, onto two 6 cm long and 2 cm wide paper rectangles at both ends of the fibres (Figure 6). Both paper supports were glued to two plastic holders, ensuring a distance of 10 cm from the top to the bottom of the final support. A second holder was placed on each end onto the paper supports, trapping the paper rectangle were the fibres were glued. Then, each combination of plastic holders were placed on rails, as well as vertical holders with 16 cm on each side of the support, in order to have the final support, as showed in Figure 7.

[1] For confidential reasons, the product name cannot be disclosed.

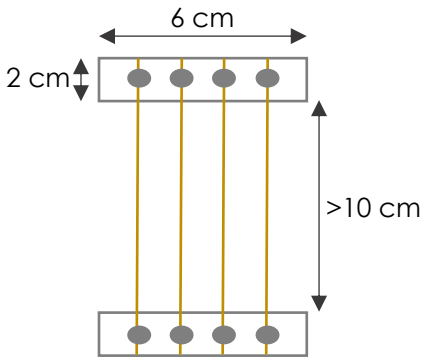



Figure 6. Exemplification of the assembly of the hair fibres.

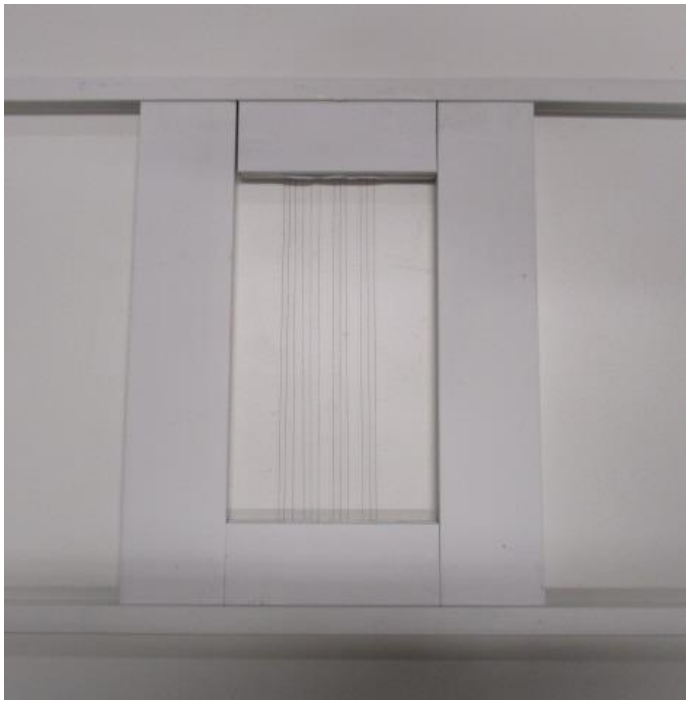


Figure 7. Example of the collected and entrapped hair fibres on the suitable support.

After the assembly, the hair fibres in the support were rinsed with tap water with a constant water flow of 2420 mL/min, for 10 seconds. After, the fibres were immersed in a 12% SLES aqueous solution, under stirring, for 30 seconds, in order to simulate the hair wash. The hair fibres were washed with tap water for 30 seconds, with constant water flow of 2420 mL/min in order to remove the 12% SLES, letting the hair fibres dry for 48 hours at room temperature, afterwards.

The heat damaged was then induced on the hair fibres assembled on one of the hair supports by stretching the hair fibres 9 times with straightening plates (Rowenta® for Elite model Look) at 200 °C for 10 seconds, while in the support.

### 3.1.5. HEAT DAMAGED SAMPLES TREATED WITH A COSMETIC SERUM

After the assembly of the hair fibres, the fibres of the other two supports were treated with two different leave on cosmetic serums: one with 0.5% of a very strong antioxidant active ingredient[2], which efficacy was being tested, and the other with the respective placebo. The cosmetic serums were applied in between each damage cycle.

For that, after the assembly, the hair fibres in each support were washed with 12% SLES, as explained on the previous point. To remove the excess of water, it was used a hand dryer (mode: cold temperature; speed: 2) for 30 seconds and then the fibres were immersed in 5 grams of the cosmetic serum for 5 seconds. The hair fibres were massaged manually for 30 seconds, to spread the product homogeneously, letting the hair fibres dry for 48 hours at room temperature, afterwards. This procedure was performed three times before the first induced damage cycle with the flat iron, as described on the previous point. Then, eight cycles of heat damage with the flat iron intercalated with seven product's applications were performed. By the end, the fibres were rinsed on the support with tap water for 60 seconds, with constant water flow of 2420 mL/min and gently massaged manually in order to remove the deposit of the product on the hair surface.

### 3.1.6. CHEMICAL, UV AND POLLUTION DAMAGED SAMPLES

First, the hair was washed with a 12% SLES and chemically damaged, as explained above.

After, the UV damage was done by submitting the hair tress to UV exposure, to induce damage, during cycles of 18 hours per day (9 hours per side of the tress), corresponding to two solar days exposure. The irradiation was done using the equipment SPF-290AS Solar Light over rectangular areas, at a defined distance UV source to hair tress (12 cm). Six cycles of UV irradiation were completed with a UV irradiation intensity of 9.22 $W/m^2$.

To mimic pollution damage, cigarette smoke was used for the exposure of the hair tress. The cigarette exposure protocol was the following: the hair tress was combed five times with a wide tooth comb and then it was placed inside a Büchner flask using an appropriate support (Figure 8). Five cigarettes were placed on the top of the Büchner flask (Figure 9) and the flask was placed in a box, used to protect the surrounding atmosphere. The water pump system used to haul the cigarette smoke was turned on (water flow: 5.5 L/min) and the five cigarettes lighted with an interval of 2 seconds each cigarette. The box was closed the water pump was turned off, as well as the smoke control tap on the Büchner flask 5 minutes

[2] For confidential reasons, the product name cannot be disclosure.

after closing the box. After 20 minutes, the cigarettes' support was removed as well as the hair tress from the Büchner flask. Six cycles of exposition to cigarette smoke were completed (120 minutes), where the Büchner flask and the box used to protect the surrounding atmosphere were cleaned with alcohol between each cycle and the water pump was turned on during 15 minutes, in order to circulate the air in all the system, promoting the removal of residual smoke present on the system tubes.

Figure 8. Support of the hair tresses on the Büchner flask.

Figure 9. Cigarettes placed on the top of the Büchner flask.

### 3.1.7. SAMPLES TRANSPORTATION

Each tress or support was properly wrapped in aluminium foil individually and appropriately identified and labelled on the outside of the aluminium foil for the correct identification of each sample.

## 3.2. EQUIPMENT AND TECHNIQUES

### 3.2.1. OPTICAL PROFILOMETER CHARACTERISTICS [42]

Samples were imaged using a Zeta-20 Optical Microscope (Zeta Instruments, CA, U.S.A) (Figure 10), a light microscope technique which can take 3D topography measurements from a sample and provides a non-contact method to measure surface topography and height variation across a sample. The instrument does not use a split light source, which provides high light throughput for imaging. This enables true colour images to be acquired for a wide range of sample types, including very dark surfaces and those with low reflectivity.

The other advantage provided by the Zeta-20 instrument is the ZDot™ technology, an advanced precision focusing pattern based on a confocal grid illumination which simultaneously collects high-resolution 3D data and a True Colour infinite focus image. This generates contrast irrespective of the sample type and therefore allows the system to accurately detect and provide height measurements for virtually all surfaces, including those that are transparent, tilted or display large variations in height. The instrument uses this focusing pattern to accurately map multiple focal planes across the user specified Z range, which can then be rebuilt to create an accurate 3D profile of the sample.

One of the proprieties that the Zeta-20 measures is 3D texture, quantifying the sample's roughness and waviness. The equipment enables measurement of roughness ranging from tens of nanometres to very rough surfaces and from angstroms to microns to smooth surfaces. It also enables visualizing very fine surface detail by revealing small changes in the slope. With this equipment, it is possible to evaluate the roughness of the hair fibre surface, allowing to conclude the damage degree of the samples, as when being submitted to a type of damage, the hair fibres surface has a more irregular profile and consequently an increase of the surface roughness is expected.

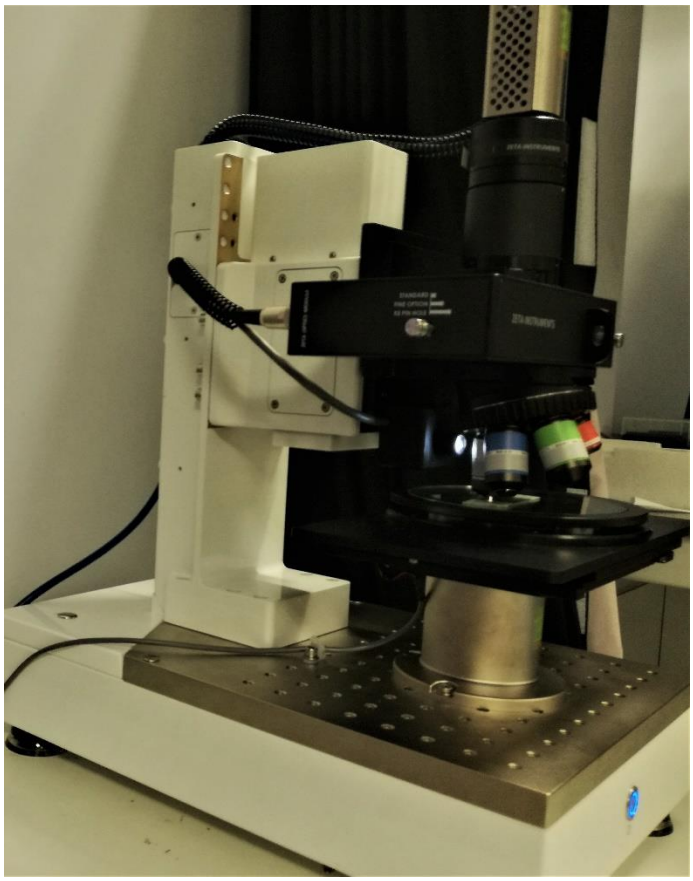


Figure 10. Zeta-20 Optical Microscope (Zeta Instruments, CA, U.S.A).

### 3.2.2. SAMPLES PREPARATION FOR OPTICAL PROFILOMETRY ANALYSIS

For this technique, hair fibres were fixed straight and parallel onto glass slides with tape on the ends of the fibres, being the glass slides loaded directly into the instrument (Figure 11). For the validation of the methodology, mainly two types of damage were analysed: chemical and heat damage. Both damaged and respective virgin hair fibres were analysed, being analysed 5 different areas of 3 different fibres of each type of hair (n=15).


Figure 11. Samples assembling on the glass slides for the Optical profilometry analysis.

### 3.2.3. IMAGES AND DATA COLLECTION AND ANALYSIS

Images were collected at ×50 magnification. The samples were vertically scanned at regular depth intervals between user-defined upper and lower reference surfaces. The Z resolution was defined as the total depth scanned divided by the number of vertical scans acquired. Both 2D and 3D optical images were produced and accurate step height measurements could then be acquired for any feature in the image respective to the underlying flat substrate. 2D optical images were used to perform the image analysis.

Firstly, two types of analysis were performed in order to understand which of the analysis was the most reliable for the optimization of the methodology: box roughness and line roughness. With the box roughness, a rectangular area (100 x 30 μm) of an individual hair fibre surface was selected and the software calculated the roughness of that selected area. The line roughness allowed to draw as much lines as it was intended (5 horizontal lines per image, all across the image) and the software calculated the mean roughness of the areas where the lines were drawn.

A descriptive statistical analysis of the results was performed using Microsoft Office Excel® and IBM® SPSS® Statistics Version 24 (IBM Corp., U.S.A.), including the calculation of mean, standard deviation, outliers identification and graphic representations. When observing outliers, their influence on results analysis was studied by performing one analysis with the actual values and at least one other analysis eliminating or reducing the outlier effect. Normality tests (Shapiro-Wilk test) were performed in order to assure normal distribution of the objective data obtained. If normal distribution of the data was verified, independent samples t test was applied to compare the values obtained for the damaged hair *versus* respective virgin hair. For non-normal distributions, a non-parametric test (Mann-Whitney test) was performed. The significance value was established at 0.05 and a power of 0.95.

### 3.2.4. TOF-SIMS ("TIME-OF-FLIGHT" + SECONDARY ION MASS SPECTROMETRY) EQUIPMENT CHARACTERISTICS [36]

All analysis was conducted using an IONTOF ToF-SIMS IV (IONTOF, GmbH) (Figure 12) equipped with a single-stage reflectron mass analyser which gives amass resolution up to 7000 at m/z = 29. ToF-SIMS equipment used on this project have a liquid metal ($Bi_n^{+n}$) ion gun (LMIG) for spectroscopy and imaging at a spatial resolution of approximately 1 µm, an argon gas cluster source for the high-resolution depth profiling of organic materials (polymers and biological samples) and 3D chemical characterisation, a sensitivity down to ppm (femtomole) and a 5-axis multi-sample stage fully automated which provides rotation for high resolution (nm) depth profiling ($Cs^+$ or $C_{60}^+$ sources). It is possible to obtain images from surface areas from the µm to cm scale and also 3D elemental mapping from samples with sizes ranging from a few mm up to approximately 10 cm.

Figure 12. ToF-SIMS IV instrument (IONTOF, GmbH). [36]

### 3.2.5. SAMPLES PREPARATION FOR TOF-SIMS ANALYSIS

Prior to analysis, several fragments of the hair fibres were fixed one by one to adhesive tape in order to obtain a flat and even surface for the analysis and loaded directly into the instrument using the ToF-SIMS 'backmount' stage, a proper support with individual 1 cm × 1 cm openings (Figure 13). This allows

samples to be mounted on the underside of the stage and the surface of the sample presented through. This approach can be used without the risk of contamination from the double-sided tape because the analysed peaks are native peaks and not ones which are applied from a formulation.

Three types of damaged hair were analysed with this technique: chemical damage, heat damage and chemical, UV and pollution damage, being the respective virgin hair also analysed as well as the treated hair samples for the chemical and heat damages. It was prepared one sample of each type of analysed hair in the case of the chemical damage and the chemical, UV and pollution damage and it were prepared three samples of each type of analysed hair in the case of the heat damage. Each assembled sample was analysed in triplicate.

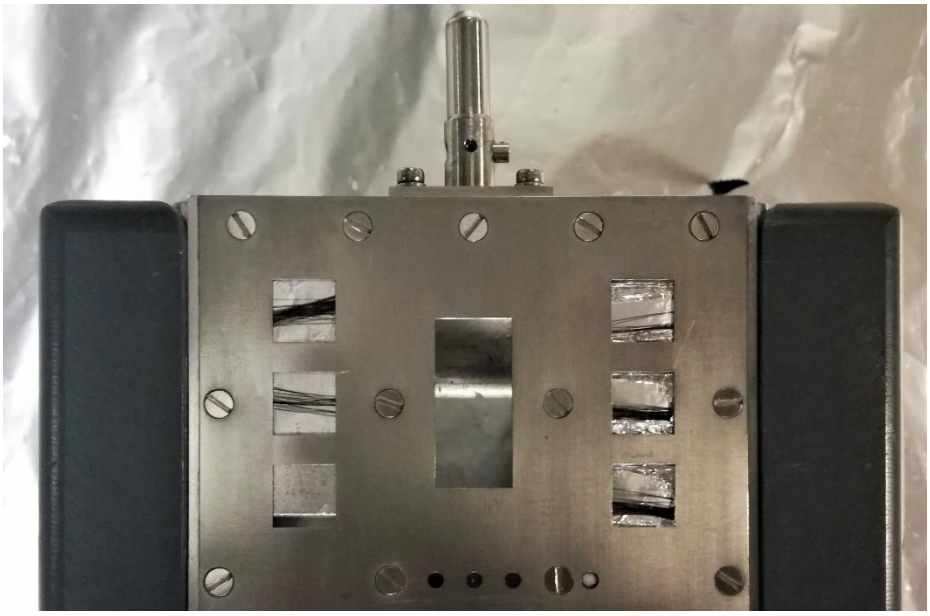


Figure 13. Samples assembling on the ToF-SIMS proper support.

### 3.2.6. IMAGES AND DATA COLLECTION AND ANALYSIS

Static analysis was conducted using a bismuth liquid metal ion gun (LMIG), with $Bi^{3+}$ clusters chosen as the primary ion source. A 25 keV beam energy, approximately 0.3 pA pulsed target current and 10 keV post-acceleration energy were employed. High current bunched (HC-BU) mode was used throughout and the primary ion dose density was always maintained at ≤ 1×1012 ions/$cm^2$ to ensure static conditions. The primary ion beam was directed at the sample at an angle of 45 °C and in HC-BU mode the bismuth LMIG had a focused beam size of 1–2 μm. Charge compensation of the sample was achieved using a low energy (lower than 20 eV) electron flood gun.

All ToF-SIMS data was acquired and analysed using SurfaceLab 6 software (IONTOF, GmbH) and all exported peak intensities were normalised to the total ion count of the spectra. For all samples, data was acquired over 500 μm × 500 μm area in negative polarity and in positive polarity at a resolution of 256 pixels/mm. Each 500 μm × 500 μm area was scanned using the macroraster stage function, using a random raster pattern. A total of 20 scans was acquired per analysis area and the total analysis time for each area was 131 seconds.

The removal of 18-MEA from the hair surface by the different damaging processes could be detected through ToF-SIMS through its negative molecular ion peak at m/z 341 (18-MEA molecular ion peak). The damage degree of the hair fibres was evaluated by the level of degradation of the 18-MEA on its surface and this is calculated through the analysis of the peak intensity ratio of the molecular ion peak of the 18-MEA and the $CN^-$ peak (m/z 26) from the protein matrix, used to evaluate the 18-MEA amount on the hair.

A descriptive statistical analysis of the results was performed using Microsoft Office Excel® and IBM® SPSS® Statistics Version 24 (IBM Corp., U.S.A.), including the calculation of mean, standard deviation, outliers identification and graphic representations. When observing outliers, their influence on results analysis was studied by performing one analysis with the actual values and at least one other analysis eliminating or reducing the outlier effect. Normality tests (Shapiro-Wilk test) were performed in order to assure normal distribution of the objective data obtained. For the results obtained for the chemical and heat damage, if normal distribution of the data was verified, one-way ANOVA was applied and Tukey's test for post-hoc analysis to compare the values obtained for the damaged hair *versus* respective virgin hair and the damaged hair *versus* respective treated hair. For non-normal distributions, a non-parametric test (Kruskal Wallis test) was performed. In the case of the chemical + UV + pollution damage results, if normal distribution of the data was verified, independent samples t test was applied to compare the values obtained for the damaged hair *versus* respective virgin hair. For non-normal distributions, a non-parametric test (Mann-Whitney test) was performed. The significance value was established at 0.05 and a power of 0.95.

# CHAPTER 4. RESULTS AND DISCUSSION

In this chapter, all the obtained results with both explored techniques are presented and discussed, presenting also the preliminary results for the optimization of the methodologies.

## 4.1. OPTICAL PROFILOMETRY

### 4.1.1. PRELIMINARY RESULTS FOR THE OPTIMIZATION OF THE METHODOLOGY

Preliminary tests for the optimization of the Optical Profilometry technique were performed, where it was compared the surfaces roughness analysis through box roughness (Figure 14) and through line roughness (Figure 15). The line roughness can give us the Ra parameter, i.e., the arithmetical mean height of a line. On the other hand, the box roughness can give us the Sa. i.e., the arithmetical mean height of an area (it is the extension of Ra).

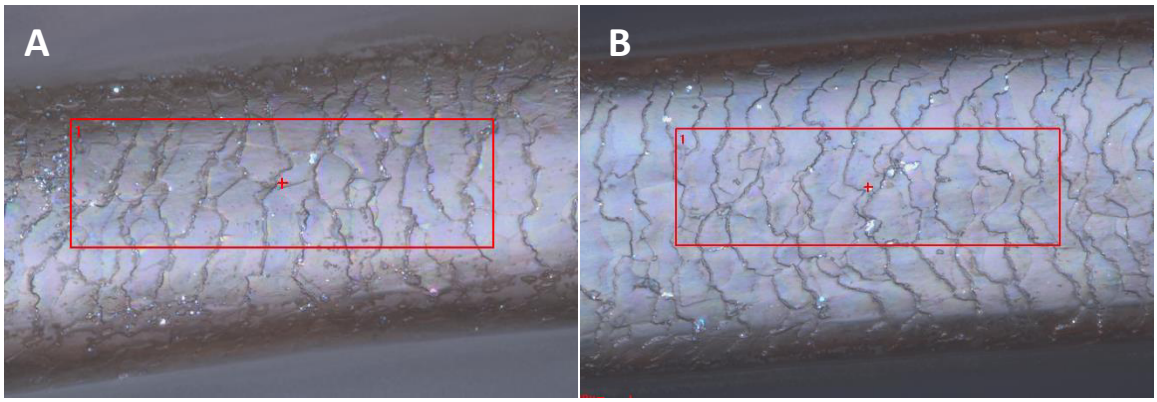



Figure 14. 2D images of a virgin hair fibre (A) and a heat damaged hair fibre (B) obtained through Optical Profilometry with a box drawn for box roughness analysis.

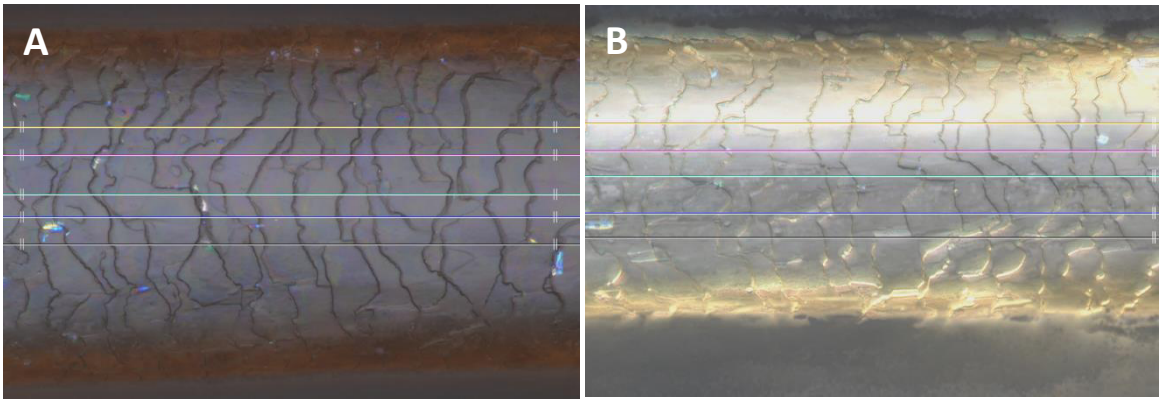



Figure 15. 2D images of a virgin hair fibre (A) and a chemically damaged (bleached) hair fibre (B) obtained through Optical Profilometry with 5 lines drawn for line roughness analysis.

It was concluded that the most promising type of analysis is the line roughness, as it can be observed some artefacts, including some crystals, on the hair fibres surface that can interfere with the box roughness analysis. These crystals can be substances that are present even in water and settle on the hair surface. With the line drawing it is possible to avoid these crystals.

### 4.1.2. METHODOLOGY VALIDATION

Optical profilometry can give us the roughness of the hair fibre surface, allowing to conclude the damage degree of the samples. With the damage, the surface has a more irregular profile, having consequently an increase of the surface roughness.

Mainly two types of damage were analysed: Chemical and Heat damage. Both damaged and respective virgin hair fibres were analysed, being analysed 5 different areas of 5 different fibres of each type of hair in analysis, drawing 5 lines on each image. 15 different areas from each type of hair were analysed; nevertheless, outliers were removed before presenting the final results. The results are shown on the following tables (Table 2 to Table 5) and 2D and 3D imagens were also obtained and presented (Figure 16 to Figure 19).

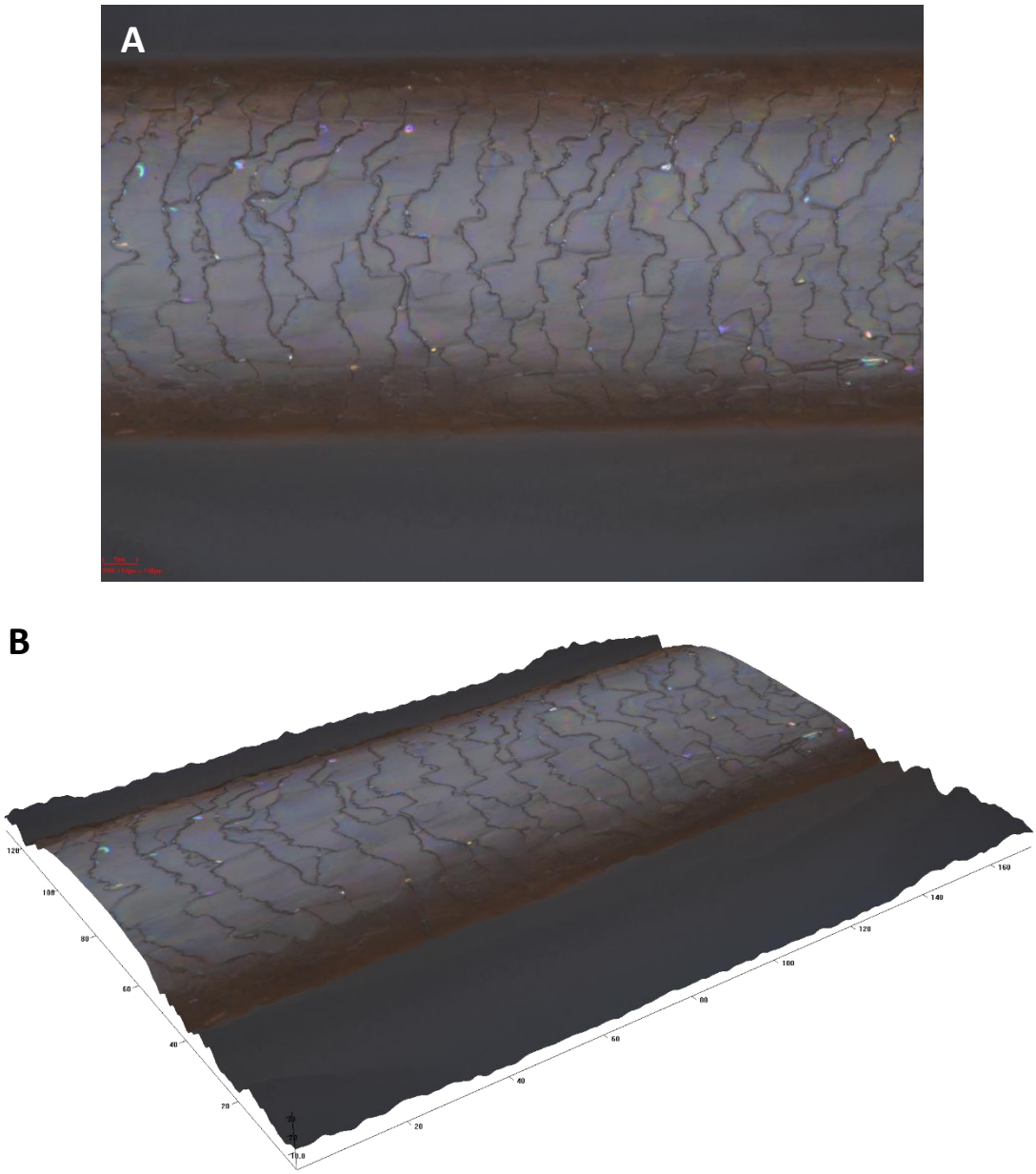



Figure 16. 2D (A) and 3D (B) images obtained from a selected area of a virgin hair fibre from the same hair used for the chemical damage.

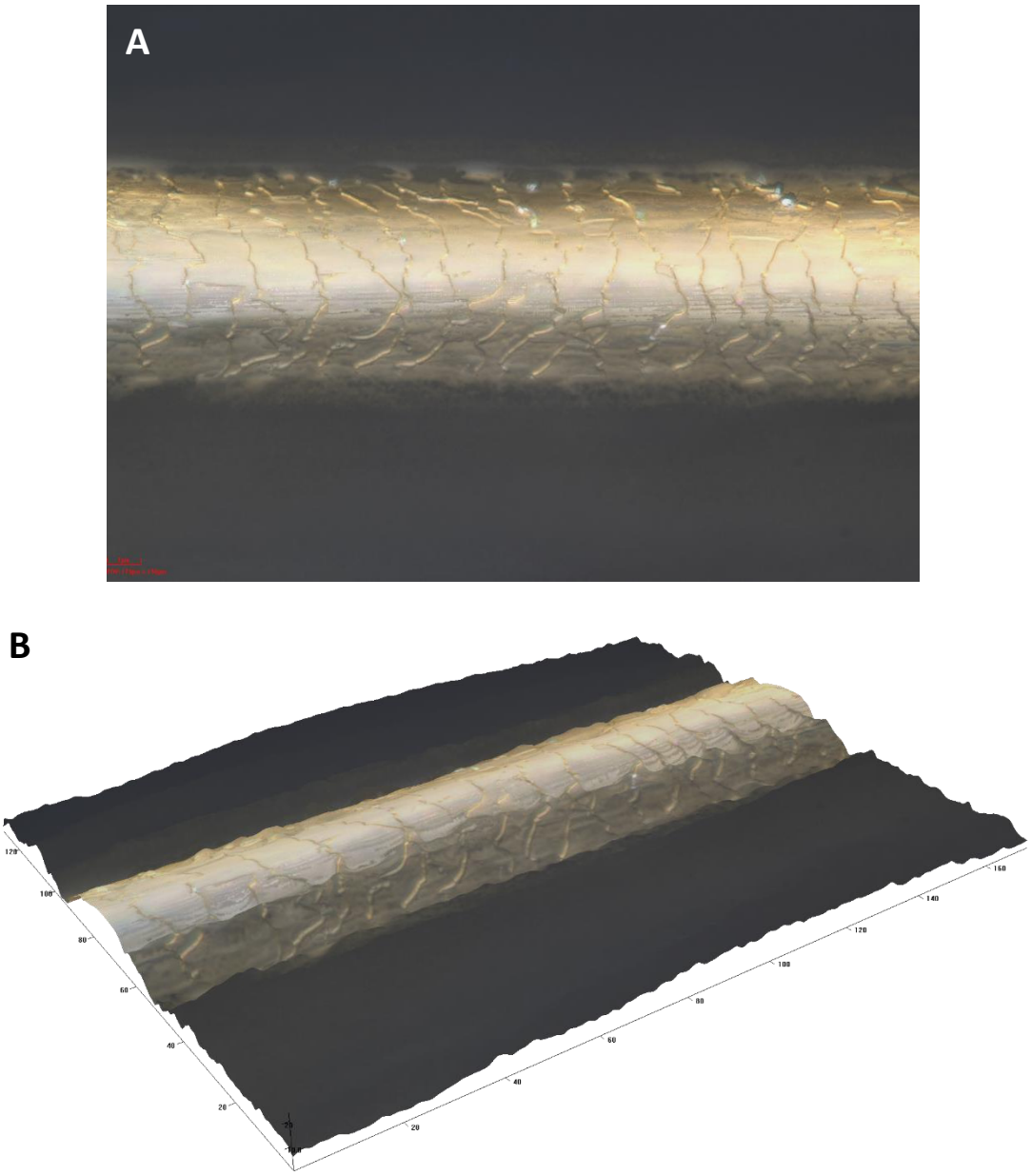



Figure 17. 2D (A) and 3D (B) images obtained from a selected area of a chemically damaged fibre.

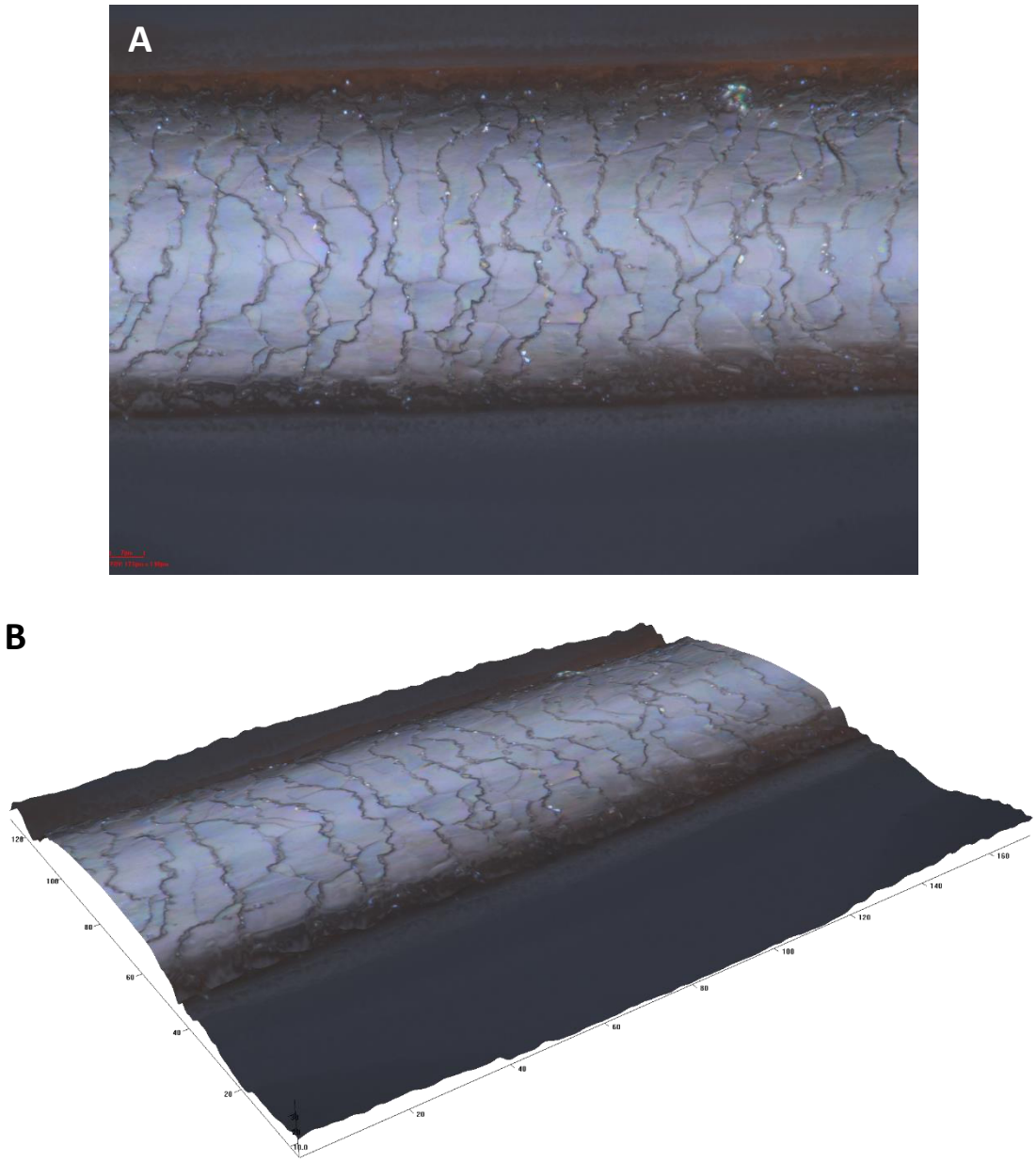



Figure 18. 2D (A) and 3D (B) images obtained from a selected area of a virgin hair fibre from the same hair used for the heat damage.

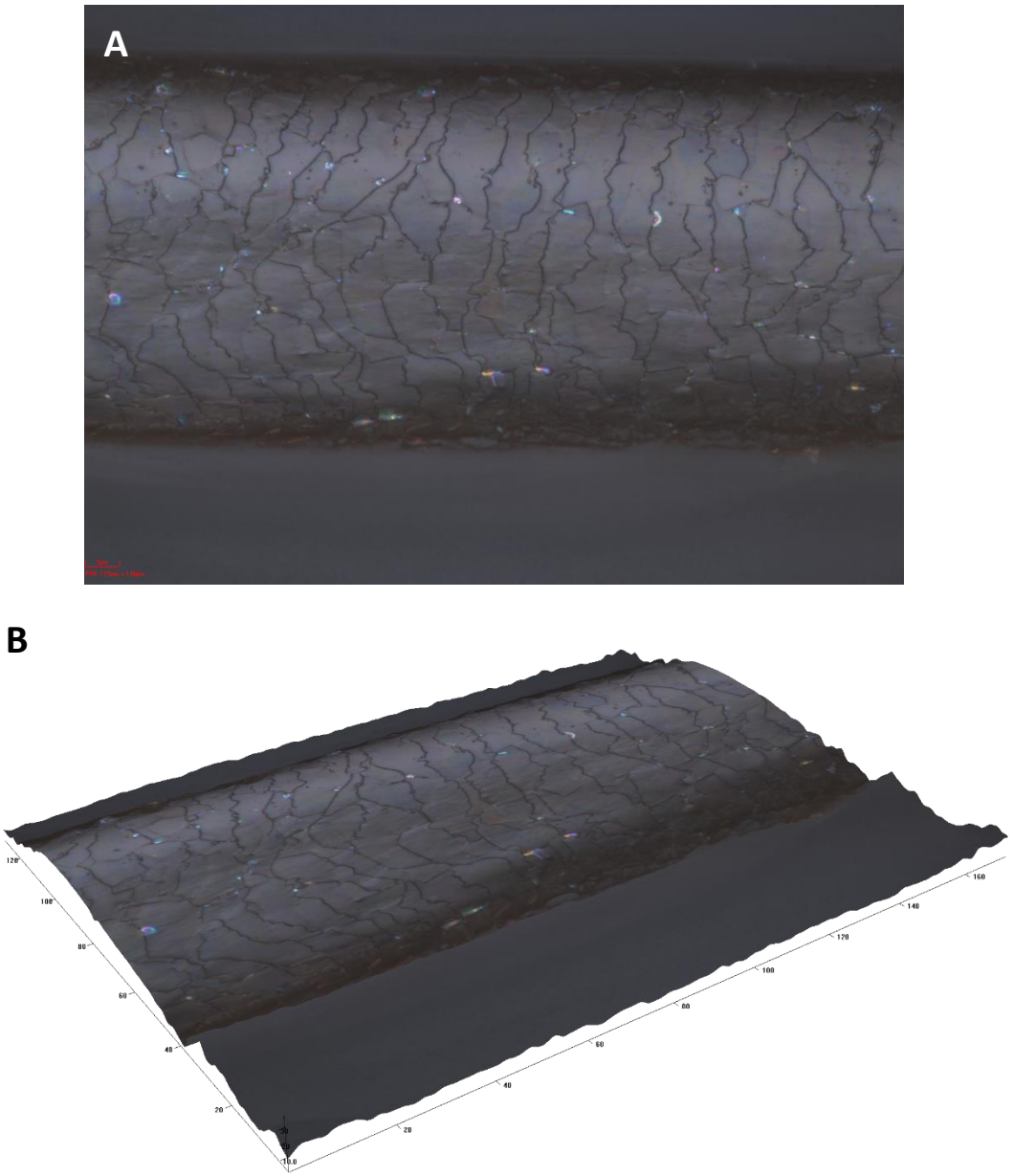



Figure 19. 2D (A) and 3D (B) images obtained from a selected area of a heat damaged fibre.

Table 2. Coefficient of variation (%) of the surface roughness (Ra) results obtained on each analysed fibre for chemical damaged hair and the respective virgin hair samples

| **Surface roughness (Ra) CV (%) – Chemical damage (n=5)** | | |
|---|---|---|
| **FIBRE** | **Virgin Hair** | **Damaged Hair** |
| 1 | 33.6% | 45.3% |
| 2 | 64.8% | 32.1% |
| 3 | 28.7% | 85.2% |

Table 3. Surface roughness results obtained for chemical damaged hair and the respective virgin hair samples

| **Surface roughness (Ra) – Chemical damage** | | |
|---|---|---|
| | **Virgin hair (n=14)** | **Damaged hair (n=12)** |
| **Roughness mean values (µm)** | 0.233 | 0.29 |
| **±SD** | 0.061 | 0.10 |
| **CV (%)** | 26.4% | 34.4% |
| **Mean differences** | 0.05 | |
| **±SD** | 0.14 | |
| **Mean differences (%)** | 33.2% | |
| **±SD** | 67.4% | |
| **p value (Virgin vs Damaged)** | 0.101* | |

* Independent Samples t Test

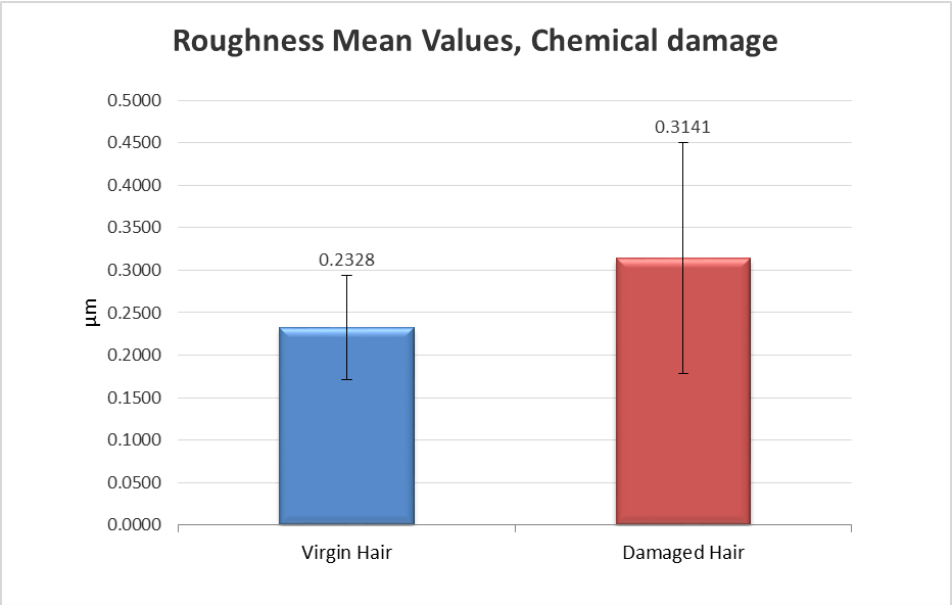



Figure 20. Mean results regarding the hair surface roughness for chemical damaged hair and the respective virgin hair samples.

Table 4. Coefficient of variation (%) of the surface roughness (Ra) results obtained on each analysed fibre for heat damaged hair and the respective virgin hair samples

| **Surface roughness (Ra) CV (%) – Heat damage (n=5)** | | |
|---|---|---|
| **FIBRE** | **Virgin Hair** | **Damaged Hair** |
| 1 | 92.2% | 91.9% |
| 2 | 73.5% | 110.2% |
| 3 | 55.6% | 56.4% |

Table 5. Surface roughness results obtained for heat damaged hair and the respective virgin hair sample

| Surface roughness (Ra) – Heat damage | | |
|---|---|---|
| | **Virgin hair (n=14)** | **Damaged hair (n=13)** |
| **Roughness mean values (µm)** | 0.290 | 0.306 |
| **±SD** | 0.079 | 0.086 |
| **CV (%)** | 27.4% | 28.1% |
| **Mean differences** | 0.021 | |
| **±SD** | 0.092 | |
| **Mean differences (%)** | 12.1% | |
| **±SD** | 36.0% | |
| **p value (Virgin vs Damaged)** | 0.629* | |

* Independent Samples t Test

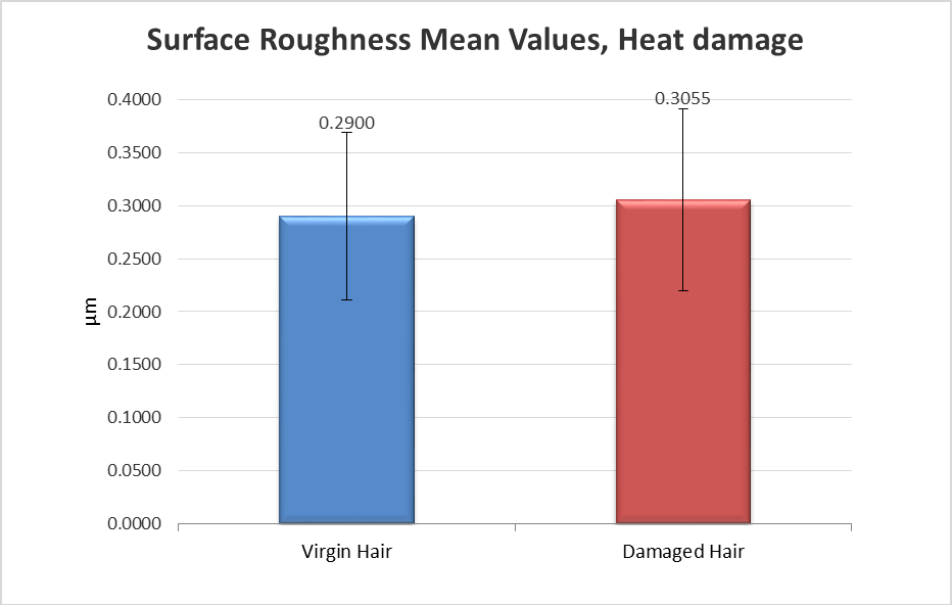



Figure 21. Mean results regarding the hair surface roughness for heat damaged hair and the respective virgin hair samples.

The results show an increase of the surface roughness for both type of damage (33.2% for the chemical damaged hair and 36.0% for the heat damaged hair), which was also noticeable on the 3D images

obtained through this technique. As a result, it is possible to see and calculate the differences between virgin hair and damaged hair roughness (Ra). However, the differences were not statistically significant ($p>0.05$) in the test conditions (Table 3 and Table 5) and the methodology needs to be polished in order to have more significant results.

This lack of significance of the results can be justified with the fact that the obtained coefficient of variation (CV) of all the obtained results are high (26.4% and 34.4% for virgin and damaged samples for the chemical damage, respectively, and 27.4% and 28.1% for virgin and damaged samples for the heat damage, respectively). Normally, for biological samples, the CV indicates a low variability of the results when it is under 20%.[43] In the case of the obtained results, all the calculated CV are higher than 20%, meaning already a medium distribution of the results. Nevertheless, the CV obtained individually for each analysed fibre (Table 2 and Table 4) are really high and dispersed (a minimum of 28.7% and a maximum of 85.2% for the samples analysed for the chemical damage and a minimum of 55.6% and a maximum of 110.2% for the samples analysed for the heat damage). Regarding the virgin hair samples, it is indicative of a naturally not regular surface. Also, when analysing the results obtained for the damaged samples, it illustrates that, when inducing any type of damage, it is hard to distribute it homogenously throughout all the fibres and over the entire area of each fibre. So, when analysing different small areas of a fibre, there will be a highly dispersed results, as these areas can be more or less regular and more or less damaged. Also, this makes it difficult to find a number of samples which can give us significant results, because there will always be a great variability.

It is possible to see the differences on the surface roughness through the images and quantify these differences, as showed by the obtained results. However, it is necessary to optimize the sampling and the number of samples necessary to obtain significant results.

## 4.2. TOF-SIMS

### 4.2.1. METHODOLOGY VALIDATION

As previously explained, 18-MEA fatty acid can be easily removed when damaging the hair and it is detected through ToF-SIMS on the negative spectrum obtained through the equipment (negative molecular ion peak at m/z 341). The peak intensity ratio of the molecular ion peak of 18-MEA and the $CN^-$ peak (m/z 26) from the matrix protein were to evaluate the 18-MEA amount on the hair.

Results obtained for chemical, heat and chemical, UV and cigarette damaged hair fibres and the respective virgin and treated hair samples, being the last ones only applicable for the chemical and heat

damages, are presented on the following tables (Table 6 to Table 10). Also, one example of each type of damage from the 18-MEA distribution throughout the fibres and the respective negative ion mass spectrums concerning 18-MEA for virgin, damaged and treated hair fibres (Figure 22 to Figure 27).

The mean differences were calculated between the damaged hair and the respective virgin hair and, when applicable, between the damaged hair and the respective treated hair.

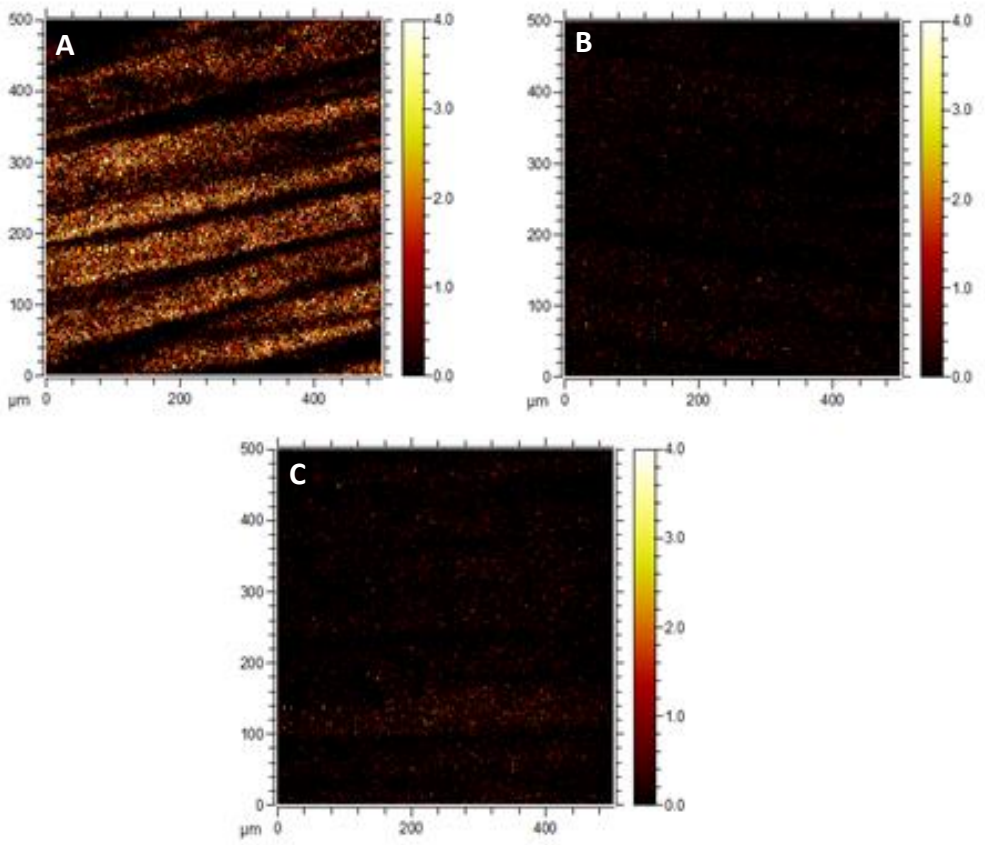



Figure 22. ToF-SIMS images of virgin (A), chemically damaged (B) and treated (C) hair fibres showing 18-MEA distribution.

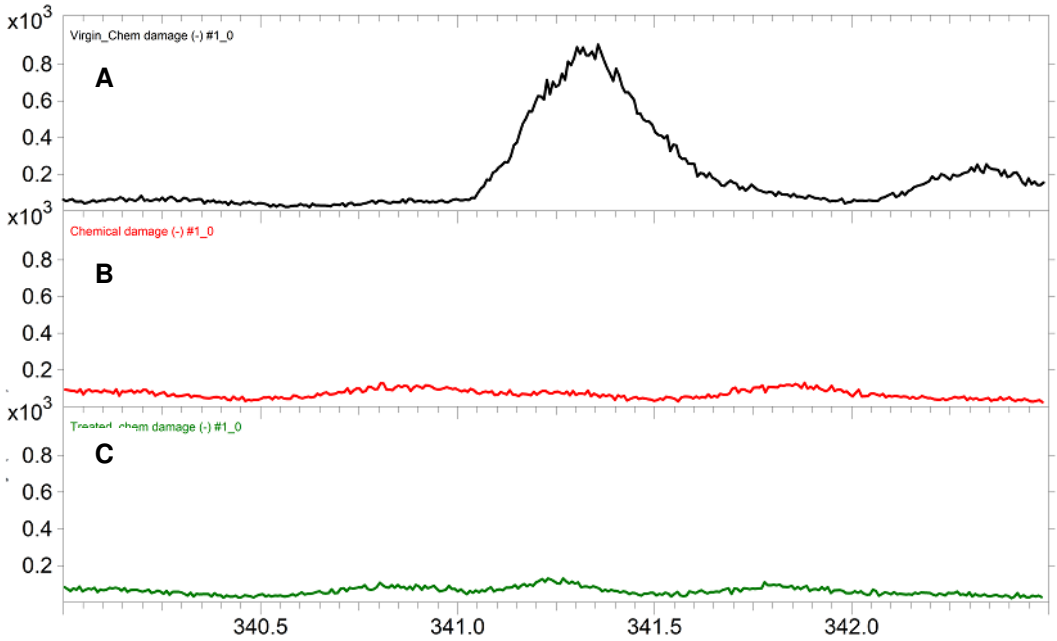



Figure 23. Negative ion mass spectrum from virgin (A), chemically (B) and treated (C) hair fibres, concerning 18-MEA.

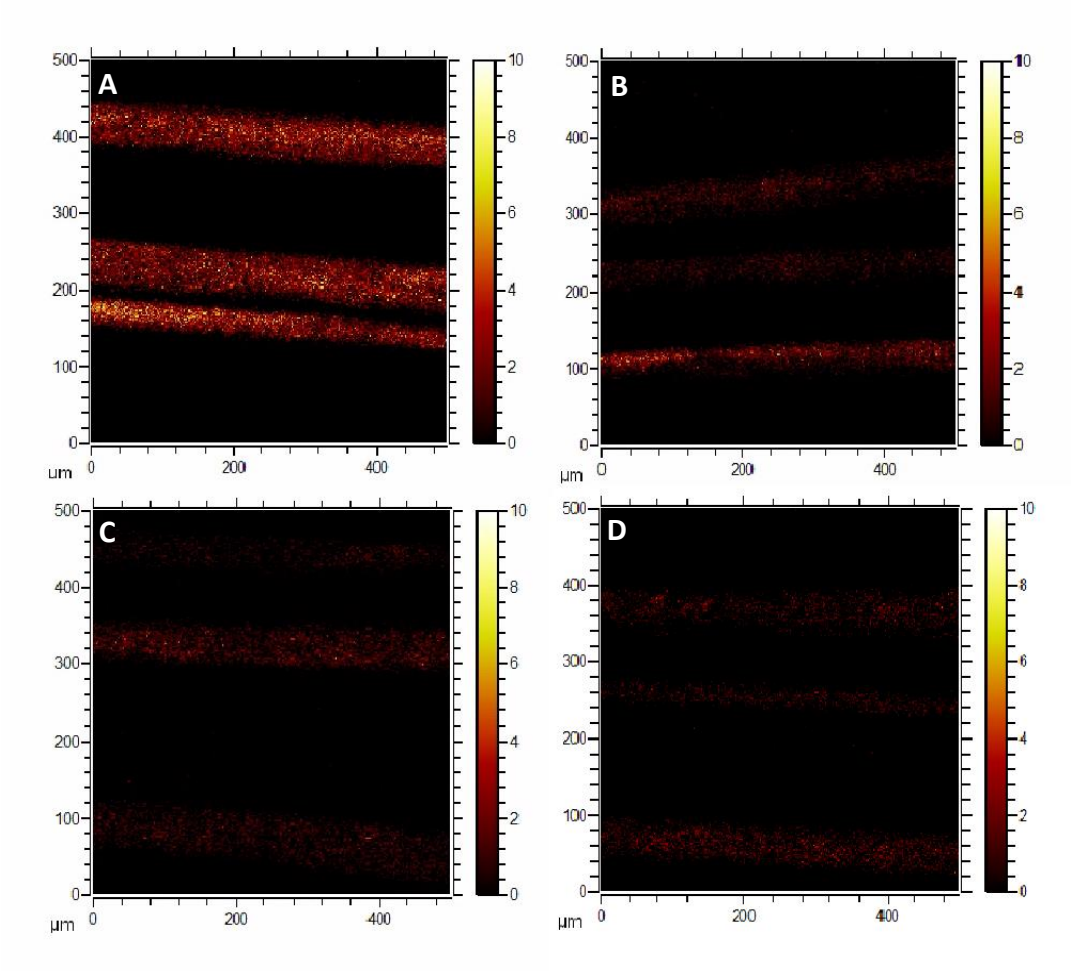



Figure 24. ToF-SIMS images of virgin (A), heat damaged (B), treated with a cosmetic product with an active ingredient (C) treated with a placebo (D) hair fibres showing 18-MEA distribution.

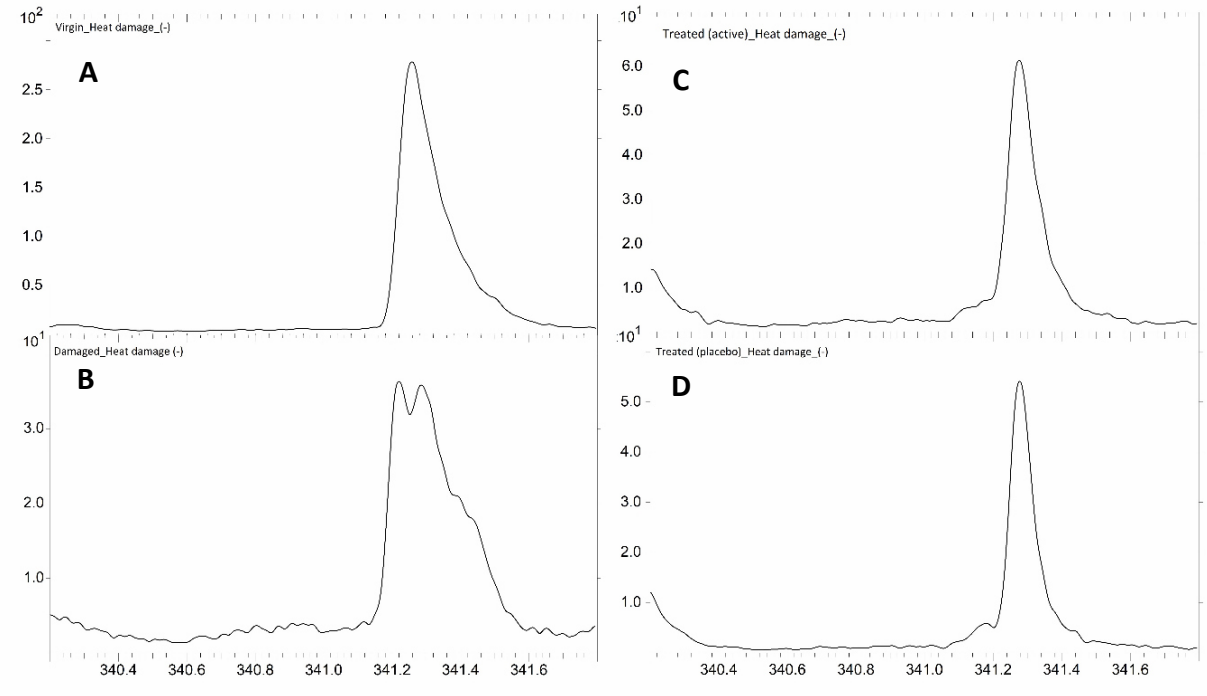



Figure 25. Negative ion mass spectrum from virgin (A), heat damaged (B), treated with a cosmetic product with an active ingredient (C) treated with a placebo (D) hair fibres, concerning 18-MEA.

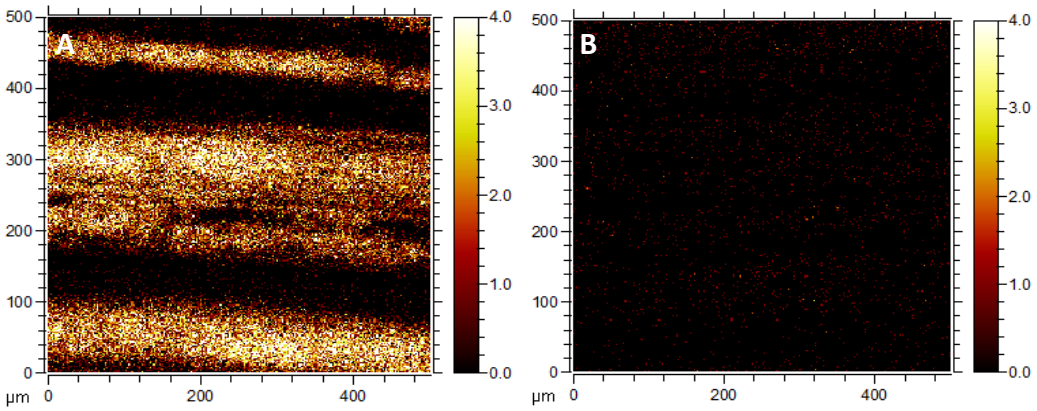



Figure 26. ToF-SIMS images of virgin (A) and damaged (bleached + UV + smoke exposure) (B) hair fibres showing 18-MEA distribution.

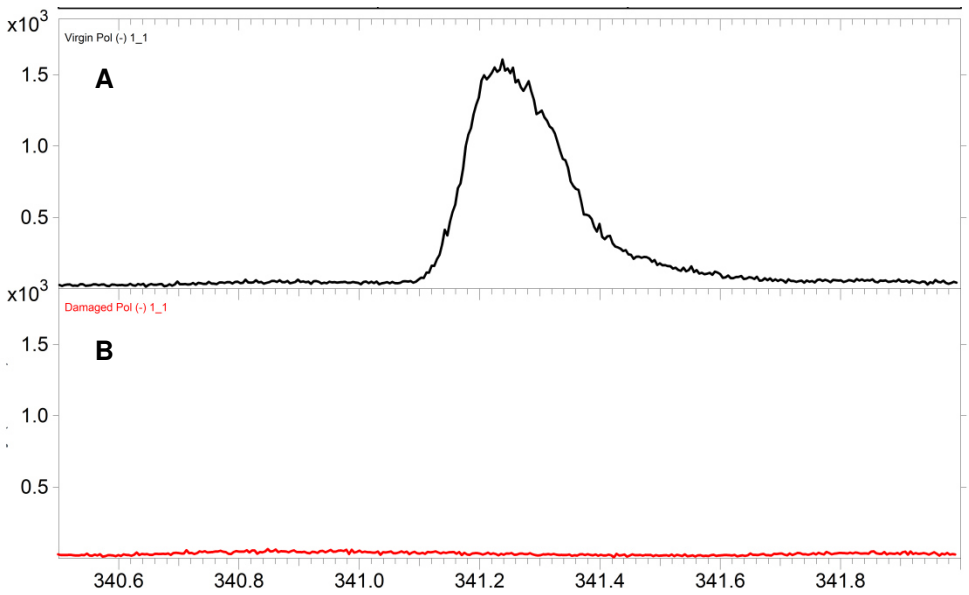



Figure 27. Negative ion mass spectrum from virgin (A) and damaged (bleached + UV + smoke exposure) (B) hair fibres, concerning 18-MEA.

Table 6. Peak intensity ratio 18-MEA/$CN^-$ results obtained for chemical damaged hair and the respective virgin hair sample

| **Peak intensity ratio 18-MEA/$CN^-$ – Chemical damage (n=1 with triplicates)** | | |
|---|---|---|
| | **Virgin hair** | **Damaged hair** |
| **Peak intensity ratio mean values** | 0.1403 | 0.00614 |
| **±SD** | 0.0086 | 0.00028 |
| **CV (%)** | 6.2% | 4.6% |
| **Mean differences** | | -0.1341 |
| **±SD** | | 0.0088 |
| **Mean differences (%)** | | -95.60% |
| **±SD** | | 0.44% |
| **p value (Virgin vs Damaged)** | | <0.001** |

** One-way ANOVA with Post Hoc Tukey's Test

Table 7. Peak intensity ratio 18-MEA/$CN^-$ results obtained for chemical damaged hair and the respective treated hair sample

| **Peak intensity ratio 18-MEA/$CN^-$ – Chemical damage (n=1 with triplicates)** | | |
|---|---|---|
| | **Damaged hair** | **Treated hair** |
| **Peak intensity ratio mean values** | 0.00614 | 0.0105 |
| **±SD** | 0.00028 | 0.0023 |
| **CV (%)** | 4.6% | 21.8% |
| **Mean differences** | | 0.0044 |
| **±SD** | | 0.0026 |
| **Mean differences (%)** | | 72.6% |
| **±SD** | | 45.9% |
| **p value (Damaged vs Treated)** | | 0.584** |

** One-way ANOVA with Post Hoc Tukey's Test

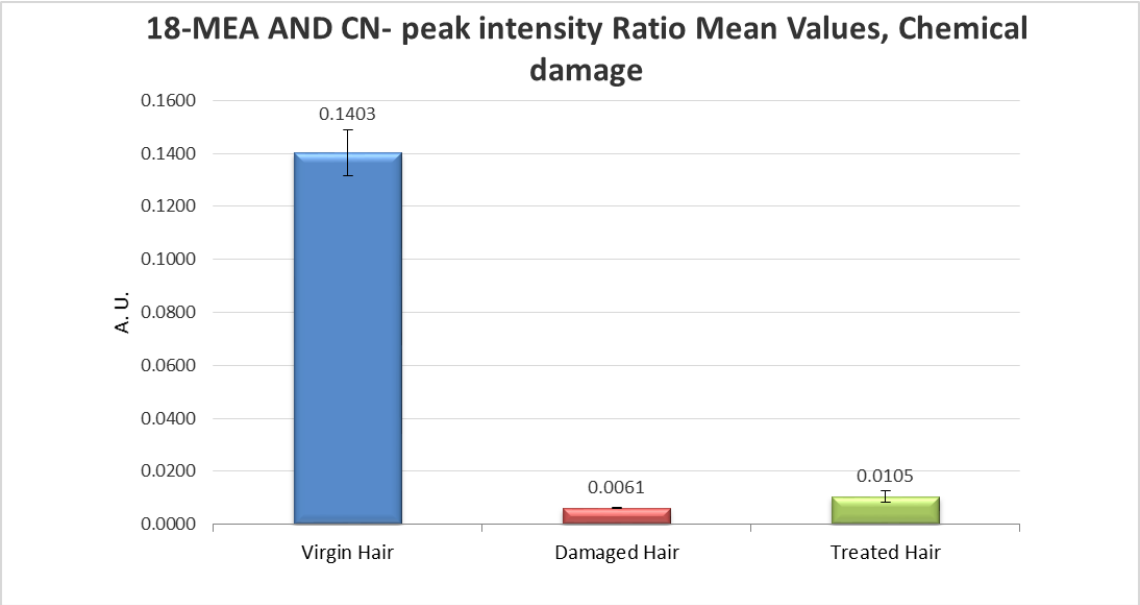



Figure 28. Mean results regarding the peak intensity ratio 18-MEA/CN- for chemical damaged hair and the respective virgin and treated hair samples.

Table 8. Peak intensity ratio 18-MEA/CN- results obtained for heat damaged hair and the respective virgin hair samples (one outlier removed from the damaged hair results)

| **Peak intensity ratio 18-MEA/CN- – Heat damage** | | |
|---|---|---|
| | **Virgin hair** | **Damaged hair** |
| **Peak intensity ratio mean values** | 0.138<br>(n=6) | 0.0363<br>(n=5) |
| **±SD** | 0.039 | 0.0060 |
| **CV (%)** | 28.5% | 16.4% |
| **Mean differences** | | -0.112 |
| **±SD** | | 0.030 |
| **Mean differences (%)** | | -74.9% |
| **±SD** | | 4.5% |
| **p value (Virgin vs Damaged)** | | <0.001** |

** One-way ANOVA with Post Hoc Tukey's Test

Table 9. Peak intensity ratio 18-MEA/CN- results obtained for heat damaged hair and the respective treated hair samples (one outlier removed from the damaged hair results)

| **Peak intensity ratio 18-MEA/CN- – Heat damage** | | | |
|---|---|---|---|
| | **Damaged hair** | **Treated hair (active treatment)** | **Treated hair (placebo treatment)** |
| **Peak intensity ratio mean values** | 0.0363 (n=5) | 0.0191 (n=6) | 0.021 (n=6) |
| **±SD** | 0.0060 | 0.0058 | 0.010 |
| **CV (%)** | 16.4% | 30.6% | 48.7% |
| **Mean differences** | | -0.018 (n=5) | -0.019 (n=5) |
| **±SD** | | 0.012 | 0.010 |
| **Mean differences (%)** | | -47.2% | -50.2% |
| **±SD** | | 23.5% | 24.6% |
| **p value (Damaged vs Treated)** | | 0.549*** | 0.623** |

** One-way ANOVA with Post Hoc Tukey's Test

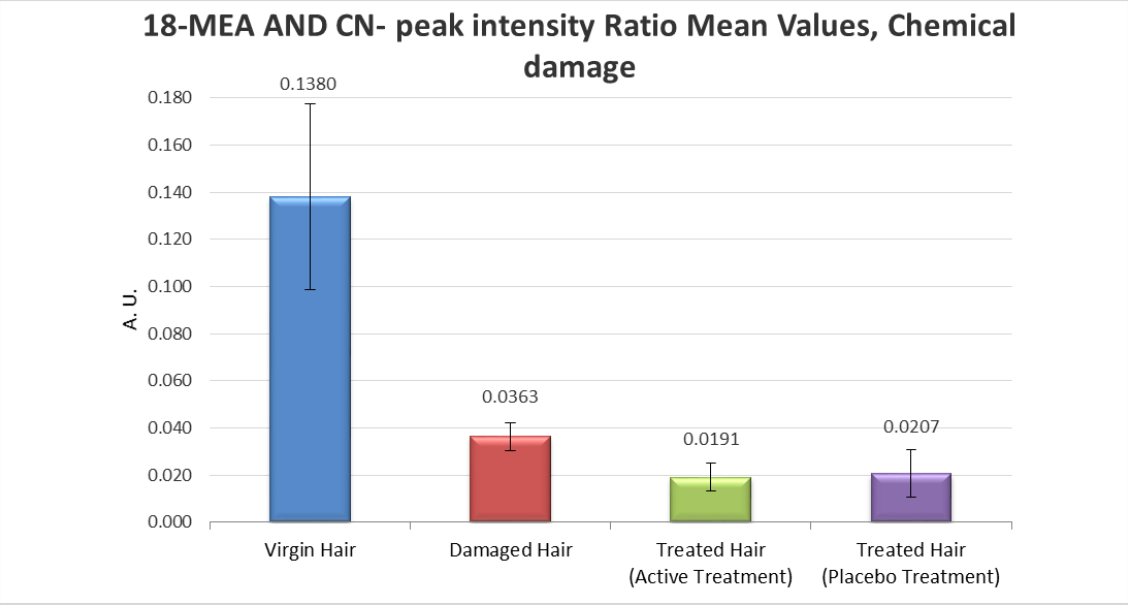



Figure 29. Mean results regarding the peak intensity ratio 18-MEA/CN- for heat damaged hair and the respective virgin and treated hair samples.

Table 10. Peak intensity ratio 18-MEA/CN- results obtained for chemical + UV + pollution damaged hair and the respective virgin hair sample

| **Peak intensity ratio 18-MEA/CN- – Chemical + UV + pollution damage (n=1 with triplicates)** | | |
|---|---|---|
| | **Virgin hair** | **Damaged hair** |
| **Peak intensity ratio mean values** | 0.086 | 0.00952 |
| **±SD** | 0.012 | 0.00030 |
| **CV (%)** | 13.8% | 3.2% |
| **Mean differences** | -0.076 | |
| **±SD** | 0.012 | |
| **Mean differences (%)** | -88.8% | |
| **±SD** | 1.8% | |
| **p value (Virgin vs Damaged)** | 0.008*** | |

*** Independent Samples t Test

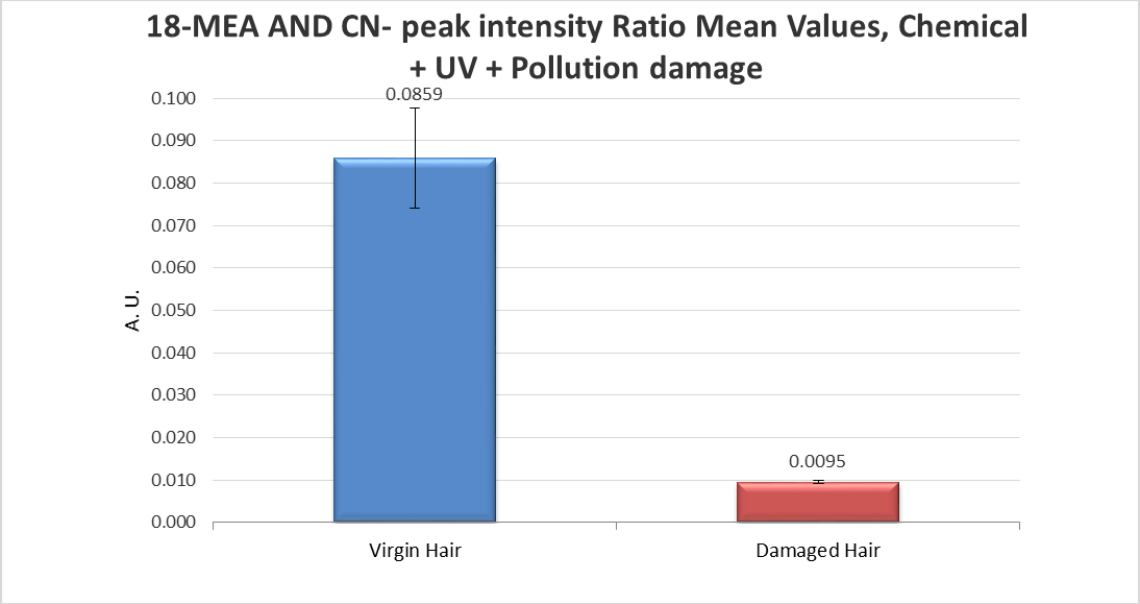



Figure 30. Mean results regarding the peak intensity ratio 18-MEA/CN- for Chemical + UV + Pollution damaged hair and the respective virgin hair sample.

Regarding the ToF-SIMS technique, results show an expected significant decrease of the mean peak area normalized intensity values due to the induced damages compared to virgin hair for all three types of damage: -95.60% for the chemical damage ($p<0.001$), -74.9% for the heat damage ($p<0.001$) and -88.8% for the chemical + UV + pollution damage ($p=0.008$) (Table 6, Table 8 and Table 10).

Concerning the treated samples, for the chemical damaged hair, it is possible to see that the applied treatment had an effect on the hair fibres condition, as there is an increase of the peak intensity ration for the treated sample comparatively to the damaged sample, although the differences were not statistical significant (Table 7). For the heat damaged hair, the differences between the results obtained for the damaged hair without treatment and for both treated hair (with the product with the active ingredient and with the placebo) were not statistically significant, not being possible to see any improvement of the condition of hair cuticle induced by either the placebo (as it was expected) nor the cosmetic serum with the active ingredient (Table 9).

All these results are in accordance with the CV values obtained for all the type of samples analysed, being the ones with higher CV values the ones that had not statistical significant results, except for the virgin hair correspondent to the heat damage, probably because this hair was naturally more irregular. Probably a higher number of samples would help to have statistical significant results and to have a less dispersed results. Yet, one of the things that was visible on the optical profilometry methodology that it is not visible on this methodology is high CV values for the damaged samples. In fact, on the case of the ToF-SIMS results, all the CV values obtained for the damaged samples are below 20%, so the results' distribution is low, indicating a higher homogeneity of the results with this technique.

All the calculated differences were also visible on the images obtained through the equipment (Figure 22, Figure 24 and Figure 26) and they can be correlated. The intensity of the colour present on the images is indicative of the presence of the 18-MEA on the hair surface. With this, visually, it is possible to see a decrease on the 18-MEA distribution on the damaged hair fibres relatively to the respective virgin hair. Also, comparing the treated hair fibres with the chemically damaged hair fibres, there is an increase of the 18-MEA distribution that, although it was not statistically significant, it was detected and quantified through the intensity peak ration calculation (Figure 22 and Table 7). Also, the non-statistical significant differences of the results obtained for the treated hair fibres (with the active cosmetic product in study and the placebo) comparing with the hair damaged with the chemical + UV + pollution procedure can also be visually detected (Figure 24 and Table 9). Although the differences between these samples are

small, visually it is possible to correlate the significance of the results, as the damaged hair images reveal a higher colour intensity on the hair fibres surface than the ones obtained for the treated hair fibres.
One disadvantage of this technique is that in order to obtain more accurate images, the respective ion mass spectrum will be obtained with higher noise, and vice-versa. So, in order to obtain more specific chemical differences it is better to obtain more defined spectrums; in order to obtain a better distribution of an element it is better to get a higher resolution image. In the case of the efficacy testing of a cosmetic product, it is recommended to have well-defined spectrums from all the analysed samples and just a representative high definition image. Nevertheless, this will depend on the objective of the efficacy test and the claims to be demonstrated.

This protocol was validated, as differences between normal hair and damaged hair were statistically significant and also the differences visually verified through the images obtained correlates with the obtained results.

# CHAPTER 5. CONCLUSION AND FUTURE WORK

After a bibliographic research, it was possible to understand that the majority of the techniques used for the evaluation of the hair damage are not sensitive nor precise enough to show damages on the hair surface, not being aligned with the constant and fast cosmetic industry evolution. This project was developed accordingly to inovapotek's mission of meeting the clients and final consumers' expectations, and aimed to explore the most appropriate techniques and validate new methodologies that could give more sensitive and precise information about the hair surface.

Two techniques were tested: Optical Profilometry (which can give the hair surface roughness) and "Time-of-Flight" + Secondary Ion Mass Spectrometry (ToF-SIMS) (which can give the level of lipids on the hair surface) using different samples submitted to different types of damage: chemical, heat and chemical + UV + pollution. All the samples were prepared accordingly to inovapotek's internal procedures, previously developed and validated. Preliminary tests with the selected techniques were performed before the final validation of the designed methodologies.

It is possible to achieve quantitative and qualitative results with the designed protocol for the surface hair analysis with the Optical Profilometer, through the line roughness calculation (Ra) and the 2D and 3D obtained images, respectively. Nevertheless, the obtained differences between the chemical and heat damaged samples and the respective virgin samples were not statistically significant ($p>0.05$) in the test conditions and the CV values indicates a high distribution of the results. This methodology needs to be polished in order to have more significant results. So, the methodology was not yet validated, but a higher number of samples could probably increase the significance of the results, reducing the high distribution of the results.

The protocol designed for the ToF-SIMS analysis helped to understand and quantify the damage degree of the hair fibres through the 18-MEA level of degradation. This protocol was validated, as differences between normal hair and damaged hair were statistically significant and also the differences visually verified through the images obtained correlates with the obtained results. Also, it was possible to verify a lower distribution of the obtained results, meaning that it is possible to analyse the same type of samples, but with a greater precision. In conclusion, ToF-SIMS is a great technique to evaluate the damage degree of hair and the analysis is relatively simple, showing significant differences on the hair surface that are not detected with the traditional techniques normally used. This technique showed to be very sensitive, accurate and precise to evaluate the lipid changes on the hair surface induced by the different damages.

In fact, ToF-SIMS' samples preparation is a somehow a little time-consuming and thorough and more expensive than the Optical Profilometry protocol. However, the first can give more precise results with less number of samples in order to show lower differences between different types of samples and also the samples analysis is simpler. Yet, the Optical Profilometry protocol can be validated and probably a cheaper option for efficacy testing hair care products.

In the future, the aim is to evaluate more samples though Optical Profilometry in order to validate the technique, including the analysis of treated samples with very low differences from the damaged samples, in order to evaluate the precision degree of the methodology. Other objective for the future is to analyse the same treated samples with very low differences from the damaged samples with the ToF-SIMS methodology. In order to do that, firstly it is necessary to study which are the best cosmetic product to be used in order to obtain the necessary results for the ultimate validation of the designed methodologies.

# CHAPTER 6. REFERENCES


[1] "inovapotek Homepage," *inovapotek*. [Online]. Available: https://inovapotek.com/. [Accessed: 18-Jun-2020].

[2] "University of Nottingham," *THE World University Rankings*. [Online]. Available: https://www.timeshighereducation.com/world-university-rankings/university-nottingham. [Accessed: 24-Jun-2020].

[3] "University of Nottingham Homepage," *University of Nottingham*. [Online]. Available: https://www.nottingham.ac.uk/. [Accessed: 24-Jun-2020].

[4] "ISAC - Interface and Surface Analysis Centre Nanoscale and Microscale Research Centre," *University of Nottingham*. [Online]. Available: https://www.nottingham.ac.uk/isac/index.aspx. [Accessed: 24-Jun-2020].

[5] Official Journal of the European Union, *REGULATION (EC) No 1223/2009 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 30 November 2009 on cosmetic products*. 2009.

[6] R. da Gama, A. Baby, and M. Velasco, "In Vitro Methodologies to Evaluate the Effects of Hair Care Products on Hair Fiber," *Cosmetics*, vol. 4, no. 2, pp. 1–10, Jan. 2017.

[7] M. Brandt, G. Springmann, S. Bielfeldt, and K.-P. Wilhelm, "Substantiating Claims for Hair Care Products," *sofw journal*, vol. 142, pp. 18–24, 2016.

[8] M. Valéria *et al.*, "Hair fiber characteristics and methods to evaluate hair physical and mechanical properties of Energetic and Nuclear Researches/IPEN-CNEN," *Brazilian J. Pharm. Sci.*, vol. 45, no. 1, pp. 153–162, 2009.

[9] Y. Lee, Y. D. Kim, H. J. Hyun, L. Q. Pi, X. Jin, and W. S. Lee, "Hair shaft damage from heat and drying time of hair dryer," *Ann. Dermatol.*, vol. 23, no. 4, pp. 455–462, 2011.

[10] T. Kojima, "Studies on the effects of hair cosmetics on human hair by secondary ion mass spectrometry," Nagoya, Japan, 2015.

[11] S. S. Sandhu, R. Ramachandran, and C. R. Robbins, "A simple and sensitive method using protein loss measurements to evaluate damage to human hair during combing," *J. Soc. Cosmet. Chem.*, vol. 46, pp. 39–52, 1995.

[12] M. Okamoto, K. Ishikawa, N. Tanji, and S. Aoyagi, "Investigation of the damage on the outermost hair surface using ToF-SIMS and XPS," *Surf. Interface Anal.*, vol. 44, no. 6, pp. 736–739, Jun. 2012.

[13] C. R. Robbins, "Chemical Composition of Different Hair Types," in *Chemical and Physical Behavior of Human Hair*, 5th Editio., Springer Berlin Heidelberg, 2012.

[14] R. Jachowicz and J. McMullen, “Thermal degradation of hair. I. Effect of curling irons,” *J. Cosmet. Sci.*, vol. 49, pp. 223–244, 1998.

[15] A. Kleiner, “Protecting against dirt - Anti-pollution trend for hair care,” *COSSMA*, pp. 16–18, 2015.

[16] M. C. Rheinstädter, F.-C. Yang, and Y. Zhang, “The structure of people’s hair,” *PeerJ*, vol. 2, no. e619, pp. 1–19, 2014.

[17] BASF The Chemical Company, “Puricare ™ by Beauty Creations,” *Care Creations TM*, 2012.

[18] B. M. Teipel, C. Barba, E. Fernández, B. Martínez-Teipel, R. Armengol, and L. Coderch, “Efficacy of antioxidants in human hair,” *Artic. J. Photochem. Photobiol. B, Biol.*, vol. 117, no. 5, pp. 146–156, 2012.

[19] S. Tokunaga, H. Tanamachi, and K. Ishikawa, “Degradation of hair surface: Importance of 18-MEA and epicuticle,” *Cosmetics*, vol. 6, no. 2, p. 31, Mar. 2019.

[20] M. Okamoto, N. Tanji, T. Habe, S. Inoue, S. Tokunaga, and H. Tanamachi, “ToF-SIMS characterization of the lipid layer on the hair surface. II : Effect of the 18-MEA lipid layer on surface hydrophobicity,” *Surf. Interface Anal.*, vol. 43, no. 1–2, pp. 298–301, Jan. 2011.

[21] M. D. Berthiaume, J. H. Merrifield, D. A. Riccio, G. E. Silicones, and H. R. Road, “Effects of silicone pretreatment on oxidative hair damage,” *J. Soc. Cosmet. Chem.*, vol. 46, pp. 231–245, 1994.

[22] L. Whitehouse, “Anti-pollution and the beauty industry: Part I,” *CosmeticsDesign*, 2016. [Online]. Available: https://www.cosmeticsdesign-europe.com/Article/2016/11/22/Anti-pollution-and-the-beauty-industry-Part-I. [Accessed: 22-Jan-2020].

[23] Y. Zhou *et al.*, “The effect of various cosmetic pretreatments on protecting hair from thermal damage by hot flat ironing,” *J. Cosmet. Sci.*, vol. 62, no. 2, pp. 265–282, Mar. 2011.

[24] Mintel Press Team, “Anti-pollution claims on beauty products in Asia Pacific grow by 40% in two years,” *Mintel*, 2014. [Online]. Available: https://www.mintel.com/press-centre/beauty-and-personal-care/anti-pollution-claims-on-beauty-products-in-asia-pacific-grow-by-40-in-two-years. [Accessed: 09-Jan-2020].

[25] M. Shahbandeh, “Global hair care industry 2012-2024,” *Statista*, 2019. [Online]. Available: https://www.statista.com/statistics/254608/global-hair-care-market-size/. [Accessed: 21-Jan-2020].

[26] M. F. R. G. Dias, “Hair cosmetics: An overview,” *Int. J. Trichology*, vol. 7, no. 1, pp. 2–15, Jan. 2015.

[27] E. Clementino Da Silva, M. Valéria, R. Velasco De Paola, J. Do, R. Matos, and J. R. Matos, "Análise térmica aplicada à cosmetologia," *Rev. Bras. Ciências Farm. Brazilian J. Pharm. Sci.*, vol. 43, no. 3, pp. 347–356, 2007.

[28] M. Visscher and K. G. Struik, "Optical profilometry and its application to mechanically inaccessible surfaces Part I: Principles of focus error detection," *Precis. Eng.*, vol. 16, no. 3, pp. 192–198, 1994.

[29] Laser Focus World, "OPTICAL SURFACE PROFILING: Profilometer advances benefit surface analysis, film-thickness measurement," 2010. [Online]. Available: https://www.laserfocusworld.com/test-measurement/test-measurement/article/16552227/optical-surface-profiling-profilometer-advances-benefit-surface-analysis-filmthickness-measurement. [Accessed: 29-Jan-2020].

[30] "Optical profilometer," *ST Instruments*. [Online]. Available: https://www.stinstruments.com/optical-profilometer/. [Accessed: 29-Jan-2020].

[31] "Optical profilometry for surface topography, coating, 3D imaging," *Materials Research Institut, The Pennsylvania State University*. [Online]. Available: https://www.mri.psu.edu/materials-characterization-lab/characterization-techniques/optical-profilometry. [Accessed: 19-Jan-2020].

[32] R. Kohli and K. L. Mittal, "Chapter 3: Methods for assessment and verification of cleanliness of surfaces and characterization of surface contaminants," in *Developments in surface contamination and cleaning, Volume 12*, 2019, pp. 23–105.

[33] B. Bhushan, "Chapter 2: Surface Roughness Analysis and Measurement Techniques," in *Modern Tribology Handbook*, 2001.

[34] SB Joniot, GL Grégoire, AM Auther, and YM Roques, "Three-Dimensional Optical Profilometry Analysis of Surface States Obtained After Finishing Sequences for Three Composite Resins," *Oper. Dent.*, vol. 25, no. 4, pp. 311–315, 2000.

[35] V. S. Smentkowski, "Report: Applications of Time of Flight Secondary Ion Mass Spectrometry in an Industrial Setting," Niskayuna, New York.

[36] "ToF-SIMS," *Interface and Surface Analysis Centre (ISAC) - University of Nottingham*. [Online]. Available: https://www.nottingham.ac.uk/isac/facilities/tof-sims/tof-sims.aspx. [Accessed: 29-Jan-2020].

[37] D. W. Mogk, "Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS)," *Geochemical Instrumentation and Analysis - Montana State University*. [Online]. Available: https://serc.carleton.edu/research_education/geochemsheets/techniques/ToFSIMS.html.

[Accessed: 29-Jan-2020].

[38] I. M. Kempson and W. M. Skinner, “ToF-SIMS analysis of elemental distributions in human hair,” *Sci. Total Environ.*, vol. 338, no. 3, pp. 213–227, Feb. 2005.

[39] T. Kojima, H. Kitano, M. Niwa, K. Saito, Y. Matsushita, and K. Fukushima, “Imaging analysis of cosmetic ingredients interacted with human hair using TOF-SIMS,” *Surf. Interface Anal.*, vol. 43, no. 1–2, pp. 562–565, Jan. 2011.

[40] T. Terlier, J. Lee, and Y. Lee, “Investigation of human hair using ToF-SIMS: From structural analysis to the identification of cosmetic residues,” *J. Vac. Sci. Technol. B*, vol. 36, no. 3, p. 03F131, May 2018.

[41] I. M. Kempson and P. Kirkbride, “Advanced Analysis of Metal Distributions in Human Hair,” *Environ. Sci. Technol.*, vol. 40, no. 10, pp. 3423–3428, 2006.

[42] “Zeta-20 Optical Profilometer,” *KLA*. [Online]. Available: https://www.kla-tencor.com/products/instruments/optical-profilers/zeta-20#details. [Accessed: 18-Jul-2020].

[43] G. F. Reed, F. Lynn, and B. D. Meade, “Use of coefficient of variation in assessing variability of quantitative assays,” *Clin. Diagn. Lab. Immunol.*, vol. 9, no. 6, pp. 1235–1239, Nov. 2002.

# CHAPTER 7. ANNEXES

## ANNEX I – RA RAW DATA – CHEMICAL DAMAGE

| AREA | Virgin Hair | Damaged Hair |
|---|---|---|
| 1 | 0.2172 | 0.3286 |
| 1 | 0.1793 | 0.3443 |
| 1 | 0.1295 | 0.3595 |
| 1 | 0.1677 | 0.3436 |
| 1 | 0.1558 | 0.3032 |
| 2 | 0.2631 | 0.1970 |
| 2 | 0.2463 | 0.2469 |
| 2 | 0.1633 | 0.2436 |
| 2 | 0.2554 | 0.2394 |
| 2 | 0.4137 | 0.1708 |
| 3 | 0.1450 | 0.3773 |
| 3 | 0.1687 | 0.2384 |
| 3 | 0.3150 | 0.2430 |
| 3 | 0.1354 | 0.2232 |
| 3 | 0.1416 | 0.2329 |
| 4 | 0.2479 | 0.6378 |
| 4 | 0.1362 | 0.2000 |
| 4 | 0.1774 | 0.2427 |
| 4 | 0.2054 | 0.2614 |
| 4 | 0.1911 | 0.6730 |
| 5 | 0.1455 | 0.1683 |
| 5 | 0.1680 | 0.2105 |
| 5 | 0.1973 | 0.2364 |
| 5 | 0.1394 | 0.1453 |
| 5 | 0.1904 | 0.1965 |
| 6 | 0.3055 | 0.2246 |

| AREA | Virgin Hair | Damaged Hair |
|---|---|---|
| 6 | 0.1962 | 0.2544 |
| 6 | 0.2592 | 0.2833 |
| 6 | 0.3321 | 0.1778 |
| 6 | 0.3400 | 0.2040 |
| 7 | 0.6739 | 0.2685 |
| 7 | 0.1625 | 0.4745 |
| 7 | 0.3303 | 0.4038 |
| 7 | 0.5369 | 0.3635 |
| 7 | 0.4769 | 0.3418 |
| 8 | 0.6201 | 0.2939 |
| 8 | 0.2504 | 0.2000 |
| 8 | 0.2151 | 0.1888 |
| 8 | 0.2126 | 0.2309 |
| 8 | 0.4622 | 0.1502 |
| 9 | 0.9236 | 0.2635 |
| 9 | 0.1666 | 0.2161 |
| 9 | 0.1922 | 0.1853 |
| 9 | 0.1982 | 0.1649 |
| 9 | 0.1270 | 0.1468 |
| 10 | 0.1579 | 0.2127 |
| 10 | 0.1382 | 0.2408 |
| 10 | 0.1300 | 0.2767 |
| 10 | 0.1756 | 0.1900 |
| 10 | 0.1767 | 0.2534 |
| 11 | 0.1855 | 1.4630 |
| 11 | 0.2197 | 0.1375 |
| 11 | 0.2298 | 0.1618 |
| 11 | 0.1665 | 0.4663 |
| 11 | 0.1627 | 0.3275 |

| AREA | Virgin Hair | Damaged Hair |
|---|---|---|
| 12 | 0.4175 | 0.2468 |
| 12 | 0.2108 | 0.3167 |
| 12 | 0.2389 | 0.2584 |
| 12 | 0.2761 | 0.2588 |
| 12 | 0.2517 | 0.3120 |
| 13 | 0.2703 | 0.3031 |
| 13 | 0.1732 | 0.2011 |
| 13 | 0.2035 | 0.3197 |
| 13 | 0.1832 | 0.9468 |
| 13 | 0.2804 | 1.4240 |
| 14 | 0.4018 | 0.5243 |
| 14 | 0.2874 | 0.4448 |
| 14 | 0.1864 | 0.3615 |
| 14 | 0.1856 | 2.2910 |
| 14 | 0.1833 | 0.9387 |
| 15 | 0.1944 | 1.3280 |
| 15 | 0.3038 | 0.8730 |
| 15 | 0.2174 | 0.5658 |
| 15 | 0.2020 | 1.5690 |
| 15 | 0.1893 | 0.3458 |

## ANNEX II – RA RAW DATA – HEAT DAMAGE

| AREA | Virgin Hair | Damaged Hair |
|---|---|---|
| 1 | 1.7990 | 0.1959 |
| 1 | 0.7447 | 1.6900 |
| 1 | 0.3186 | 0.7067 |
| 1 | 0.1359 | 0.4703 |
| 1 | 0.1594 | 0.2667 |
| 2 | 0.2835 | 0.7689 |
| 2 | 0.2063 | 0.1490 |
| 2 | 0.1831 | 0.3769 |
| 2 | 0.1806 | 0.5908 |
| 2 | 0.2150 | 0.1917 |
| 3 | 0.1547 | 0.8996 |
| 3 | 0.2665 | 0.3665 |
| 3 | 0.4705 | 0.2479 |
| 3 | 0.5751 | 0.1469 |
| 3 | 0.5819 | 0.1475 |
| 4 | 0.2524 | 0.1802 |
| 4 | 0.2124 | 0.1963 |
| 4 | 0.2251 | 0.2157 |
| 4 | 0.3112 | 0.2961 |
| 4 | 0.3348 | 0.3365 |
| 5 | 0.3189 | 0.1640 |
| 5 | 0.3449 | 0.1561 |
| 5 | 0.3034 | 0.2455 |
| 5 | 0.2206 | 0.2453 |
| 5 | 0.2535 | 0.1360 |
| 6 | 0.1736 | 0.2076 |
| 6 | 0.1588 | 0.1759 |

| AREA | Virgin Hair | Damaged Hair |
|---|---|---|
| 6 | 0.1644 | 0.1218 |
| 6 | 0.1893 | 0.2262 |
| 6 | 0.2566 | 0.3640 |
| 7 | 0.2854 | 0.3668 |
| 7 | 0.2058 | 0.2071 |
| 7 | 0.1544 | 0.2123 |
| 7 | 0.1579 | 0.2218 |
| 7 | 0.1299 | 0.4487 |
| 8 | 0.1839 | 0.2736 |
| 8 | 0.2123 | 0.2099 |
| 8 | 0.1877 | 0.2311 |
| 8 | 0.1584 | 0.2545 |
| 8 | 0.2983 | 0.1999 |
| 9 | 0.8525 | 1.9410 |
| 9 | 0.2191 | 0.2014 |
| 9 | 0.1398 | 0.1916 |
| 9 | 0.2120 | 0.2314 |
| 9 | 0.3885 | 1.5490 |
| 10 | 0.9103 | 0.2379 |
| 10 | 0.2043 | 0.5391 |
| 10 | 0.2588 | 0.2008 |
| 10 | 0.2645 | 0.2624 |
| 10 | 0.2603 | 1.0630 |
| 11 | 0.2779 | 0.9406 |
| 11 | 0.3015 | 0.1762 |
| 11 | 0.2027 | 0.2870 |
| 11 | 0.2115 | 0.1957 |
| 11 | 0.2593 | 0.1745 |
| 12 | 0.4791 | 0.4319 |

| AREA | Virgin Hair | Damaged Hair |
|---|---|---|
| 12 | 0.7075 | 0.2733 |
| 12 | 0.4769 | 0.1844 |
| 12 | 0.2415 | 0.5787 |
| 12 | 0.2693 | 0.2591 |
| 13 | 0.4702 | 0.4243 |
| 13 | 0.1802 | 0.4594 |
| 13 | 0.2202 | 0.1538 |
| 13 | 0.2080 | 0.3826 |
| 13 | 0.1630 | 0.4643 |
| 14 | 0.1644 | 0.3605 |
| 14 | 0.3223 | 0.2817 |
| 14 | 0.2692 | 0.1993 |
| 14 | 0.1782 | 0.1943 |
| 14 | 0.1228 | 0.2703 |
| 15 | 0.7241 | 0.3297 |
| 15 | 0.1733 | 0.1519 |
| 15 | 0.1485 | 0.1477 |
| 15 | 0.1767 | 0.2315 |
| 15 | 0.3232 | 0.2238 |

## ANNEX III – 18-MEA PEAK INTENSITY RAW DATA – CHEMICAL DAMAGE

| SAMPLE | Virgin Hair | Damaged Hair | Treated Hair |
|---|---|---|---|
| 1 | 0.0038 | 0.00033 | 0.00045 |
| 2 | 0.0037 | 0.00033 | 0.00044 |
| 3 | 0.0037 | 0.00032 | 0.00057 |

## ANNEX IV – CN- PEAK INTENSITY RAW DATA – CHEMICAL DAMAGE

| SAMPLE | Virgin Hair | Damaged Hair | Treated Hair |
|---|---|---|---|
| 1 | 0.0258 | 0.0537 | 0.0479 |
| 2 | 0.0285 | 0.0516 | 0.0487 |
| 3 | 0.0259 | 0.0542 | 0.0433 |

## ANNEX V – 18-MEA PEAK INTENSITY RAW DATA – HEAT DAMAGE

| SAMPLE | Virgin Hair | Damaged Hair | Treated Hair (Active Treatment) | Treated Hair (Placebo Treatment) |
|---|---|---|---|---|
| 1 | 0.0051 | 0.0034 | 0.00053 | 0.00072 |
| 1 | 0.0066 | 0.0020 | 0.00053 | 0.00056 |
| 1 | 0.0068 | 0.0011 | 0.00048 | 0.00055 |
| 2 | 0.0047 | 0.0034 | 0.00111 | 0.00155 |
| 2 | 0.0004 | 0.0036 | 0.00108 | 0.00144 |
| 2 | 0.0057 | 0.0029 | 0.00108 | 0.00124 |
| 3 | 0.0060 | 0.0021 | 0.00116 | 0.00073 |
| 3 | 0.0064 | 0.0026 | 0.00102 | 0.00085 |
| 3 | 0.0004 | 0.0003 | 0.00126 | 0.00064 |
| 4 | 0.0075 | 0.0020 | 0.00049 | 0.00048 |
| 4 | 0.0069 | 0.0020 | 0.00061 | 0.00042 |
| 4 | 0.0062 | 0.0023 | 0.00061 | 0.00048 |
| 5 | 0.0065 | 0.0012 | 0.00068 | 0.00035 |
| 5 | 0.0075 | 0.0008 | 0.00060 | 0.00033 |
| 5 | 0.0081 | 0.0028 | 0.00050 | 0.00033 |
| 6 | 0.0057 | 0.0013 | 0.00074 | 0.00080 |
| 6 | 0.0053 | 0.0016 | 0.00057 | 0.00104 |
| 6 | 0.0047 | 0.0019 | 0.00076 | 0.00142 |

## ANNEX VI – $CN^-$ PEAK INTENSITY RAW DATA – HEAT DAMAGE

| SAMPLE | Virgin Hair | Damaged Hair | Treated Hair (Active Treatment) | Treated Hair (Placebo Treatment) |
|---|---|---|---|---|
| 1 | 0.0394 | 0.04680 | 0.04780 | 0.02920 |
| 1 | 0.0379 | 0.04770 | 0.04830 | 0.02820 |
| 1 | 0.0377 | 0.05100 | 0.04860 | 0.03230 |
| 2 | 0.0468 | 0.04920 | 0.04450 | 0.04010 |
| 2 | 0.0416 | 0.04970 | 0.04570 | 0.03790 |
| 2 | 0.0400 | 0.05190 | 0.04590 | 0.04230 |
| 3 | 0.0423 | 0.05420 | 0.04840 | 0.03280 |
| 3 | 0.0392 | 0.05610 | 0.04790 | 0.03690 |
| 3 | 0.0390 | 0.05610 | 0.05050 | 0.03790 |
| 4 | 0.0408 | 0.05520 | 0.03700 | 0.03450 |
| 4 | 0.0401 | 0.05330 | 0.04350 | 0.03360 |
| 4 | 0.0391 | 0.05210 | 0.04600 | 0.03750 |
| 5 | 0.0394 | 0.04880 | 0.02580 | 0.04190 |
| 5 | 0.0396 | 0.04370 | 0.02410 | 0.05410 |
| 5 | 0.0419 | 0.04890 | 0.02370 | 0.05170 |
| 6 | 0.0428 | 0.04970 | 0.03400 | 0.03950 |
| 6 | 0.0428 | 0.04570 | 0.03790 | 0.04100 |
| 6 | 0.0413 | 0.04580 | 0.03860 | 0.03850 |

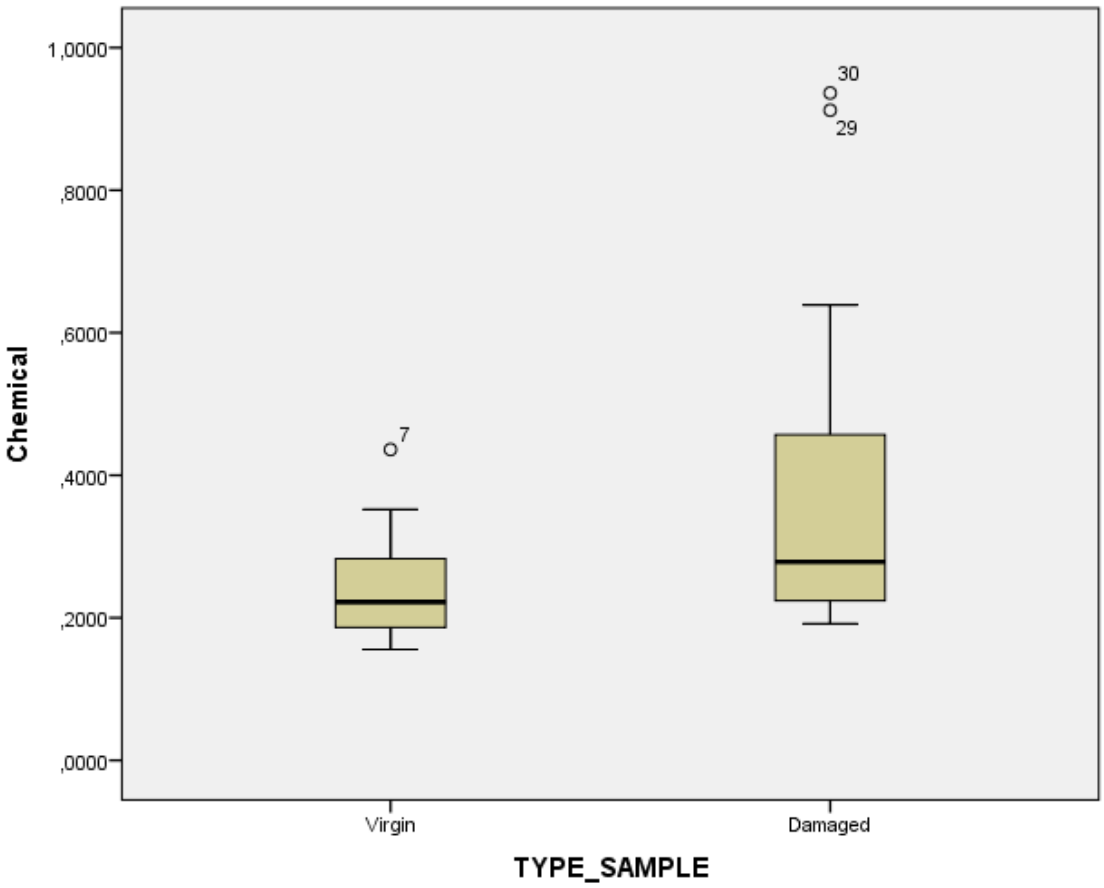
## ANNEX VII – 18-MEA PEAK INTENSITY RAW DATA – CHEMICAL + UV + POLLUTION DAMAGE

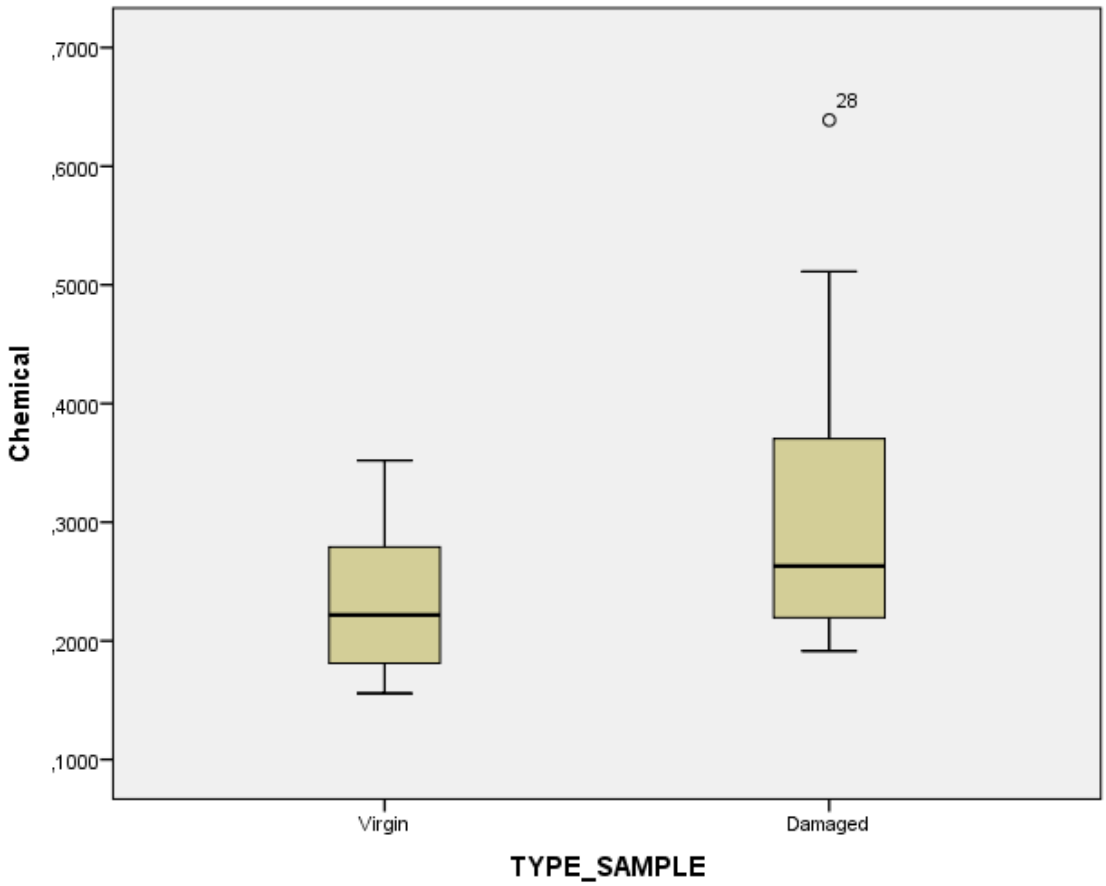
| SAMPLE | Virgin Hair | Damaged Hair |
|---|---|---|
| 1 | 0.0039 | 0.00031 |
| 2 | 0.0045 | 0.00029 |
| 3 | 0.0040 | 0.00030 |

## ANNEX VIII – $CN^-$ PEAK INTENSITY RAW DATA – CHEMICAL + UV + POLLUTION DAMAGE

| SAMPLE | Virgin Hair | Damaged Hair |
|---|---|---|
| 1 | 0.0495 | 0.0318 |
| 2 | 0.0447 | 0.0314 |
| 3 | 0.0505 | 0.0304 |

## ANNEX IX – RA STATISTICAL ANALYSIS – CHEMICAL DAMAGE






**Tests of Normality**

| | | Kolmogorov-Smirnov[a] | | | Shapiro-Wilk | | |
|---|---|---|---|---|---|---|---|
| | TYPE_SAMPLE | Statistic | df | Sig. | Statistic | df | Sig. |
| Chemical | Virgin | ,171 | 14 | ,200* | ,935 | 14 | **,363** |
| | Damaged | ,202 | 12 | ,191 | ,866 | 12 | **,058** |

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

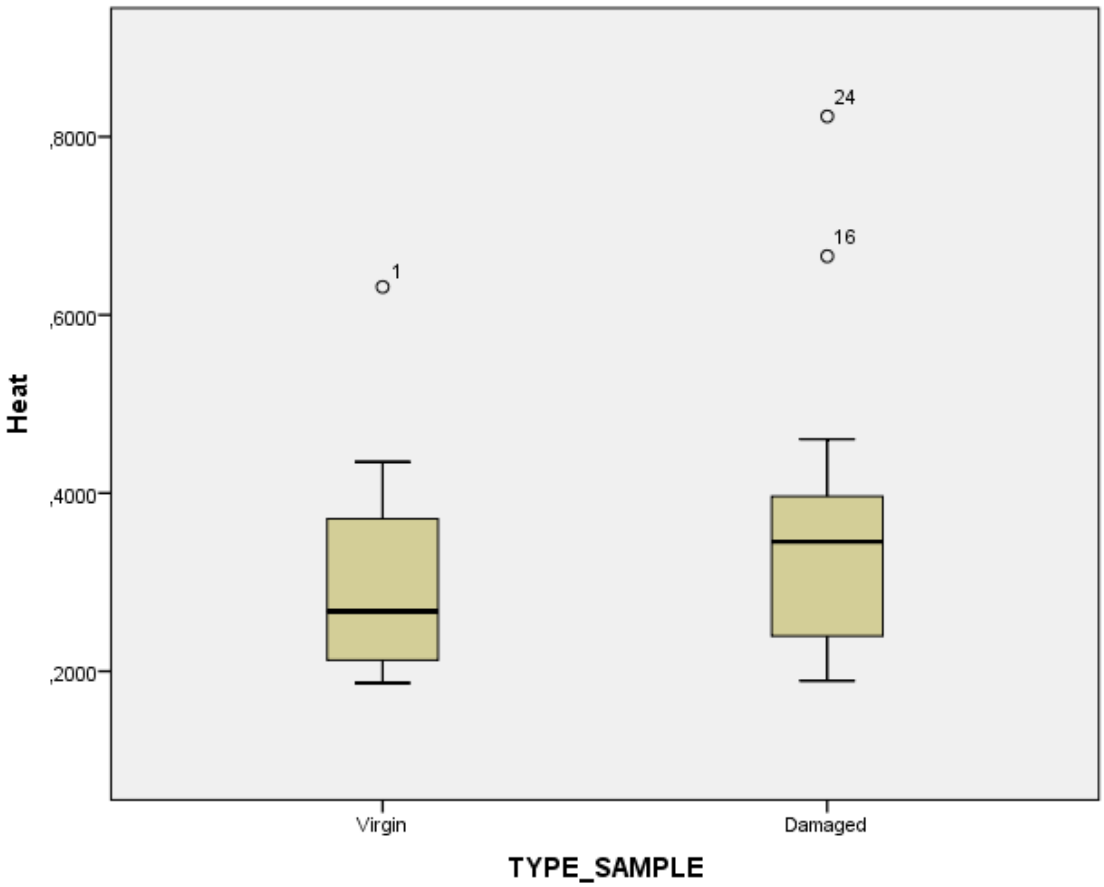
**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | |
|---|---|---|---|---|---|
| | | F | Sig. | t | df |
| Chemical | Equal variances assumed | 2,568 | ,122 | -1,708 | 24 |
| | Equal variances not assumed | | | -1,648 | 17,825 |

| | | t-test for Equality of Means | | |
|---|---|---|---|---|
| | | Sig. (2-tailed) | Mean Difference | Std. Error Difference |
| Chemical | Equal variances assumed | **,101** | -,0542333 | ,0317571 |
| | Equal variances not assumed | ,117 | -,0542333 | ,0329079 |

| | | t-test for Equality of Means | |
|---|---|---|---|
| | | 95% Confidence Interval of the Difference | |
| | | Lower | Upper |
| Chemical | Equal variances assumed | -,1197767 | ,0113100 |
| | Equal variances not assumed | -,1234191 | ,0149524 |

## ANNEX X – RA STATISTICAL ANALYSIS – HEAT DAMAGE




**Tests of Normality**

| | | Kolmogorov-Smirnov[a] | | | Shapiro-Wilk | | |
|---|---|---|---|---|---|---|---|
| | TYPE_SAMPLE | Statistic | df | Sig. | Statistic | df | Sig. |
| Heat | Virgin | ,152 | 14 | ,200* | ,906 | 14 | **,138** |
| | Damaged | ,159 | 13 | ,200* | ,940 | 13 | **,452** |

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

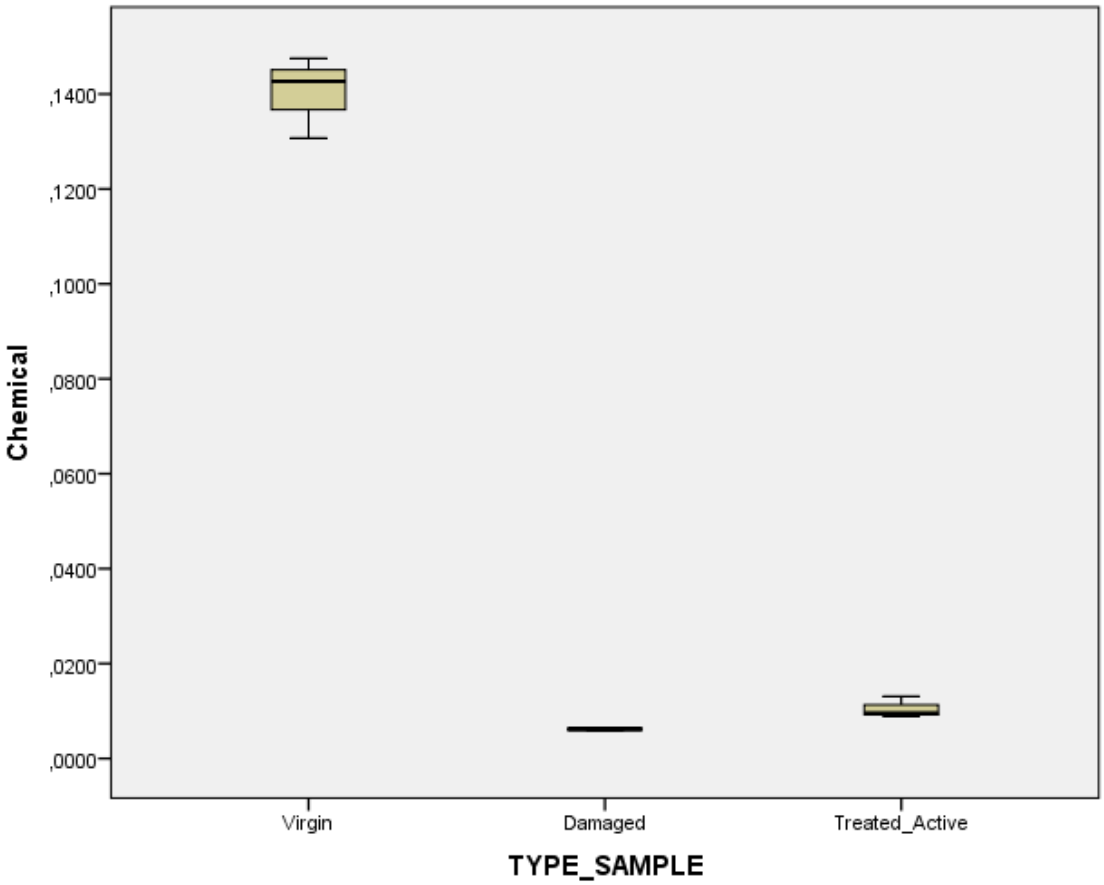
**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | |
|---|---|---|---|---|---|
| | | F | Sig. | t | df |
| Heat | Equal variances assumed | ,440 | ,513 | -,489 | 25 |
| | Equal variances not assumed | | | -,487 | 24,418 |

| | | t-test for Equality of Means | | | |
|---|---|---|---|---|---|
| | | Sig. (2-tailed) | Mean Difference | Std. Error Difference | 95% Confidence Interval of the Difference |
| | | | | | Lower |
| Heat | Equal variances assumed | **,629** | -,0155297 | ,0317608 | -,0809423 |
| | Equal variances not assumed | ,630 | -,0155297 | ,0318559 | -,0812175 |

| | | t-test for Equality of Means |
|---|---|---|
| | | 95% Confidence Interval of the Difference |
| | | Upper |
| Heat | Equal variances assumed | ,0498829 |
| | Equal variances not assumed | ,0501582 |

## ANNEX XI – PEAK INTENSITY RATIO STATISTICAL ANALYSIS – CHEMICAL DAMAGE




**Tests of Normality**

| | | Kolmogorov-Smirnov[a] | | | Shapiro-Wilk | | |
|---|---|---|---|---|---|---|---|
| | TYPE_SAMPLE | Statistic | df | Sig. | Statistic | df | Sig. |
| Chemical | Virgin | ,276 | 3 | . | ,942 | 3 | **,537** |
| | Damaged | ,253 | 3 | . | ,964 | 3 | **,637** |
| | Treated_Active | ,337 | 3 | . | ,855 | 3 | **,253** |

a. Lilliefors Significance Correction

## Oneway

### Test of Homogeneity of Variances

Chemical

| Levene Statistic | df1 | df2 | Sig. |
|---|---|---|---|
| 6,614 | 2 | 6 | ,030 |

### ANOVA

Chemical

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | ,035 | 2 | ,017 | 652,683 | ,000 |
| Within Groups | ,000 | 6 | ,000 | | |
| Total | ,035 | 8 | | | |

### Post Hoc Tests

### Multiple Comparisons

Dependent Variable: Chemical

Tukey HSD

| (I) TYPE_SAMPLE | (J) TYPE_SAMPLE | Mean Difference (I-J) | Std. Error | Sig. | 95% Confidence Interval Lower Bound |
|---|---|---|---|---|---|
| Virgin | Damaged | ,1341667* | ,0042199 | **,000** | ,121219 |
| | Treated_Active | ,1298000* | ,0042199 | ,000 | ,116852 |
| Damaged | Virgin | -,1341667* | ,0042199 | **,000** | -,147114 |
| | Treated_Active | -,0043667 | ,0042199 | **,584** | -,017314 |

| | | | | | |
|---|---|---|---|---|---|
| Treated_Active | Virgin | -,1298000* | ,0042199 | ,000 | -,142748 |
| | Damaged | ,0043667 | ,0042199 | **,584** | -,008581 |

**Multiple Comparisons**

Dependent Variable: Chemical

Tukey HSD

| (I) TYPE_SAMPLE | (J) TYPE_SAMPLE | 95% Confidence Interval Upper Bound |
|---|---|---|
| Virgin | Damaged | ,147114 |
| | Treated_Active | ,142748 |
| Damaged | Virgin | -,121219 |
| | Treated_Active | ,008581 |
| Treated_Active | Virgin | -,116852 |
| | Damaged | ,017314 |

*. The mean difference is significant at the 0.05 level.

**Homogeneous Subsets**

**Chemical**

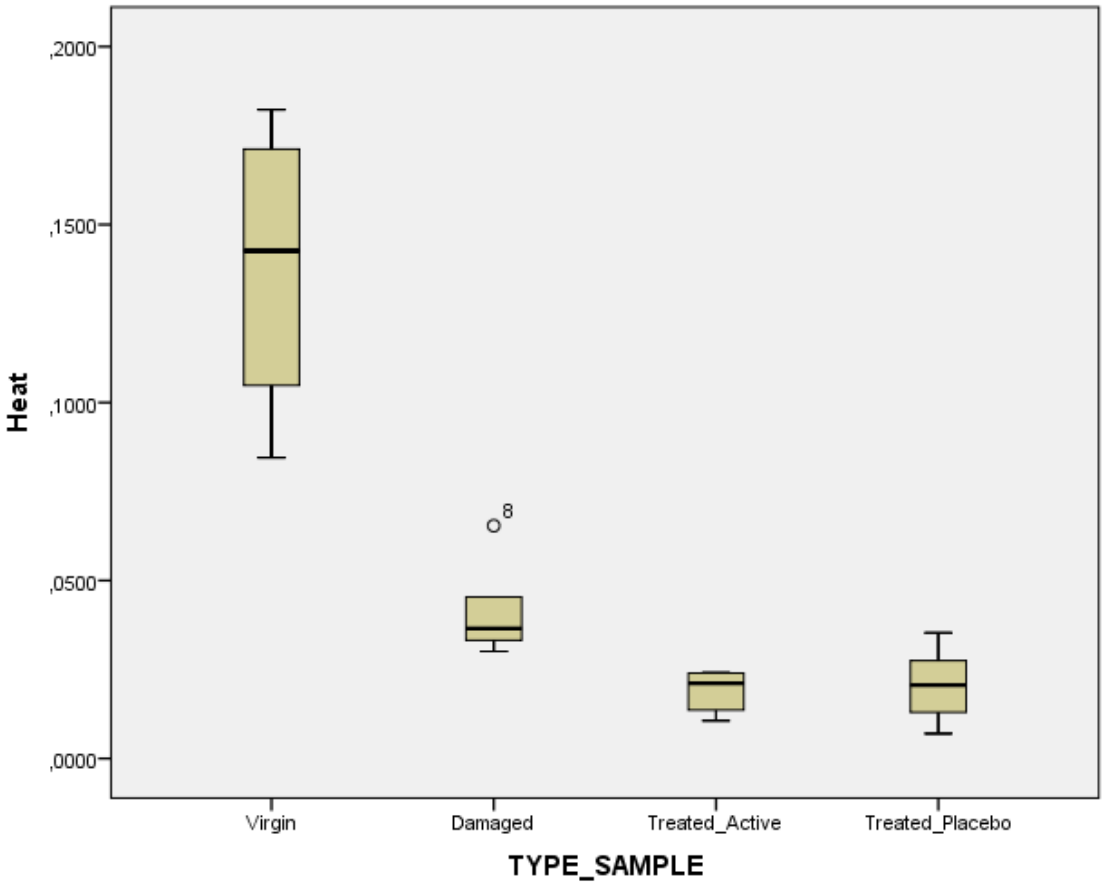
Tukey HSD[a]

| | | Subset for alpha = 0.05 | |
|---|---|---|---|
| TYPE_SAMPLE | N | 1 | 2 |
| Damaged | 3 | ,006133 | |
| Treated_Active | 3 | ,010500 | |
| Virgin | 3 | | ,140300 |
| Sig. | | ,584 | 1,000 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3,000.

## ANNEX XII – PEAK INTENSITY RATIO STATISTICAL ANALYSIS – HEAT DAMAGE




**Tests of Normality**

| | | Kolmogorov-Smirnov[a] | | | Shapiro-Wilk | | |
|---|---|---|---|---|---|---|---|
| | TYPE_SAMPLE | Statistic | df | Sig. | Statistic | df | Sig. |
| Heat | Virgin | ,221 | 6 | ,200* | ,927 | 6 | **,557** |
| | Damaged | ,246 | 5 | ,200* | ,936 | 5 | **,635** |
| | Treated_Active | ,270 | 6 | ,194 | ,847 | 6 | **,150** |
| | Treated_Placebo | ,165 | 6 | ,200* | ,981 | 6 | **,954** |

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

## Oneway

### Test of Homogeneity of Variances

Heat

| Levene Statistic | df1 | df2 | Sig. |
|---|---|---|---|
| 17,245 | 3 | 19 | ,000 |

## ANOVA

Heat

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | ,058 | 3 | ,019 | 42,772 | ,000 |
| Within Groups | ,009 | 19 | ,000 | | |
| Total | ,066 | 22 | | | |

## Post Hoc Tests

### Multiple Comparisons

Dependent Variable: Heat

Tukey HSD

| (I) TYPE_SAMPLE | (J) TYPE_SAMPLE | Mean Difference (I-J) | Std. Error | Sig. | 95% Confidence Interval Lower Bound |
|---|---|---|---|---|---|
| Virgin | Damaged | ,1016767* | ,0128619 | **,000** | ,065511 |
| | Treated_Active | ,1189333* | ,0122634 | ,000 | ,084451 |
| | Treated_Placebo | ,1173500* | ,0122634 | ,000 | ,082867 |
| Damaged | Virgin | -,1016767* | ,0128619 | **,000** | -,137842 |

| | | | | | |
|---|---|---|---|---|---|
| | Treated_Active | ,0172567 | ,0128619 | **,549** | -,018909 |
| | Treated_Placebo | ,0156733 | ,0128619 | **,623** | -,020492 |
| Treated_Active | Virgin | -,1189333* | ,0122634 | ,000 | -,153416 |
| | Damaged | -,0172567 | ,0128619 | **,549** | -,053422 |
| | Treated_Placebo | -,0015833 | ,0122634 | ,999 | -,036066 |
| Treated_Placebo | Virgin | -,1173500* | ,0122634 | ,000 | -,151833 |
| | Damaged | -,0156733 | ,0128619 | **,623** | -,051839 |
| | Treated_Active | ,0015833 | ,0122634 | ,999 | -,032899 |

**Multiple Comparisons**

Dependent Variable: Heat

Tukey HSD

| | | 95% Confidence Interval |
|---|---|---|
| (I) TYPE_SAMPLE | (J) TYPE_SAMPLE | Upper Bound |
| Virgin | Damaged | ,137842 |
| | Treated_Active | ,153416 |
| | Treated_Placebo | ,151833 |
| Damaged | Virgin | -,065511 |
| | Treated_Active | ,053422 |
| | Treated_Placebo | ,051839 |
| Treated_Active | Virgin | -,084451 |
| | Damaged | ,018909 |
| | Treated_Placebo | ,032899 |
| Treated_Placebo | Virgin | -,082867 |
| | Damaged | ,020492 |
| | Treated_Active | ,036066 |

*. The mean difference is significant at the 0.05 level.

## Homogeneous Subsets

### Heat

Tukey HSD[a,b]

| | | Subset for alpha = 0.05 | |
|---|---|---|---|
| TYPE_SAMPLE | N | 1 | 2 |
| Treated_Active | 6 | ,019083 | |
| Treated_Placebo | 6 | ,020667 | |
| Damaged | 5 | ,036340 | |
| Virgin | 6 | | ,138017 |
| Sig. | | ,530 | 1,000 |

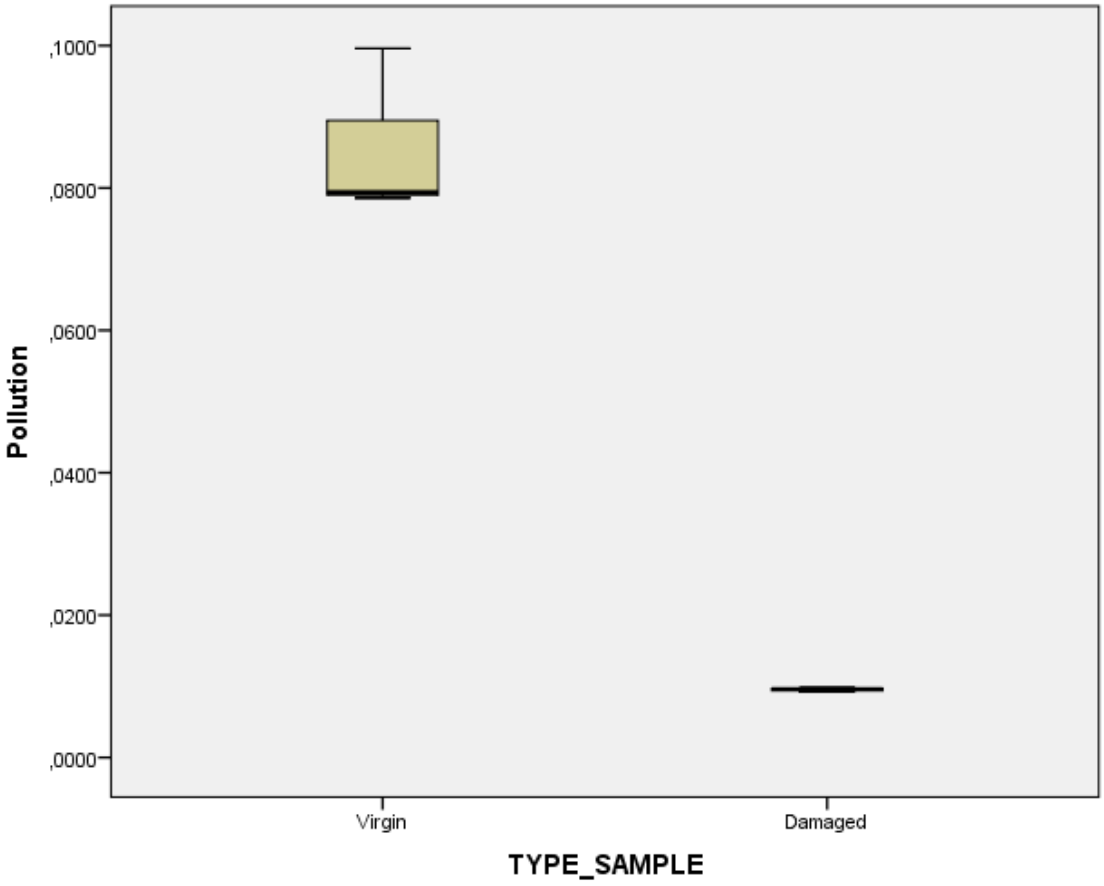
Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5,714.

b. The group sizes are unequal. The harmonic mean of the group sizes is used.

Type I error levels are not guaranteed.

## ANNEX XIII – PEAK INTENSITY RATIO STATISTICAL ANALYSIS – CHEMICAL + UV + POLLUTION DAMAGE




**Tests of Normality[b]**

| | | Kolmogorov-Smirnov[a] | | | Shapiro-Wilk | | |
|---|---|---|---|---|---|---|---|
| | TYPE_SAMPLE | Statistic | df | Sig. | Statistic | df | Sig. |
| Pollution | Virgin | ,373 | 3 | . | ,779 | 3 | **,064** |
| | Damaged | ,253 | 3 | . | ,964 | 3 | **,637** |

a. Lilliefors Significance Correction

b. There are no valid cases for Pollution when TYPE_SAMPLE = 3,000. Statistics cannot be computed for this level.

**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | |
|---|---|---|---|---|---|
| | | F | Sig. | t | df |
| Pollution | Equal variances assumed | 15,061 | ,018 | 11,107 | 4 |
| | Equal variances not assumed | | | 11,107 | 2,003 |

| | | t-test for Equality of Means | | |
|---|---|---|---|---|
| | | Sig. (2-tailed) | Mean Difference | Std. Error Difference |
| Pollution | Equal variances assumed | ,000 | ,0763333 | ,0068728 |
| | Equal variances not assumed | **,008** | ,0763333 | ,0068728 |

| | | t-test for Equality of Means | |
|---|---|---|---|
| | | 95% Confidence Interval of the Difference | |
| | | Lower | Upper |
| Pollution | Equal variances assumed | ,0572513 | ,0954153 |
| | Equal variances not assumed | ,0467993 | ,1058674 |