Hair Substantivity Assay

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Tradename: AC Curezyme

Code: 20562

CAS #: 100209-45-8

Test Request Form #: 12604

Lot #: N250131A

Sponsor: *Active Concepts, LLC – 107 Technology Drive, Lincolnton, North Carolina 28092*
Study Director: *Daniel Shill*
Principal Investigator: *Hannah Stade*

Test Performed:
Hair Substantivity Assay

Introduction

Haircare substantivity is the ability of a product to deposit onto the hair during use. Ideally a product will be resistant to rinsing out, but not so substantive that it cannot be washed out. Products lacking substantivity do not impart significant benefits to the hair as they wash out too easily. Alternatively, products that are too substantive are resistant to washing out and build up on the hair causing hair damage over time.

Accordingly, an *ex vivo* study was conducted to evaluate the ability of **AC Curezyme** to provide substantive benefits without product build up on human hair tresses. In addition to **AC Curezyme**, AC Hydrolyzed Vegetable Protein PF was evaluated as a non-enzymatically modified protein control to demonstrate the performance enhancement achieved through **AC Curezyme's** optimal enzyme-to-protein technology, while a Quaternized Plant Protein was included as a representative quaternized conditioning agent to assess product deposition and buildup behavior relative to traditional quat-based systems.

Assay Principle

This modified Rubine Red Test method uses the electrostatic properties of hair to determine how substantive a cosmetic ingredient is by exploiting the properties of Direct Red 80 to demonstrate product deposition on hair. Successful product deposition results in pink to red toned hair, with more pigmentation indicative of a stronger interaction between the hair treatment and the dye. Water rinses test the strength of the interaction between the hair and cosmetic ingredients, where a strong interaction retains more pigmentation. Furthermore, the hair should easily release the color when washed with a simple shampoo and should not hold the dye if exposed again.

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Materials

A. **Equipment:** Canon EOZ Rebel T3 Digital Camera; Virgin Blonde Hair Tresses
B. **Reagents & Products:** Direct Red 80 (Sigma-Aldrich); 31% HCl Solution; Base Shampoo (Table 1)
C. **Software:** ImageJ (NIH); Excel Analysis ToolPak (Microsoft)
D. **Other:** 3mL Plastic Syringes

Table 1. Base Shampoo Compositional Breakdown.

Base Shampoo Formulation	
INCI	**%**
Water	41.0
Guar Hydroxypropyltrimonium Chloride	1.0
Sodium Methyl 2-Sulfolaurate (and) Disodium 2-Sulfolaurate	35.0
Cocamidopropyl Betaine	15.0
Lactobacillus Ferment & Lactobacillus & Cocos Nucifera (Coconut) Fruit Extract	4.0
Polysorbate 20	2.0
Fragrance	2.0

Methods

Five virgin blonde hair tresses were used as this shade shows the greatest level of staining and wash-out. All hair tresses were washed with 0.5 mL of base shampoo. Following the wash, the tresses were fully saturated in their respective conditions as described in Table 2. After all treatments were completed, the tresses were dipped into a 2.5 mM solution of Direct Red 80 Dye. Excess dye solution was allowed to drip off before the tresses were rinsed thoroughly with water. Tresses were hung and allowed to airdry overnight.

Table 2. Descriptions of the Conditions for each Tress .

Condition	Treatment Description	INCI
Untreated	DI Water	Water
Diluted HVP	Equivalent AC Hydrolyzed Vegetable Protein PF found in **AC Curezyme** diluted in DI Water	Water & Hydrolyzed Vegetable Protein & Leuconostoc/Radish Root Ferment Filtrate
HVP	100% AC Hydrolyzed Vegetable Protein PF	Water & Hydrolyzed Vegetable Protein & Leuconostoc/Radish Root Ferment Filtrate
QPP	100% Quaternized Plant Protein	Hydroxypropyltrimonium Corn/Wheat/Soy Amino Acids
AC Curezyme	100% **AC Curezyme**	Vegetable Protein Keratinoligase

In order to further demonstrate the substantivity of the products, all hair tresses were rinsed again with water, demonstrating the ability of the products to resist rinses. Each tress was then washed with the base shampoo again to remove the dye and products. Finally, all tresses were re-dipped in the dye solution and rinsed to understand potential build up on the hair.

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Images were taken before and after dye treatment, after the water rinse, after the final shampoo wash, and after the final dye treatment. The entire experiment was performed at room temperature and water rinses were approximately 37-45°C to reflect common shower temperatures. ImageJ histogram analysis was performed to evaluate changes in red, green, and blue (R+G+B) present in each tress after each treatment step. Percent change from initial was calculated using the formula below:

$$Percent\ Change\ (\%) = \frac{Mean_{Treatment\ Step} - Mean_{Before\ Treatment}}{Mean_{Before\ Treatment}} \times 100$$

Three separate experiments were performed, and average values were recorded. Data was analyzed using a one-way ANOVA with statistical significance accepted at $p \leq 0.05$.

Results

The data obtained from this study met criteria for a valid assay and the controls performed as anticipated. All four experimental tresses exhibited varying levels of product deposition on the hair and withstood the rinse out application. Moreover, AC Hydrolyzed Vegetable Protein PF and **AC Curezyme** easily washed out after one shampoo treatment while Quaternized Plant Protein did not.

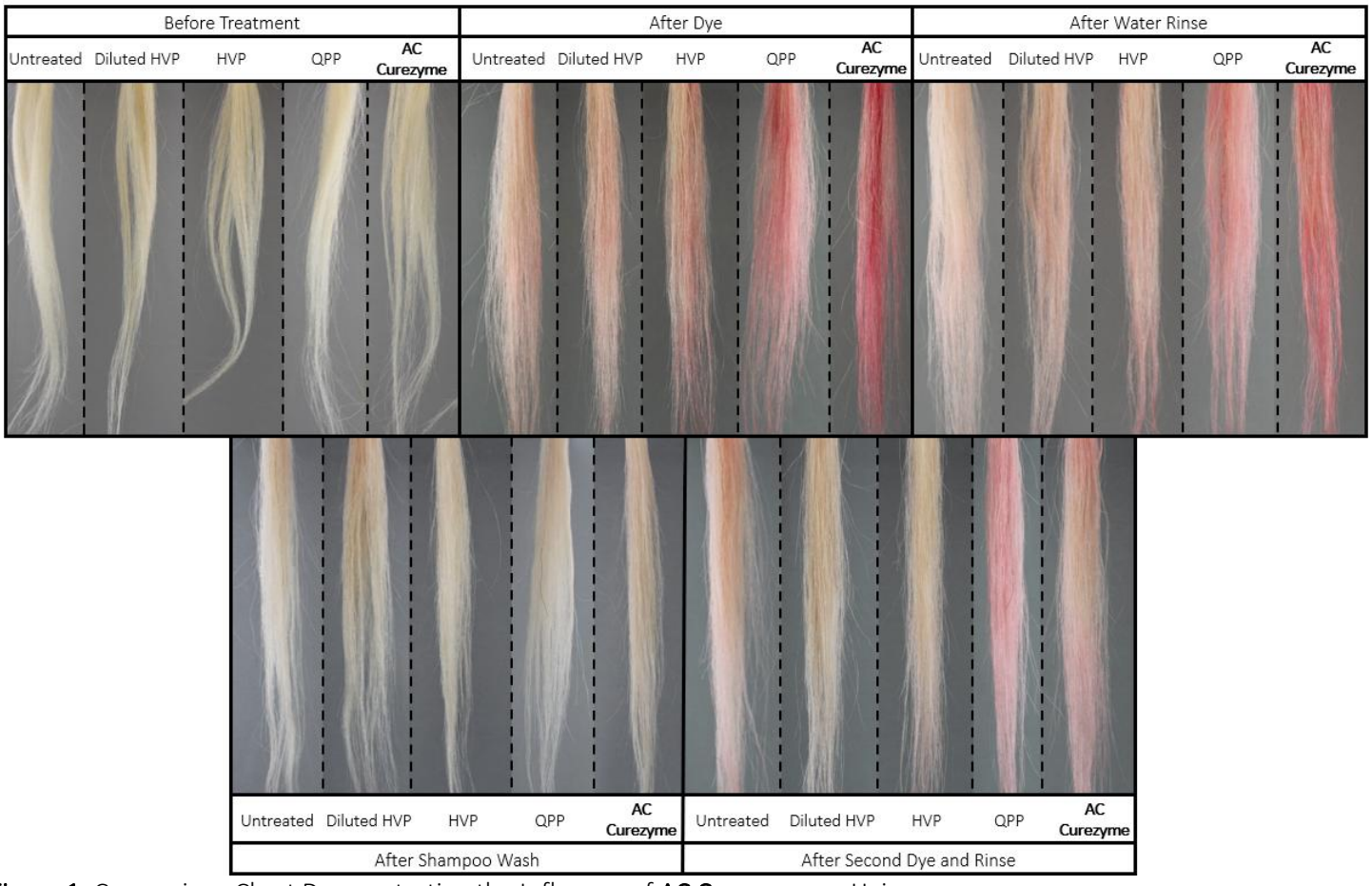

Figure 1. Comparison Chart Demonstrating the Influence of **AC Curezyme** on Hair.

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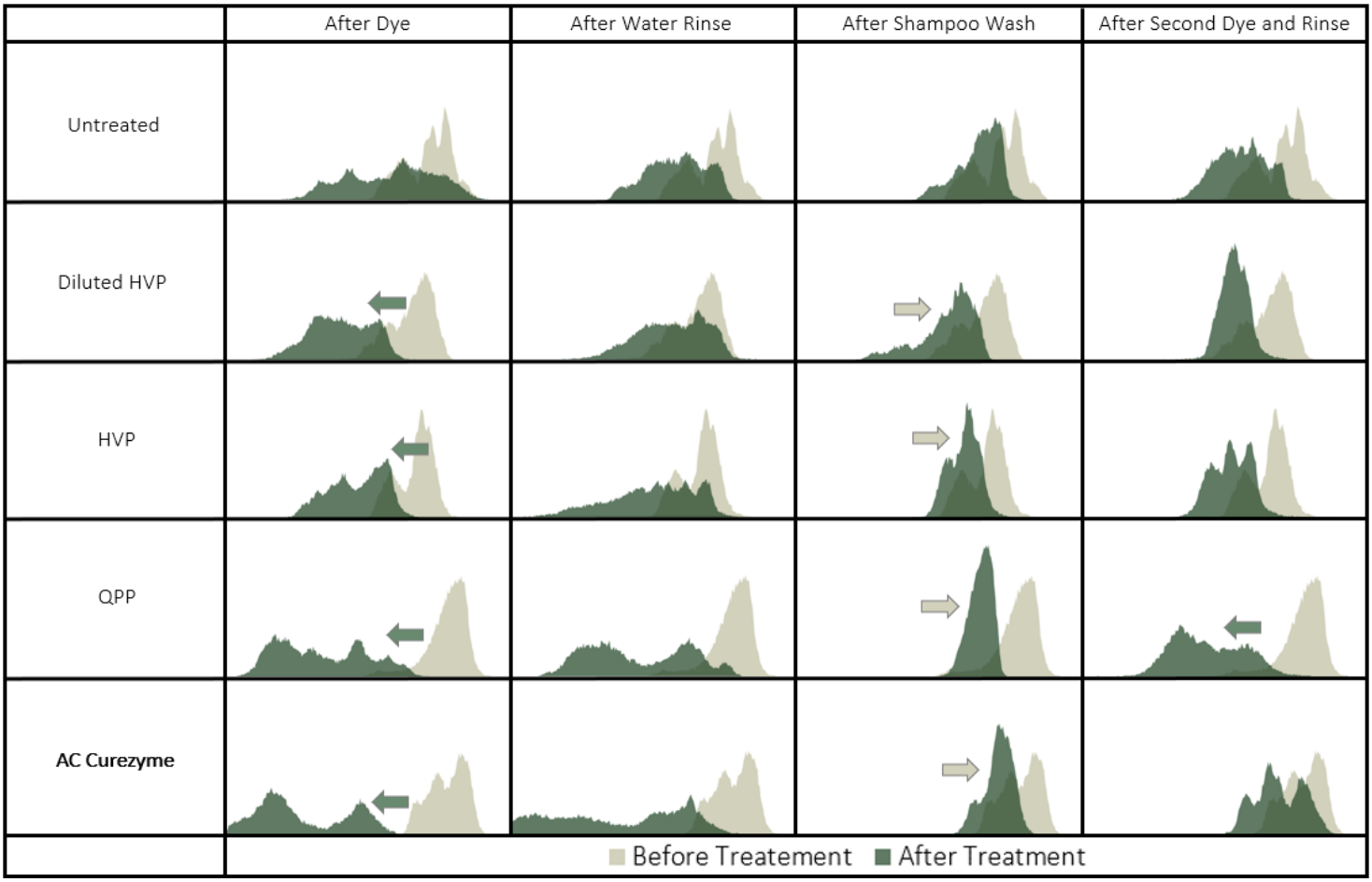

Figure 2. R+G+B histograms of each tress at each treatment step compared to before treatment. The green arrows indicate a shift towards the red end of threshold while the beige arrows indicate a shift back towards initial color. R+G+B color threshold set from 0 (left) to 255 (right), where 0 reflects exclusively red pixels and 255 reflects exclusively blue pixels.

Table 3. Results from one-way ANOVA Statistical Analysis from Before Treatment to After Dying, After Rinsing, After Washing and After Second Dying for Each Condition. * indicates significance ($p \leq 0.05$) compared to Before Treatment

	Untreated	Diluted HVP	HVP	QPP	AC Curezyme
After Dying	> 0.05	< 0.001*	< 0.001*	< 0.001*	< 0.001*
After Rinsing	> 0.05	0.001*	< 0.001*	< 0.001*	< 0.001*
After Washing	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05
After Second Dying	> 0.05	> 0.05	> 0.05	< 0.001*	> 0.05

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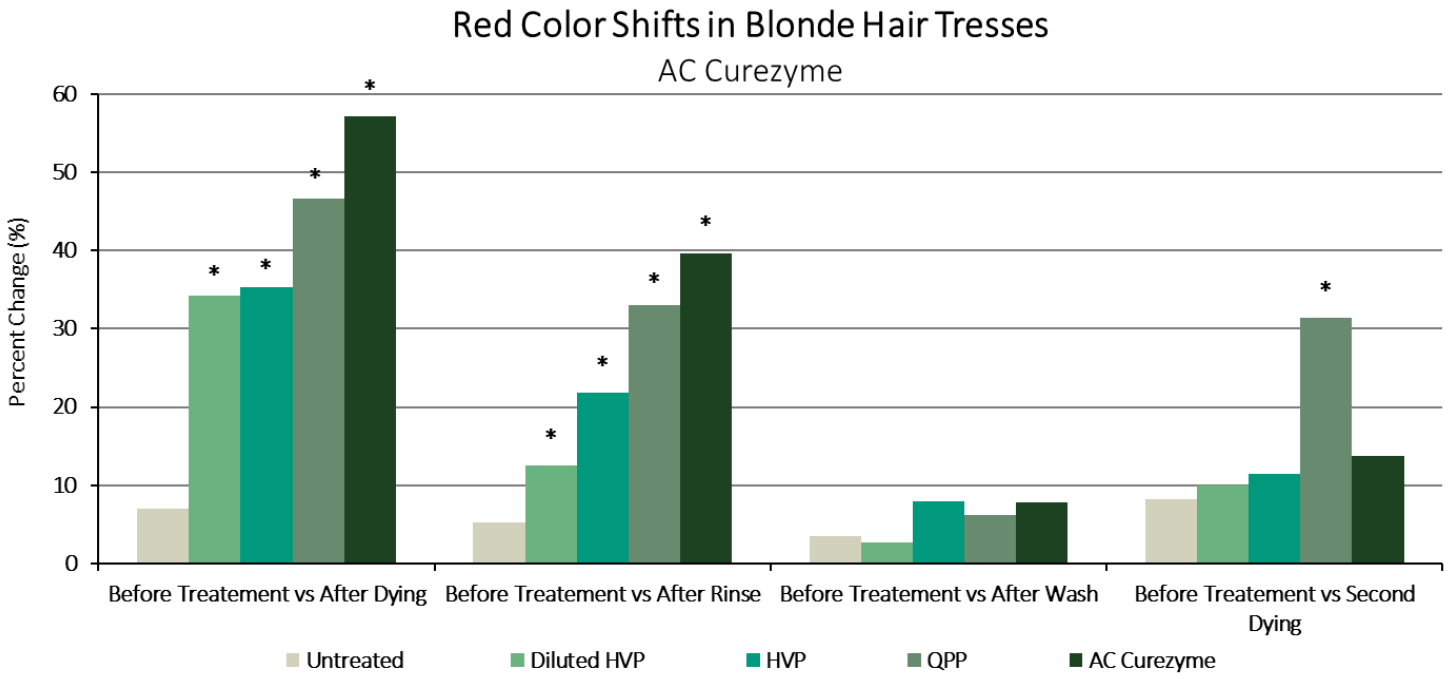

Figure 3. Virgin blonde hair shifts in red color (R+G+B intensity) after treatments for each condition. A large percent change indicates a shift towards more red tones, whereas a small percent change indicates a shift away from red tones back towards baseline. * indicates significance ($p \leq 0.05$) compared to Before Treatment.

Table 4. Results from one-way ANOVA Statistical analysis for Red Color of Hair Tresses between Conditions at Each Treatment Step. * indicates significance ($p \leq 0.05$) between the two conditions compared.

Percent Change (%)	Before Treatment	After Dying	After Rinsing	After Washing	After Second Dying
Untreated vs Diluted HVP	> 0.05	< 0.001*	0.028*	> 0.05	> 0.05
Untreated vs HVP	> 0.05	< 0.001*	0.004*	> 0.05	> 0.05
Untreated vs QPP	> 0.05	< 0.001*	< 0.001*	> 0.05	0.001*
Untreated vs **AC Curezyme**	> 0.05	< 0.001*	< 0.001*	> 0.05	> 0.05
Diluted HVP vs **AC Curezyme**	> 0.05	< 0.001*	< 0.001*	> 0.05	> 0.05
HVP vs **AC Curezyme**	> 0.05	< 0.001*	0.001*	> 0.05	> 0.05
QPP vs **AC Curezyme**	> 0.05	0.003*	0.003*	> 0.05	0.039*

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Discussion

The results indicate **AC Curezyme** provides substantive benefits to hair and does not build up overtime. Moreover, **AC Curezyme** elicits significantly better interactions with the hair than AC Hydrolyzed Vegetable Protein PF while being easier to wash out than Quaternized Plant Protein.

Tresses treated with DI water did not experience significant changes in color after dying. Alternatively, **AC Curezyme** provided substantive benefits as demonstrated by the deeper red color seen in the experimental tresses compared to the untreated tresses (Figure 1, Tables 3 & 4). This is confirmed by the histogram shifts in Figure 2 indicating that more red pigment is present in the tresses (green arrow). The R+G+B histogram intensity significantly shifted by 57% towards the red end of the spectrum for the tresses treated with **AC Curezyme** (Figure 3, Table 3). Similarly, the tresses treated with the Equivalent AC Hydrolyzed Vegetable Protein PF, 100% AC Hydrolyzed Vegetable Protein PF, and 100% Quaternized Plant Protein experienced shifts of 34%, 35%, and 47% towards the red end of the spectrum, respectively. The color shifts in tresses treated with Equivalent AC Hydrolyzed Vegetable Protein PF, 100% AC Hydrolyzed Vegetable Protein PF, and 100% Quaternized Plant Protein were significantly less drastic than those treated with **AC Curezyme** (Table 4). All experimental tresses maintained significant red tones when rinsed again under warm water.

Despite the tresses treated with **AC Curezyme** having a dark red color, all tresses lost any trace of color when washed with the base shampoo again which is highlighted by the beige arrows in Figure 2. Similarly, tresses treated with Equivalent AC Hydrolyzed Vegetable Protein PF, 100% AC Hydrolyzed Vegetable Protein PF, and 100% Quaternized Plant Protein lost all color when washed with base shampoo. When re-introduced to the dye tresses treated with Equivalent AC Hydrolyzed Vegetable Protein PF, 100% AC Hydrolyzed Vegetable Protein PF, and **AC Curezyme** remained blonde and did not significantly retain the red dye (Figure 3, Tables 3 & 4). This indicates that **AC Curezyme** is easy to remove and will not create buildup on hair. Comparatively, 100% Quaternized Plant Protein retained a significant amount of dye indicating product build up.

In summary, **AC Curezyme** enhances a conditioning agent's protection and moisturization properties by strengthening hair-product interactions without creating build up and compromising hair health.