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A New, Rapid Method for Examining Potential Skin-Brightening Ingredients Using Apple Slices

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Synopsis

Darkening of fruits is the result of the oxidative activation of polyphenol oxidase converting low–molecular weight phenols present in the fruit body into quinone intermediates. Then, through polymerization, these reactive quinones convert to light yellow and red low–molecular weight melanin and, given enough time, to darker, higher molecular weight brown and black melanin. The process that occurs in the flesh of cut fruit is very similar to the process that human skin cells use to make melanin: the oxidative activation of tyrosinase and conversion of tyrosine to dopaquinone and eventually to darker melanin. The conversion of the phenols by tyrosinase to quinones is the rate-limiting step in the biochemical manufacture of melanin. This article will discuss a new and cost effective way to screen skin-brightening ingredients by the use of apple slices as a model for skin using a chromameter to measure the change in color that occurs in apple slices over a short time course. Such measurements have been popularly used by food manufacturers to examine ingredients that inhibit fruit browning. Interestingly, as will be noted, many of the ingredients used commercially to inhibit food browning are also popular skin-brightening ingredients. We have found that a DermaLab (Cortex Technologies, Hadsund, Denmark) chromameter measuring the erythema index of apple slice darkening appears to be able to differentiate the benefit of a formulation containing azelaic acid, a known skin-lightening ingredient, to minimize the darkening effects that occur in sliced apples. We will discuss how different apples behave differently when cut and how to best use the chromameter to analyze the changes that occur that can potentially help rapidly screen ingredients for their skin-brightening benefits.

INTRODUCTION

Development of new topical ingredients that can support skin-brightening and -calming claims is constantly expanding. In particular, because of the propensity of many Asian populations to seek ingredients that can help brighten complexion, ongoing research in this area is aggressive. The best way to confirm whether an ingredient has potential skin-brightening benefits is to conduct a clinical screening on a population of individuals with darker skin. However, such robust clinical studies are typically quite expensive to run and can take time to note effective results. A more rapid method to investigate potential topical ingredient benefits involves irradiating volunteer's skin with simulated solar radiation, UV energy, or possibly even high-energy visible light to cause a small spot that

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initially starts with an erythemal response (redness) and then over time moves to a tanned appearance, which can be used to investigate possible skin-brightening and -calming ingredients that might function to help reduce the Reactive Oxygen Species (ROS)-initiated skin effects of the energy exposure. Here, the problem is that this kind of study requires irradiating people to the point of redness, which some view as being problematic because it potentially exposes humans to harmful radiation effects. Again, this method can provide a more rapid way to investigate ingredients, but it is still somewhat limited because it requires humans and offers a limited number of testing sites (typically six). The use of skin explants and human skin tissue equivalents, such as MatTek's Melanoderm (Ashland, MA), is a good way to test skin-brightening ingredients *in vitro* but requires a laboratory that can grow and handle human tissues or tissue equivalents, and the necessary spectrophotometric analytical equipment to measure melanin expression. A much more rapid and convenient method has been developed that uses human- or mushroom-derived tyrosinase which is typically mixed with tyrosine and then analyzed using a UV-Visible spectrophotometer to look for the development of melanin precursors (1). The method does allow for rapid screening, but it is a kinetic *in situ* assay that requires some skill in using the spectrophotometer to gain meaningful data. Interestingly, the fact that mushrooms make a type of tyrosinase that can be purchased and used to analyze potential human skin-brightening ingredients raises an interesting point that the tyrosinase enzyme is highly conserved across humans, plants, fungi, and bacteria. In plants, tyrosinase is more often called polyphenol oxidase (PPO) (2). The presence of PPO in fruits such as apples, pears, and bananas is the primary reason that, on opening the fruits, they begin to rapidly darken.

Food browning is a very common problem for many commercial foods, which results in expensive disposal of vast amounts of food because the appearance of the food becomes undesirable for culinary consumption (3). Fruits are highly sensitive to the browning reaction, and popular fruits such as apples, pears, avocados, and bananas are especially sensitive to browning effects (4). A tremendous amount of food research has, therefore, been directed toward ways to minimize the browning of fruits and vegetables (5,6). Interestingly, from a chemical perspective, application of certain chemicals such as ascorbic acid, kojic acid, *N*-acetylcysteine, and 4-hexylresorcinol to sliced fruits to minimize the browning reaction is interesting because these popular food ingredients also turn out to be quite popular skin-brightening ingredients when used topically (7). Although the food science literature is replete with the use of such chemicals to minimize food browning, it has been noted that there is a very curious dearth in the literature on the possible use of fruit browning as a model examining melanin inhibition as it relates to skin. One article of interest by Zhao et al. examines the benefits of vanillyl cinnamate analogues on reducing the browning effects that occur in apples (8). Cinnamate derivatives are well known skin lightening ingredients. The fact that on slicing a fruit, such as apple, results in the immediate commencement of browning effects prompted us to examine more closely what, exactly, is happening inside the apple fruit that causes the browning to occur.

In apples, an enzyme called PPO exists (9). This enzyme is called tyrosinase in fungi such as mushrooms and is functionally like human tyrosinase. In fact, PPO and tyrosinase are both categorized in the enzyme cataloguing system as EC 1.14.18.1. The enzyme is known as the rate-limiting catalyst in the conversion of phenols to quinones. In humans, the conversion of tyrosine to *L*-DOPA and then to dopaquinone are considered the rate-limiting steps in the skin-tanning response (10). Mushroom tyrosinase is commercially available

and is often used in *in vitro* studies to examine potential skin-brightening ingredients for topical applications (1). However, the enzyme tends to be expensive, and as noted earlier, the use of the enzyme in the kinetic *in vitro* study of tyrosinase inhibition is challenging because the study is kinetic (i.e., requires attention to time) and uses expensive spectrophotometric equipment that is not always available in skin care product formulating laboratories.

In the recent work reported by Lin et al. (11,12), multiple scanning electrochemical microscopic mapping was used to examine tyrosinase activity in banana slices and in mushroom-derived tyrosinase directly, and in a later publication, the same technique was used to examine melanin in human skin melanoma biopsy samples. This work demonstrates the similarity between the enzymatic activity of tyrosinase in banana skins and in human skin. The work is intended to try and expand the use of electrochemical microscopic mapping as a means of detecting and staging melanoma tumors in human skin.

We wish to report here efforts to use apple slices as a model for skin melanin production as a straightforward way to screen potential skin-brightening ingredients. We will discuss some parameters that were found to be important in using apple slices as a substrate to test skin-brightening ingredients. As will be shown, it does appear that this simple method could also potentially work to screen various types of skin-brightening ingredients including, potentially, finished formulations that are difficult to analyze with tissue equivalents or the *in vitro* tyrosinase assay.

MATERIALS AND METHODS

APPLE SLICES

Apples used in the current study were purchased from a local supermarket and included two varieties, McIntosh apples and honey crisp apples. Initial work started by simply cutting the apples in half, but it was quickly realized that using a standard apple corer worked much better, Figure 1. The slicer allowed for the quick creation of up to eight slices, and, using only six appears adequate to obtain statistically significant testing results using the chromameter.

CHROMAMETER

A DermaLab chromameter (Cortex Technologies) was used to measure the apple slice color. This instrument is well known for its use in skin melanin work (13). The proper placement of the chromameter probe on the apple slices is shown in Figure 2. The instrument measures in the CIE L*a*b* color space. The instrument also calculates an "erythema index" (a measure of redness) and a "melanin index" (a measure of brownness). As will be shown, for the purposes of examining darkening in apple slices, the erythema index appears to work best. Reasons for this will be discussed in more detail in the following paragraphs. The differences in the measurements for the single apple studies were analyzed for statistical significance using the TTEST function in Excel (two-tailed and two-sample paired). The differences in the measurements for the multiple apple studies (azelaic acid study) were analyzed for statistical significance using the TTEST function in Excel (two-tailed, two-sample unpaired unequal variance).

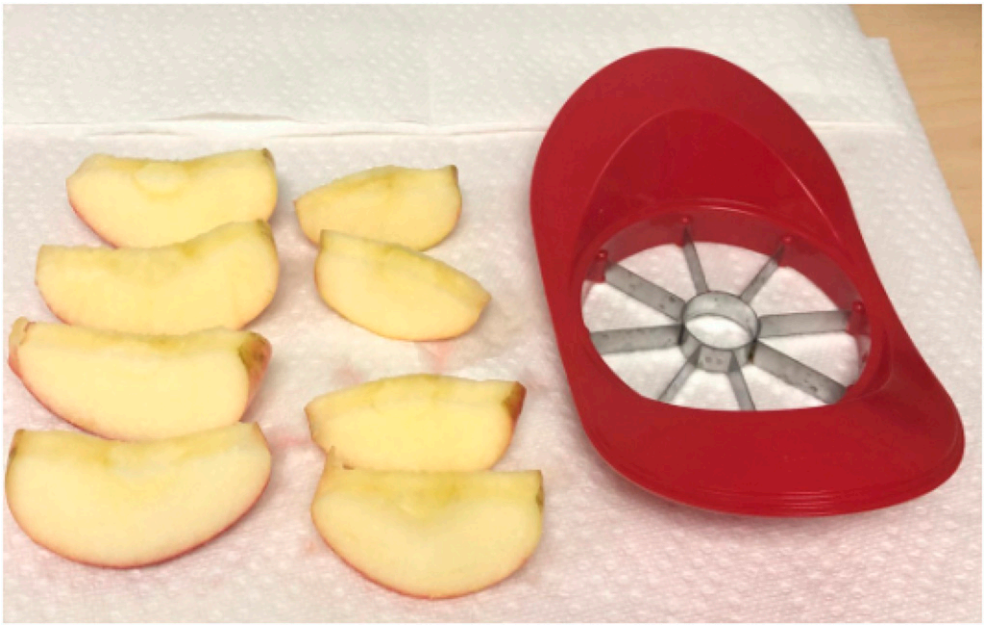

Figure 1. Apple slicing apparatus.

AZELAIC ACID

The source of azelaic acid used in the present study was Jeesperse® OptiDerm™ A-Z (Jeen International, Fairfield, NJ). The product is available as a water-dispersible powder containing 25% azelaic acid. When introduced into water at 10%, the Jeesperse® OptiDerm™ A-Z disperses to provide a white, low-viscosity emulsion containing 2.5% azelaic acid (Figure 3).

RESULTS AND DISCUSSION

INITIAL EXAMINATION OF VARIOUS KINDS OF APPLE SLICES

Initial efforts to examine apple slices as potential melanin creation substrates began by examining simple slices of apples. It was found that in examining two initial apples using the chromameter, using the erythema index measurement, there was a rapid but predictable increase in response from the surface of the apples as measured over a time course of 24 h. The McIntosh apple slices showed statistically significant increases in the erythema index response over the course of 24 h (Figure 4). However, it was noted that the initial response was very rapid, happening within a 60-min time frame. This was a concern because it would mean that the treatment and measurement of the apple slices would have to take place very quickly to be able to detect a meaningful response. To take the measurements using the DermaLab chromameter, the device is placed on the surface of the apple and then on activation, the chromameter flashes two light-emitting diodes and records the resulting color response in a photodiode in the instrument head. Each apple slice is large enough to easily gather the four measurements per slice that the instrument

Figure 2. DermaLab chromameter placed on an apple surface.

allows. So, for six apple slices, a total of 24 measurements are made of the apple color. This allows for very precise measurement of the apple color as can be seen by the statistically significant increases of the color measurement up to the 75th minute. Between measurements at 60 and 75 min, the erythema index no longer shows statistical significance. This suggested that for this apple type, work with these apples would be somewhat problematic because the time frame for measurements is very rapid.

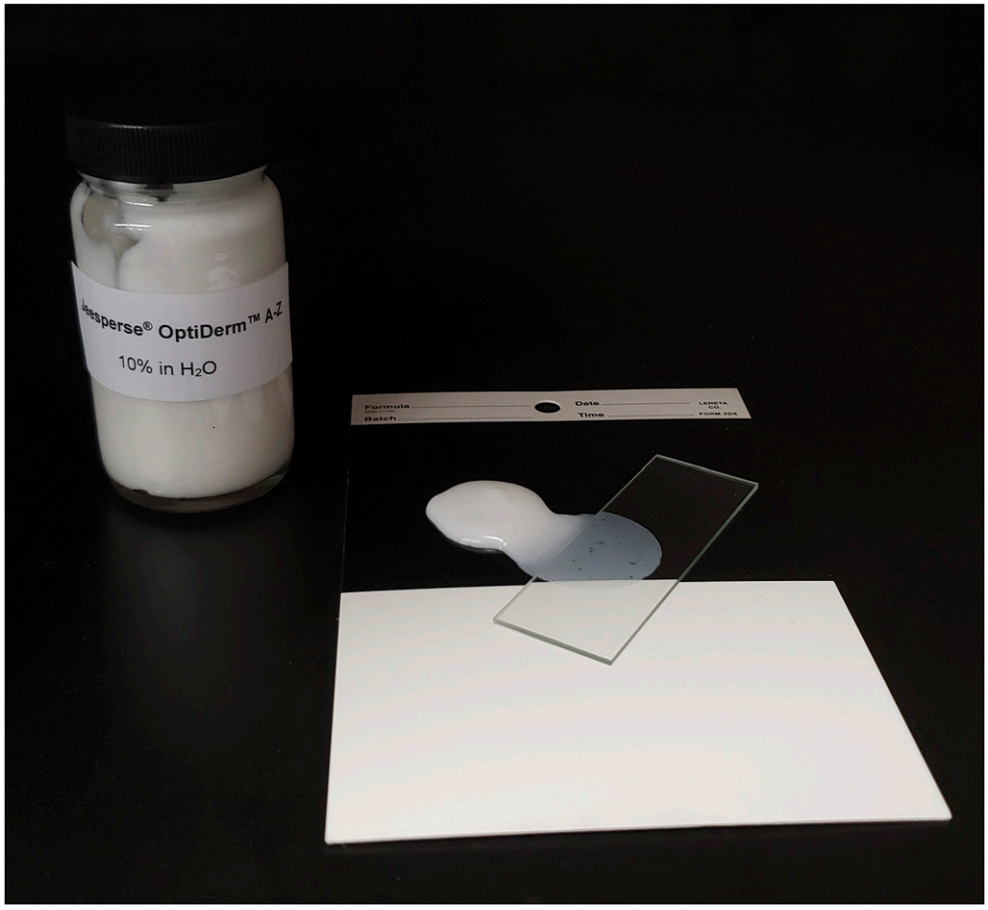

Figure 3. 2.5% azelaic acid dispersion.

For this reason, we elected to examine another variety of apples, honey crisp (Figure 5). These apple slices initially show a darker color. The initial erythema index of McIntosh apples at T(0) was around 1.5 units versus a measure at T(0) of about 5 units for honey crisp apple slices. What was encouraging for honey crisp apples, however, was the more gradual change in color that took approximately 300 min to reach nonstatistical similarity to the previous measurement. This allows more time for product application before significant color changes occur and gives ample time for measurements when numerous apple slices are being examined. To proceed with examination of azelaic acid, we elected, therefore, to proceed with honey crisp apples.

EXAMINATION OF BRIGHTENING EFFECTS OF AZELAIC ACID

Azelaic acid has already been proven to have clinical skin-brightening benefits (14,15). However, formulating with azelaic acid can be problematic because the ingredient is sparingly soluble in both water and commonly used nonpolar cosmetic ingredients. Jeen International has developed a form of azelaic acid that is contained within its Jeesperse® technologies (16). The ingredient, which is available as powder, is quickly dispersible in water,

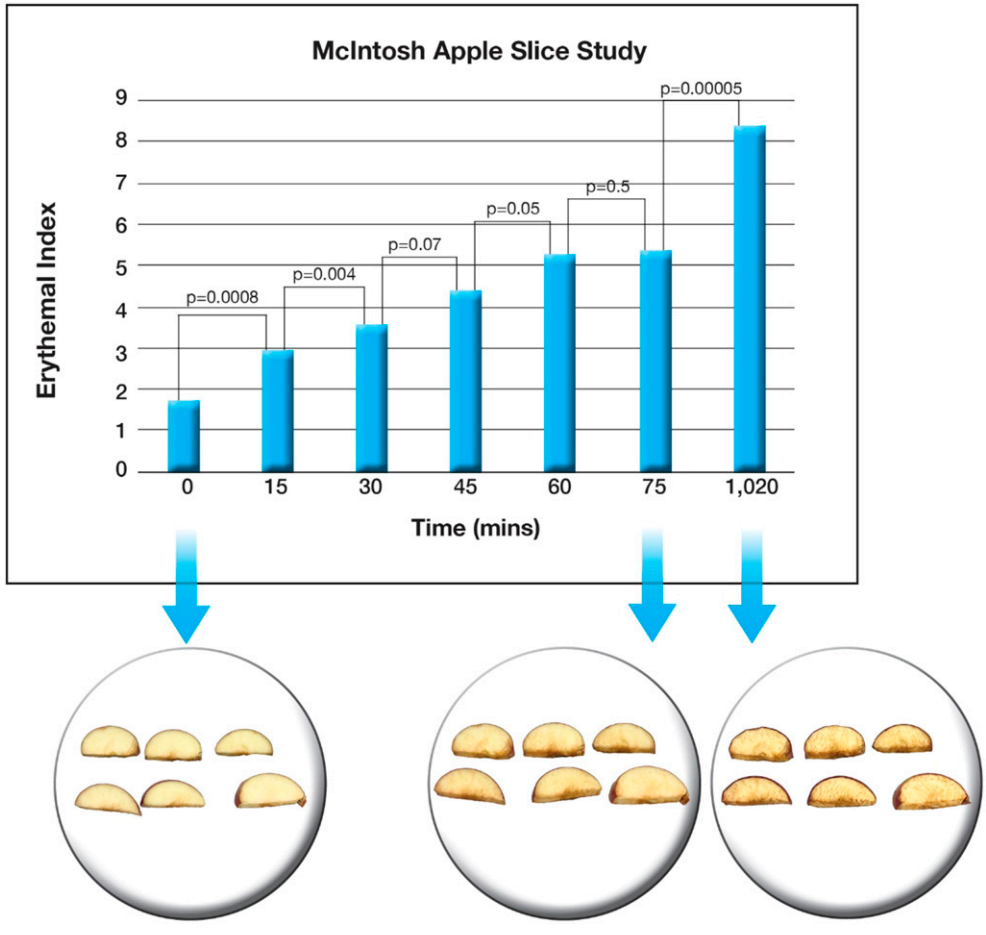

Figure 4. Initial erythema index on McIntosh apple slices. Images show apple slices at T(0), T(75), and T(1,020) min.

and for the purposes of these studies, a 10% dispersion of the Jeesperse® OptiDerm™ A-Z was made in water. This provides an effective amount of azelaic acid of 2.5%.

For this study, two sets of six apple slices were used: one set was untreated and the other was treated with two cotton swab loadings of azelaic acid dispersion. After application of the dispersion on the six apple slices, the product was gently rubbed on the apple surface with a finger to give consistent coverage of the entire exposed surface of the apple (Figure 6). As noted in Figure 3, the ingredient is an emulsion, very similar to a finished product emulsion. Application of the product on the apple surface is straightforward, suggesting that this test method may have applications in screening finished emulsion-based formulations.

It was found that by placing the apple slices on paper towels, the pressure from the placement of the chromameter measuring head would not move the apple slices, which is important to gain adequate color measurements. Also, although coring the apples removes the seed elements, the apple slices can still maintain a small ring of color from the area around the seeds. For this reason, the color measurements were made along the upper surface of each apple slice, as shown in Figure 2, where the color was more uniform and consistent. As

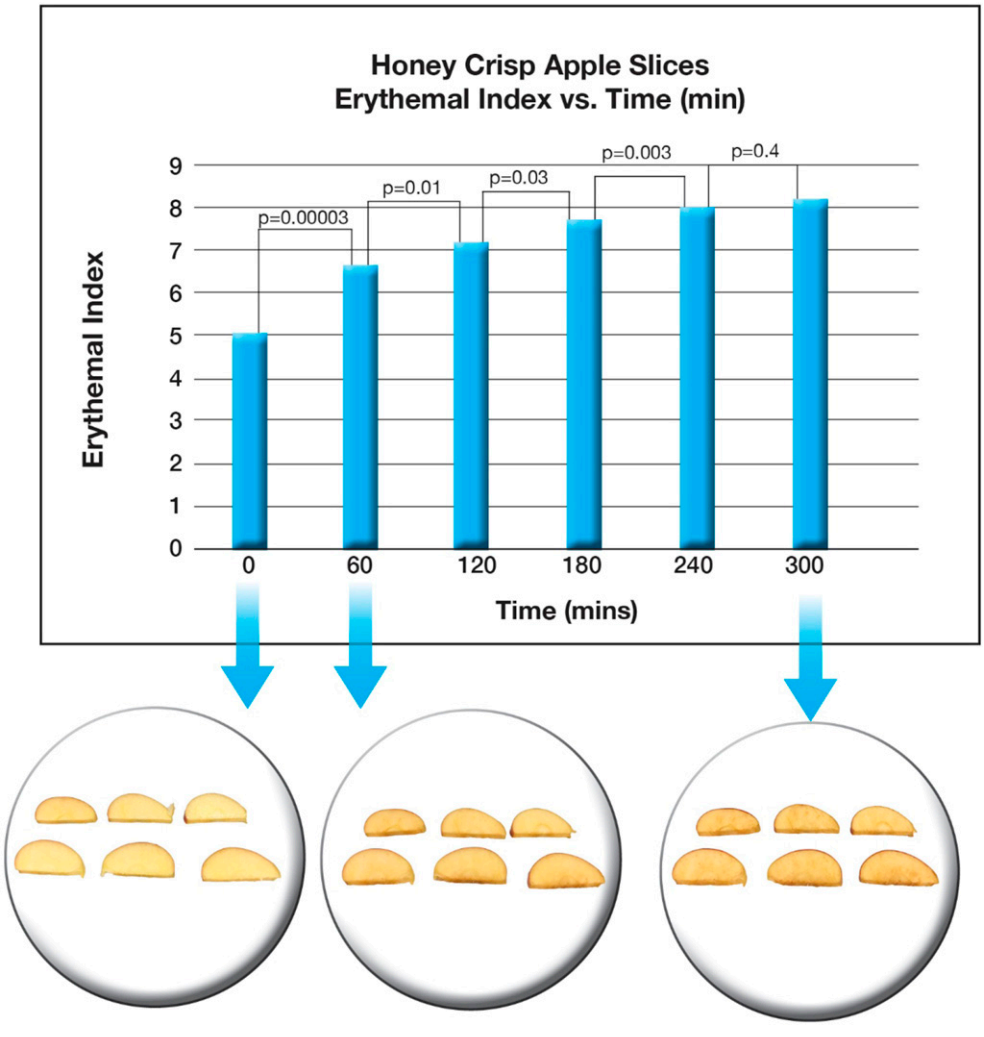

Figure 5. Initial erythema index on honey crisp apple slices. Images show apples slices at T(0), T(60), and T(300) min.

noted, for each set of measurements, a total of four measurements were made on each apple slice, and six apple slices were used per treatment type. This provided 24 measurements of color for each treatment. In this way, variations in apple slice color consistency are adequately minimized.

We measured both the erythema index response and the melanin index (not shown). It was found that the erythema index mimics very well what the eye sees of the apples, that is, darkening of color. Interestingly, the melanin index (which one might think would be a better measure of melanin formation) was relatively inconsistent. However, in reflecting on this apparent anomaly, we believe that the reason the erythema index is a better measure of the development of melanin in the apple slices is because the time frame we are operating in does not allow formation of the higher molecular weight dark brown and black melanin. It is more likely that within the eight hours of these studies, we observed formation

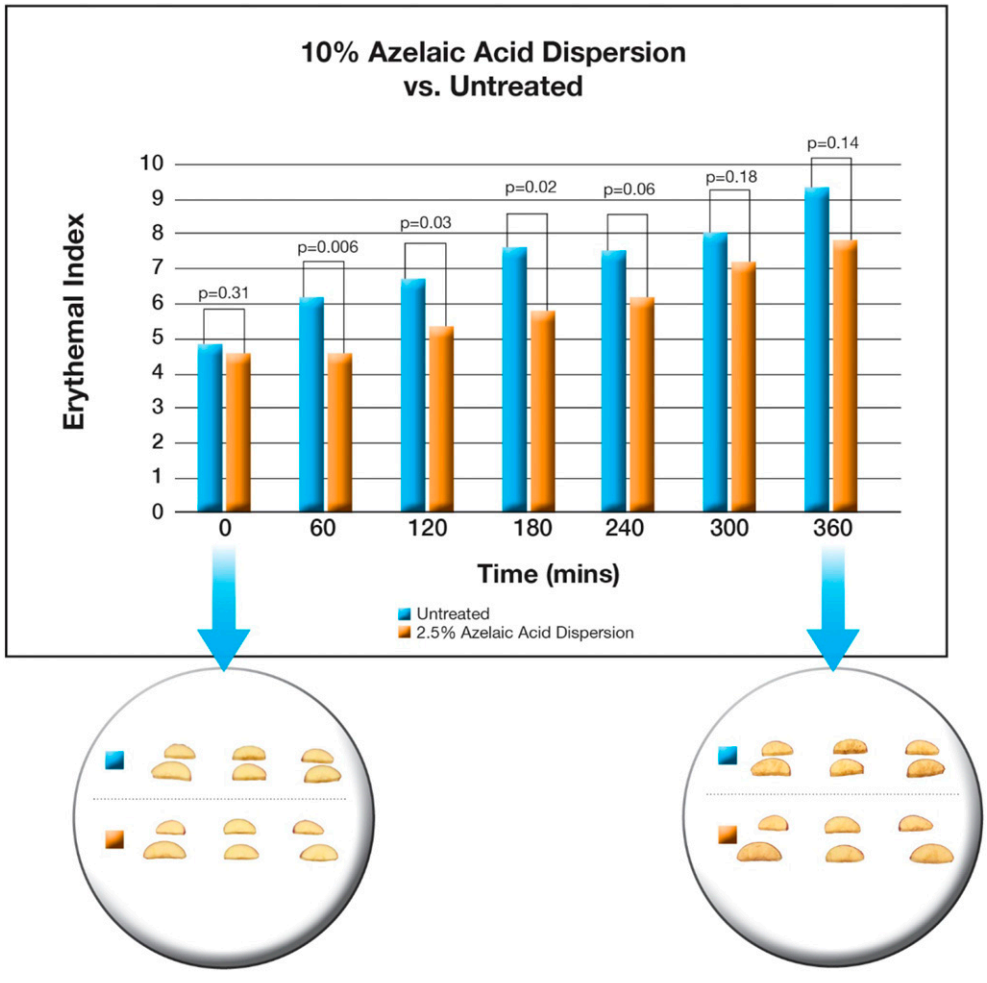

Figure 6. Erythema index measurements of honey crisp apple slices. Results show untreated slices (blue) versus slices treated with 2.5% azelaic acid as described. Images show the apple slices at T(90) and T(360) min.

of lower molecular weight melanin components that would tend to be more reddish or even slightly yellowish. These colors are more sensitively measured with the DermaLab chromameter using the erythema index. One area where this will be a problem in using apple slices to measure potential skin-brightening effects will be for ingredients that contribute a reddish color to the surface of the apples. In this case, it may be more difficult to distinguish the melanin response from the product color. In the case of azelaic acid dispersion examined here, that was not considered an issue because the dispersion is very white (Figure 3), and as can be seen by the initial T(0) measurement comparing the treated and untreated apple slices, there was no statistical difference in the overall color of the slices.

Two additional concerns that need to be addressed further are pH and temperature, both of which can impact the PPO enzyme. The temperature effects were not considered a problem in the present study because the untreated apples and the treated apples were effectively tested side-by-side. Also, inhibiting apple tyrosinase would require temperatures

that would exceed 50°C, which was not the case. The pH effects are more of a concern as it is known, for example, that citric acid is a popular food treatment for sliced apples. However, it has recently been reported that the effects of citric acid on PPO activity may not be solely related to pH as was noted in potatoes (17). Certainly, more studies are needed to make sure the influence of azelaic acid is not just a pH effect. However, azelaic acid has already been clinically proven to be an effective skin-brightening ingredient. Currently we are studying nonacidic skin-brightening ingredients to confirm that the effects are related to the inhibition of PPO activity.

CONCLUSION

We have reported here what we believe is the first instance of the use of fruit slices to measure the effectiveness of a skin-brightening ingredient by direct topical application. Although it is easy to note that human skin and sliced apples are quite different and that, skin pigmentation is an extremely complex process, the model offers what may be a quick and easy way to screen numerous formulations or ingredient benefits for potential skin-brightening claims (18). The fact that human tyrosinase and fruit PPO are the same enzyme and would function similarly in human skin and fruits by controlling the rate-limiting step in the conversion of phenols to quinones in the production of melanin suggests that these models may work well for initial evaluation of ingredients that would impact early melanin production in the skin. It is likely no coincidence that ingredients popular in preserving food color, such as ascorbic acid and kojic acid, are also very popular skin-brightening ingredients. The methods described here are easy and could be run simply in any application laboratory that has access to a chromameter such as the DermaLab instrument used here. This preliminary summary describes in detail a potential for fruit slice measurements to be a way to examine skin-brightening technologies, while additional work is needed to define the value and limitations of this technique.

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