

## Role of Internal Lipids in Hair Health

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### Synopsis

Saturated and unsaturated fatty acids make up 85% of the total hair lipid content and are found in the cuticle and cortical cell membrane complex. Although these lipids only make up 2–6% of the hair's overall weight, they play a crucial role in keeping hair healthy, influencing shine, feel, manageability, and strength. The objective of this work was to understand the mechanisms of how these lipids are lost on exposure to external stressors, such as chemical treatments, washing, and UV exposure and to understand how their loss impacts hair strength. The experimental approach was to measure these lipids and oxidation products, lipid peroxides (LPOs) and correlate their loss with fatigue strength measurements. The results show lipids are lost over time by washing, exposure to chemical treatments, such as coloring, and environmental insults, such as UV, and it was confirmed that a mechanism of degradation is via oxidation of unsaturated lipids to form LPOs. In addition, it was shown that replenishment of these lipids is possible by incorporating lipids, such as fatty alcohols (FaOHs), into a gel network with anionic surfactants to create a delivery system that can efficiently penetrate FaOHs into hair and increase internal strength as measured by fatigue.

### INTRODUCTION

Hair health is an important attribute to consumers and, thus, to the cosmetics industry. Reducing the signs of damage, such as poor shine and manageability, split ends, broken fibers, and poor feel are targets for many products in the hair care regimen, especially shampoos, conditioners, and treatments. Thus, it is important to understand the underlying causes of hair structural damage from external stressors to develop better performing products. These stressors include chemical treatments, such as coloring, perming, and relaxing, UV exposure, excessive heat exposure, and physical abrasion from washing and combing. As we consider hair changes we need to investigate all the major components of hair, the cuticle, cortex, medulla, protein, and lipid structures. Of interest in this study are the

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lipid components of hair, which play a major role in the structural integrity of hair. Specifically of interest are internal structural lipids located in cortex and cuticle membranes and in the medulla, contributing ~2–6% of the overall fiber weight. Sebum lipids can also contribute to fiber health (1) but this is outside the scope of this work.

The internal lipids in hair are mainly composed of fatty acids, cholesterol, and ceramides with fatty acids being the major components. An excellent review from Clarence Robbins in 2009 (2) details the proposed location of these lipids in the cell membrane complex (CMC). The bound lipids, including 18-methyleicosanoic acid, are located in the cuticle CMC along with both free unsaturated and saturated fatty acids predominantly of chain length C16 and C18 in a monolayer arrangement. Cholesterol and ceramides are located primarily in the cortex CMC in a bilayer arrangement along with additional free fatty acids. The literature data are somewhat inconsistent on levels of these different lipids because of substrate variability and extraction techniques but it is agreed that the lipids are important to hair health playing a major role in adhesion of cortical and cuticle cells. This makes them an important factor for determining hair strength and as a pathway for active penetration. It is also well known that daily weathering can cause loss of these internal lipids. Masukawa et al. (3) quantified this loss after repeated shampoo treatments with and without bleaching and our work was to investigate mechanisms. This work describes a multistage extraction process to quantify the lipids and used this method to investigate mechanisms by which lipids are changed during exposure to external stressors, such as bleaching and UV, and how this impacts hair strength. In addition, a strategy to replenish these lipids when lost is discussed.

## EXPERIMENTAL

### HAIR SAMPLES AND TREATMENT

Four grams, 8-inch Caucasian-source untreated hair (i.e. no chemical treatment) was purchased from International Hair Importers & Products, Inc. (Glendale, NY).

Each wash cycle consisted of applying 0.1 g/g shampoo to the hair tress and lathering for 30 s followed by a 30-s rinse repeated for a total of two shampoo applications. Hair tresses were placed in a hot box at 80°C until the hair was dry. The hair was then soaked in a solution of 6% hydrogen peroxide, 2% ammonium hydroxide adjusted to pH 10, with acetic acid for 35 min and then rinsed for 15 min to create chemically treated hair.

### INTERNAL LIPID AND FATTY ALCOHOL (FaOH) EXTRACTION

Hair samples were equilibrated in a 20% relative humidity (RH) constant humidity chamber overnight. For each sample, ~0.1 g of hair was cut in 20–40 mm segments into vials ( $n = 4$ ). First, the hair was extracted gently with hexane to remove the external surface lipid. The hexane extraction consists of extracting the hair with hexane two times then concentrating the dried residue in second solvent (mobile phase for the SFC-MS-MS and derivatizing reagent for the GC). Next free lipid was extracted using 2:1 then 1:1 chloroform:methanol. The chloroform contained 10 mM dimethylhexylamine and the methanol contained 1% formic acid. Each extraction was heated for 30 min

at 65°C with the hair and then combined and the dried residue redissolved in a second solvent (mobile phase for the SFC-MS-MS and BSTFA derivatizing reagent for the GC). Last, the bound lipids were removed with a potassium hydroxide (KOH) extraction. Hair from the free lipid extraction was digested with 50% KOH:methanol solution for 30 min at 65°C and then neutralized with 3N HCl. A final extraction with chloroform:methanol solution was performed two times on the remaining hair then concentrated and made up in a second solvent.

Lipids were separated using super critical fluid chromatography, with a normal phase silica column that separated each chemical class as a group by polarity. The mobile phase was carbon dioxide with methanol with formic acid and ammonia formate modifier. The lipids were detected with a triple quadrupole linear ion trap mass spectrometer, with multiple reaction monitoring, using atmospheric pressure ionization (APCI). Stable isotope-labelled internal standards for each chemical class were also used.

Cetyl and stearyl alcohol were quantified by gas chromatography with flame ionization detection using a polydimethylsiloxane capillary column with hydrogen mobile phase. Nonadecanoic acid and eicosanoic acid were used as internal standards.

#### OPTICAL MICROSCOPY

Hair samples were incubated in a solution of Nile Red (2 mg/ml) for 24 h at 37°C, before short acetone washing to remove external stain. Briefly, hairs were cut with a sharp razor blade perpendicular to their longitudinal axis into approximately 3-cm sections. To allow cross-sectional imaging, hairs were placed in a glass capillary needle inside a 3D printed stand, with a central hole to contain the capillary tube. This assembly was then placed in a glass-bottomed dish. Stained hairs were imaged using a Zeiss 880 Airyscan Laser Scanning Confocal Microscope with a 1.4NA 63x oil immersion objective (Carl Zeiss Microscopy GmbH, Germany). During imaging, the 514-nm laser line was used to excite the Nile Red stain.

#### LIPID PEROXIDE (LPO) MEASUREMENTS

Hair samples were equilibrated in a 20% RH constant humidity chamber overnight. For each sample, ~0.1 g of hair was cut in 20–40-mm segments into vials ( $n = 4$ ). This hair was sonicated for 2 h in a 2:1 chloroform:methanol solvent mix and then evaporated to dryness. The extract was reconstituted in isopropyl alcohol and then tested with a commercial LPO kit (KAMIYA Biomedical Company, Seattle, WA, <http://kamiyabiomedical.com/>).

#### GEL NETWORK FORMULATIONS

The gel network was made with 11% SLE1S surfactant, 8% stearyl alcohol, 4% cetyl alcohol, and 77% deionized water, where all ingredients were first heated together to a temperature between 75°C and 90°C and then cooled to room temperature. This process formed the gel premix. A small amount of this premix, 2.3% FaOH by weight of the final shampoo, was then dispersed in a typical shampoo containing other beneficial agents, such as surfactants, silicones, and polymers.

## HAIR FATIGUE MEASUREMENTS

Hair treatments were performed on 4 g 8" chemically treated hair switches sourced from International Hair Importers & Products, Inc. Fibers were cut from the middle of the tress and ends crimped at 30 mm using a Dia-stroon Auto-Assembly System (AAS 1600) (Andover, Hampshire, UK). The average cross-sectional area along each fiber was analyzed using a Dia-Stroon Fiber Dimensional Analysis System (FDAS 770), which incorporates a Mitutoyo laser micrometer (LSM-6200) (Malborough, MA). The average cross-sectional area was calculated from three diameter measurement points along each 30-mm crimped fiber. The average cross-sectional values for each of the fibers were then used to set the Dia-Stroon Cyclic Tester (CYC801) in controlled stress mode of  $0.014 \text{ g}/\mu\text{m}^2$  and rate of 40 mm/s. Environmental testing conditions were set at 50% RH and 23°C. Data were analyzed by Weibull and Kaplan–Meier statistical tools (JMP Pro 12.1.0, SAS, Cary, NC). Fibers with break cycles less than 10 were omitted from the analysis because of premature breakage.

## RESULTS AND DISCUSSION

As we consider internal structural lipids one important question is how to quantify their levels in hair. There have been several reports in the literature (4,5) but often the data is conflicting, due in part to inherent variability in hair and in part to different extraction methods used. In this work, we developed a method using a three-stage extraction. Stage one is a hexane extraction designed to remove any surface lipids, stage two is extraction using chloroform method to extract internal unbound lipids and stage three is a hydrolysis with KOH/methanol followed by an extraction with chloroform:methanol to extract the remaining lipids. The original intent was to use the second extraction to access unbound lipids and the third to access bound lipids but this proved not to be possible. Instead, 18-MEA was used as a marker for bound lipids and extraction conditions were optimized to give readily extractable unbound lipids in the second extraction and the remainder in the third extraction. Figure 1 shows data from virgin blended hair as supplied by International Hair Importers. Most lipids are saturated and unsaturated fatty acids with C16 and C18 chain lengths predominating, but cholesterols and ceramides were also measured. These results are comparable with those published by Masukawa et al. (6) in 2005, where a similar extraction protocol was used. These lipids are typically visualized using transmission electron microscopy but in this work confocal fluorescence optical

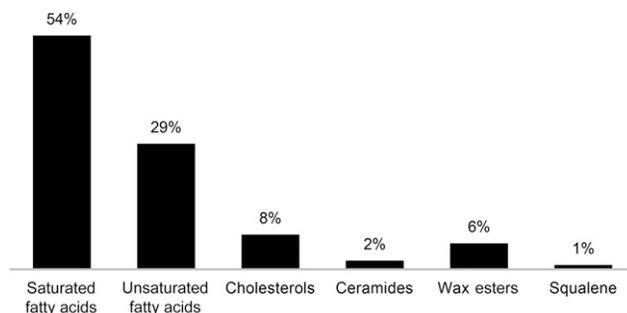


Figure 1. Internal structural lipids in hair.

microscopy was used to avoid sampling artefacts. Hair was treated with Nile Red and then imaged via confocal fluorescence microscopy in a glass capillary tube that was held vertically to enable imaging of hair cross sections in the  $x$ - $y$  plane to give optimal spatial resolution. Nile Red only fluoresces when associated with lipid structures and in Figure 2 this provides contrast for the hair images. Lipids are seen in the cuticle CMC and cortex CMC and highlights that these structures form a continuous network throughout hair. Areas of more intense contrast are seen in the medulla and in nuclear remnants, indicating high lipid concentrations in these regions.

Studies have measured changes in structural internal lipids, especially after repeated washing or coloring (3). Figure 3 shows loss of internal lipids from root to tip. Table I shows loss of internal lipids after 100 washes with a clarifying shampoo for both uncolored virgin hair and hair that had undergone five cycles of coloring with an ammonia/hydrogen peroxide colorant, with five wash cycles in between each coloring. Both coloring and washing remove lipids from hair and especially fatty acids. Coloring also significantly decreases unsaturated fatty acid levels (~40%). It is proposed that the loss of these unsaturated lipids during coloring is because of the formation of LPOs, which can either remain in hair or undergo further oxidation, e.g. in the presence of redox metals, such as copper, to form reactive oxygen species (ROS)—see Figure 4. Experiments were carried out with eight single source-untreated virgin ponytails, where initial LPO measurements were compared with LPO levels after treating, with an ammonia/hydrogen peroxide (4.5%) colorant with very low oxidative dye levels for 30 min. All the ponytails were 25–30 cm long, Caucasian hair that had not previously been colored, and all were approximately the same color (23–25 L units). To measure LPOs, hair was extracted with chloroform/methanol at room temperature and the extract measured using a LPO kit from KAMIYA Biomedical Company. In the presence of hemoglobin, LPOs are reduced to hydroxyl derivatives (lipid alcohols) and the 10-N-methylcarbonyl-3,7-bis(dimethylamino)phenothiazine chromogen is oxidatively cleaved to form methylene blue in an equal molar reaction. LPOs are then quantitated by colorimetrically measuring methylene blue at 675 nm. Figure 5 shows initial LPO data from starting substrates

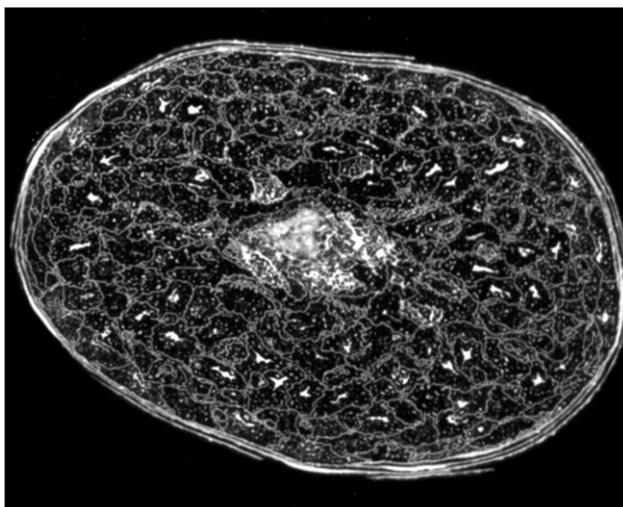


Figure 2. Confocal fluorescence microscopy image of hair stained with Nile Red dye.

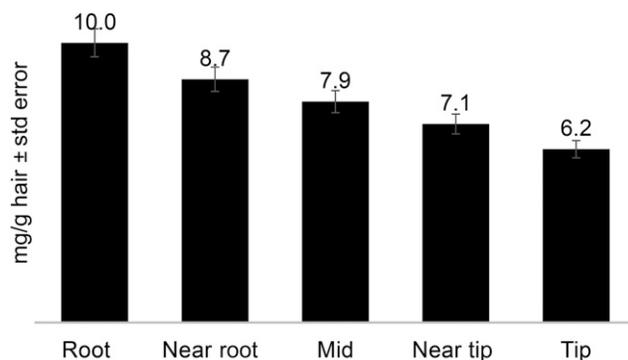


Figure 3. Lipid loss from root to tip.

and LPO levels after a 30 min colorant. LPOs were measured in almost all the starting ponytails but levels varied between ponytails. It is predicted that UV light can also form LPOs (see additional evidence below), so it is reasonable to assume these LPOs have formed from previous UV exposure by the women who donated the ponytails and may explain why levels vary from person to person. There is an increase in LPOs for all the ponytails tested apart from #1 (which had very low starting levels), providing evidence that this is a plausible mechanism for loss of unsaturated lipids after coloring. This loss is demonstrated in Figure 6, where three additional ponytails were assessed for unsaturated fatty acid levels after a 30-min coloring treatment. All three ponytails show a significant loss of unsaturated fatty acids after treatment.

The same eight ponytails were also exposed to UV irradiation that mimicked sunlight for 20 h Figure 7 shows change on LPO levels for each of the ponytails with some LPO levels increasing and some decreasing. In this case, these data illustrate that LPOs can not only be formed via UV oxidation but also be destroyed by UV oxidation. The perhydroxyl bond O-OH can be broken directly via UV absorption or more likely it reacts with other ROS formed during UV exposure. One pathway for subsequent reactivity of LPOs is shown in Figure 4 where redox metals, such as copper, can react with organic peroxides to form highly reactive alkoxy radicals, which will react further with lipids and protein structures. Previously published work has shown such mechanisms occur with hair dosed with high versus low copper levels, with added copper and UV exposure measured LPO levels are lower than with low copper levels (7).

The importance of internal structural lipids for the structural integrity of hair has been demonstrated by McMullen et al. (8), who showed removing lipids via solvent extraction caused differences in moisture management, interaction with cationic polymers and surfactants, and impact on style hold. Fatigue measurements using fixed weights showed a

Table I  
Loss of lipids with washing (100 cycles)

Hair treatment	Sat. fatty acids ( $\mu\text{g/g}$ )	Unsat. fatty acids ( $\mu\text{g/g}$ )	Wax esters ( $\mu\text{g/g}$ )	Ceramides ( $\mu\text{g/g}$ )	Chol. + Chol. sulfate ( $\mu\text{g/g}$ )	Total ( $\mu\text{g/g}$ )
Vigin	8,391 $\pm$ 779	4,522 $\pm$ 252	932 $\pm$ 50	283 $\pm$ 8	1,187 $\pm$ 150	15,308 $\pm$ 1,134
+100 Wash	7,204 $\pm$ 429	2,903 $\pm$ 140	323 $\pm$ 14	293 $\pm$ 16	994 $\pm$ 70	11,727 $\pm$ 548
5 $\times$ Colored	7,312 $\pm$ 234	2,815 $\pm$ 151	404 $\pm$ 56	277 $\pm$ 19	1,108 $\pm$ 84	11,934 $\pm$ 340
+100 Wash	6,382 $\pm$ 884	2,215 $\pm$ 168	269 $\pm$ 13	269 $\pm$ 10	1,027 $\pm$ 38	10,195 $\pm$ 977

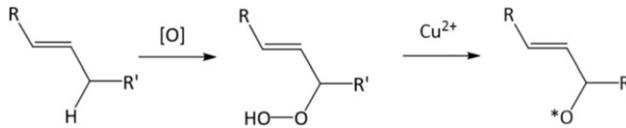


Figure 4. Oxidation of unsaturated lipids.

significant drop in cycles to break for hair, where the hair had lipids removed via extraction (66,314 for alpha value versus 18,542, where alpha value is 63.2% of fibers broken), demonstrating the importance of maintaining CMC integrity on hair strength. This importance of lipids on strength was also demonstrated in a publication by Camacho-Bragado et al. (9), where the authors suggest that breakage can occur via formation and propagation of flaws at the cell membrane locations starting at the cuticle–cortex interface. This insight sets up the question as to whether lipids can be added back into hair and specifically into the CMC to build back lipid structures and thus improve hair strength.

It is ideal to deliver lipids back into hair from every wash and thus from a daily-use product such as a shampoo. Shampoos contain high levels of anionic surfactant and are designed to remove lipid materials, i.e. sebum, so delivery of lipids from these products is challenging. A method of delivery has been developed, however, which uses a gel network structure of lipid and surfactant to deposit on hair and then on dilution release lipid into the CMC. There are several lipid classes that can be delivered by this approach and one of these is FaOHs. A gel network is made by mixing a combination of C16 FaOH (cetyl alcohol) and C18 FaOH (stearyl alcohol) together with a surfactant, SLE1S and then this gel network is added into a standard shampoo chassis. FaOHs are preferred over fatty acids for formulation stability, as at shampoo pH fatty acids will deprotonate (pKa ~ 4.8) and destabilize the gel network structure. These two classes of lipids have very similar physiochemical properties (Table II), and C16 and C18 chain lengths were specifically chosen to best match internal lipid structures. SAXS and WAXS x-ray data confirmed that an L $\beta$  lamellar structure is formed with a d-spacing of approximately 90Å in a shampoo and that this gel network remains intact over time (10). Penetration of FaOH was measured in a similar way to measuring internal structural

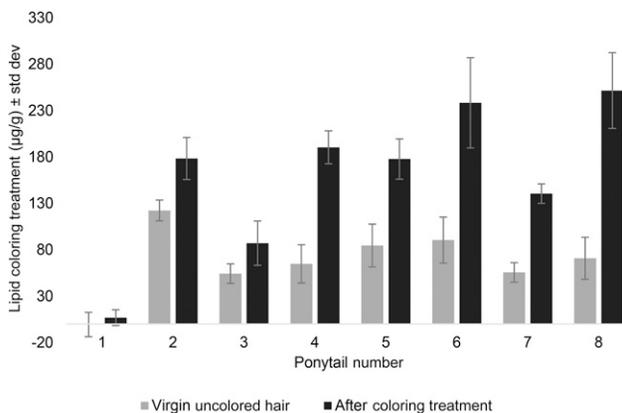


Figure 5. LPO levels in single source ponytails before and after coloring treatment.

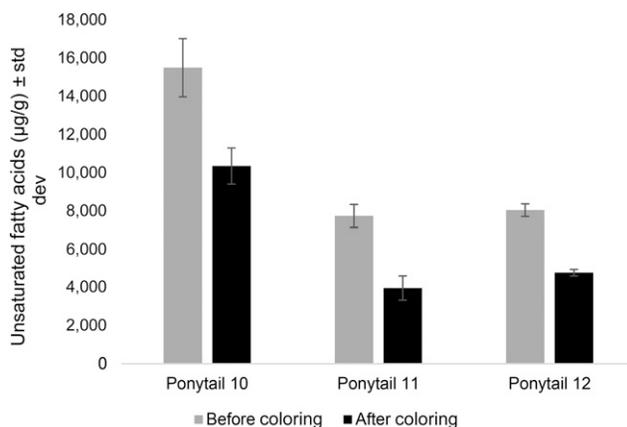


Figure 6. Unsaturated fatty acid levels before and after coloring.

lipids; an initial extraction with hexane-quantified external FaOHs and a second extraction with chloroform:methanol-quantified internal lipids. Figure 8 illustrates the importance of gel network to aid FaOH penetration comparing equal FaOH levels in a shampoo either added straight into the shampoo or as part of a gel network. Very low levels penetrated from the product with FaOH just added, but substantial penetration is measured from the gel network containing product. Table III shows fatigue data when hair treated with an ammonia/hydrogen peroxide oxidant system was washed 16 cycles in the same gel network shampoo versus an identical shampoo with no added gel network. The alpha value shown is obtained from the Weibull distribution function and is the characteristic lifetime when 63.2% of fibers have broken. The beta value is the Weibull shape parameter and provides characterization of the distribution function. The 2.3% gel network containing product showed a significantly higher alpha value than the control shampoo, i.e. more cycles are needed to break the hair after treatment with the gel network product. Weibull and Kaplan–Meier statistics using alpha values were used to determine significance between products and using both methods show that the 2.3% GN product was significantly different from the control and no gel network product. The log-rank prob > ChiSq numbers were 0.0955 (90.4%) for 2.3% gel network versus no gel network and 0.0037 (99.6%) for 2.3% gel network versus control.

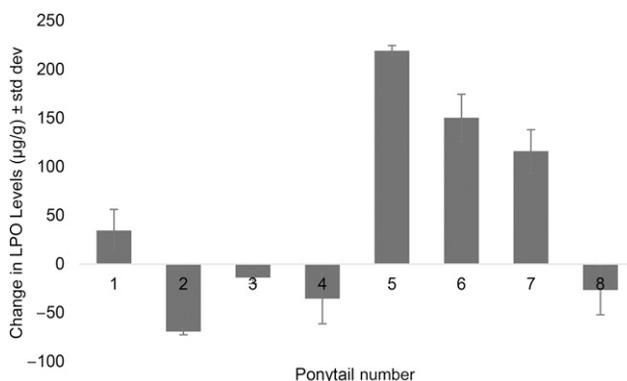


Figure 7. Change in LPO levels after UV exposure.

**Table II**  
Log P and molecular volume properties of FaOHs versus fatty acids

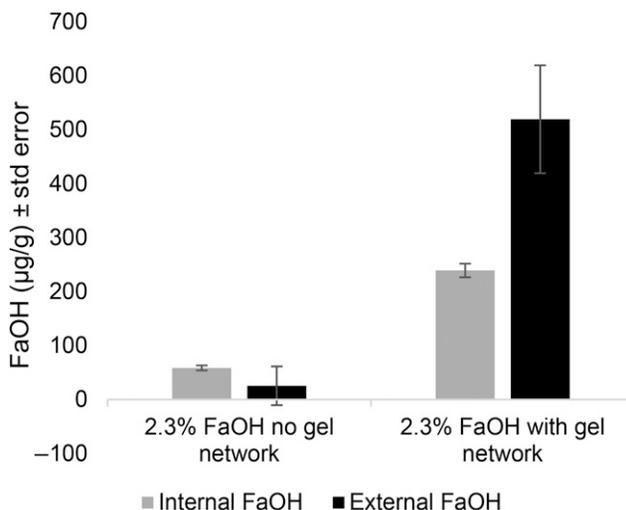
Lipid	Log P (octanol:water)	Molecular volume ( $\text{\AA}^3$ )
Cetyl alcohol (C16)	6.45	200.3
Palmitic acid (C16)	6.39	205.8
Stearyl alcohol (C18)	7.36	221.2
Stearic acid (C18)	7.31	228.8

Calculated by ACD/Labs Software (<http://www.acdlabs.com/home/>).

These data illustrate that it is feasible for lipids, such as FaOHs, to penetrate hair and increase hair strength. The exact nature of how FaOHs increase fatigue strength is not completely understood but it is hypothesized that these actives partition into the lipid-rich cortical CMC and rebuild its structural order, preventing the formation and propagation of flaws when hair is subjected to repeated extensions.

## CONCLUSIONS

Optical microscopy has been used to show how lipid structures in hair form a continuous linked structure throughout the cortex playing a major role in maintaining fatigue strength. Measurement of lipid levels has shown how these lipids are lost after washing and exposure to coloring treatments and UV. A mechanism for oxidation of unsaturated fatty acids was proposed and evidence provided by the measurement of LPOs. Replenishment of these lipids was achieved via incorporation into a gel network structure that effectively deposits on hair and releases the lipids into hair during rinsing. This penetration of internal lipids with FaOH, increasing internal strength was measured by fatigue breakage testing.



**Figure 8.** FaOH surface deposition and penetration from a shampoo formulated with a FaOH gel network and from shampoo where FaOH was added straight into shampoo (2.3% total FaOH for both shampoos).

Table III  
Fatigue breakage data for hair treated with gel network shampoo

Treatment	Alpha ( $\alpha$ ) value	Beta ( $\beta$ ) value
Control chemically treated hair (no treatment)	6,955	0.693
16 cycles shampoo with no gel network	8,328	0.682
16 cycles shampoo with gel network (total FaOH in shampoo = 2.3%)	12,142	0.569

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