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Storage Stability of Ascorbic Acid Incorporated in Edible Whey Protein Films

Theeranun Janjarasskul,[†,#] Sea C. Min,[§] and John M. Krochta*[,†]

[†]Department of Food Science and Technology, University of California, Davis, California 95616, United States

[§]Department of Food Science and Technology, Seoul Women's University, Seoul 139-774, South Korea

[#]Department of Food Technology, Chulalongkorn University, Bangkok 10330, Thailand

ABSTRACT: The stability of ascorbic acid (AA) incorporated in whey protein isolate (WPI) film and the related color changes during storage were studied. No significant loss of AA content was found in any films prepared from pH 2.0 casting solution stored at 30% relative humidity (RH) and 22 °C over 84 days. Total visible color difference (ΔE^*_{ab}) of all films slowly increased over storage time. The ΔE^*_{ab} values of pH 3.5 films were significantly higher than those of pH 2.0 films. The stability of AA−WPI films was found to be mainly affected by the pH of the film-forming solution and storage temperature. Oxidative degradation of AA−WPI films followed Arrhenius behavior. Reduction of the casting solution pH to below the pK_{a1} (4.04 at 25 °C) of AA effectively maintained AA−WPI storage stability by greatly reducing oxidative degradation, whereas anaerobic and nonenzymatic browning were insignificant. The half-life of pH 2.0 AA−WPI film at 30% RH and 22 °C was 520 days.

KEYWORDS: edible films, whey protein, ascorbic acid, stability

INTRODUCTION

The concept of oxygen-scavenger-incorporated edible whey protein films and coatings for food packaging has been explored as an alternative to synthetic scavenger systems, to avoid accidental consumption of synthetic scavengers by consumers and to improve overall recyclability of packaging systems. Ascorbic-acid-incorporated whey protein isolate (AA−WPI) films cast at pH 3.5 demonstrated oxygen-scavenging function proportional to ascorbic acid (AA) content when adjusted to pH >7.[1,2] Furthermore, the antioxidative function of AA−WPI film coating has been proven effective against lipid oxidation in roasted peanuts.[3,4] Thus, AA−WPI films and coatings have potential to further extend the stability of foods beyond what would be achieved with the already excellent oxygen barrier of WPI films and coatings.[5] AA−WPI films and coatings could accomplish this by eliminating oxygen initially present in the package headspace and providing an active barrier to oxygen permeation.

However, the effectiveness of AA−WPI film is possibly limited by AA degradation during AA−WPI film storage. To produce AA−WPI films/coatings with significant oxygen-scavenging capacity to protect foods from oxidation, it is necessary to stabilize AA during the casting/drying procedure and for a reasonably long storage time before being used. AA is highly susceptible to various modes of degradation. Oxidative degradation of AA to dehydroascorbic acid (DHAA) is the most important reaction pathway for loss of AA in foods. Under anaerobic conditions, no significant amount of DHAA was formed.[6] Anaerobic degradation of AA was found to occur simultaneously with the aerobic pathway in the presence of oxygen, but at a much lower rate than aerobic degradation.[7−9] However, a significant amount of anaerobic degradation is still possible during long storage in acidic AA−WPI films.[1]

Besides loss of antioxidant function, AA degradation is associated with discoloration reactions in both the presence and absence of amines. L-Ascorbic acid is a characteristic reductone that can enter into the nonenzymatic Maillard browning reaction. Regardless of the mechanism of AA destruction, DHAA, 2,3-diketo-L-gulonic acid (DKG), and other α-dicarbonyls formed during its degradation can degrade to furfural, 2-furoic acid, 3-hydroxy-2-pyrone, and a wide variety of other unsaturated compounds, as well as participate in Strecker degradation with amino acids.[10,11]

We hypothesized that the antioxidant activity of AA−WPI films can be stabilized during storage and that the AA oxygen-scavenging ability of AA−WPI films can be triggered when they are placed in contact with foods. Therefore, the objective of this research was to investigate AA stability in AA−WPI films, prepared at various conditions, and the related color change of films during storage.

MATERIALS AND METHODS

Materials. WPI was supplied by Davisco Foods International (Le Sueur, MN). Glycerol (used as a plasticizer to improve film flexibility), hydrochloric acid and sodium hydroxide (used to adjust the pH of film-forming solutions), potassium acetate (used as saturated salt solution to equilibrate films at 30 ± 5% relative humidity (RH) in an environmental chamber during storage), and orthophosphoric acid (used as mobile phase for HPLC analysis) were purchased from Fisher Scientific Inc. (Fair Lawn, NJ). L-Ascorbic acid was purchased from Sigma-Aldrich (St. Louis, MO). The lot analysis indicated <0.0005% iron and <0.0005% copper. Water used in any solution preparations was purified with a B-pure water purification system (Barnstead Thermolyne Corp., Dubuque, IA) (resistivity = 18 MΩ cm^{-1}). Film release papers from Gardco (Paul N. Gardner Co., Inc., Pompano Beach, FL) were used during film storage to allow easy stripping of dried film.

Preparation of Casting Solution. Preliminary experiments showed that 0.2 M AA is the maximum concentration possible in WPI film-forming

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solutions to produce glossy, transparent, colorless films with good handling properties.[1] Incorporation of 0.2 M AA reduces the film-forming solution pH from 6.7−7.5 to ∼3.5. The pH greatly influences the oxidative stability of AA, because the various ionic forms of AA differ in their susceptibility to oxidation: fully protonated (AH_2) < ascorbate monoanion (AH^-) < ascorbate dianion (A_2^-).[11,12] At pH ≥4.04 (pK_{a1} of AA), the oxidation-susceptible AH^- form is prevalent. AA oxidation would then occur rapidly in the presence of molecular oxygen. Although an oxygen-limited condition (OLC) can help prevent oxidative degradation of AA in WPI films, it cannot prevent AA degradation via anaerobic pathway or nonenzymatic browning, both of which have a maximum rate at pH ∼4.[13,14] Therefore, to produce AA−WPI films with good storage ability, it is necessary to lower the pH of the film-forming solution to substantially below the pK_{a1} of AA, in order to get the most stable form against oxidation, AH_2, as the predominant species as well as to avoid anaerobic degradation and nonenzymatic browning. Thus, for our experiments, AA at 0.2 M concentration was added to 5% (w/w) heat-denatured (at 90 °C, 30 min) WPI film-forming solutions with a WPI/glycerol ratio of 1:1. The pH of the solution was then adjusted to 2.0 or 3.5 (i.e., below the AA pK_{a1} of 4.04) to stabilize AA against oxidation and nonenzymatic browning. The pH-adjusted solution was then degassed under vacuum to remove dissolved air.

Film Casting and Storage. Degassed, pH-adjusted casting solutions, each with 3 g of total solids to achieve consistent dried film thicknesses, were pipetted onto Teflon casting plates (15.5 cm diameter) resting on a leveled granite surface. Cast solutions were then dried under either OLC (<3% O_2) or aerobic condition (AC, ∼21% O_2) at 30 ± 5% RH and 22 ± 3 °C for 48 h. After this time, dried films could be released intact from the plates. The OLC was obtained by continuous N_2 flushing of a Flexiglass chamber. Headspace oxygen at the OLC was monitored by using a dual headspace analyzer (Pak Check model 650, Mocon, Minneapolis, MN). Dried films were transparent and ∼0.1 mm thick. Films were then stored under AC at 30 ± 5% RH and 22 ± 3 °C to simulate possible commercial storage conditions, as shown in Table 1. A control film sample was prepared from pH 2.0 solution cast under OLC and then stored in a dark OLC at 30 ± 5% RH and 22 ± 3 °C. To study the effect of storage temperature on the stability of AA−WPI film, pH 2.0 films formed at AC were stored at 22, 35, and 50 °C AC. Three replications of each film and condition were tested for statistical analysis.

HPLC Analysis of AA Content of AA−WPI. For HPLC analysis of AA, each film sample was cut into small disks, using a circular die cutter (4.1 cm diameter), and weighed on an electronic analytical balance (d = 0.0001 g) (model ER-180A, A&D Co., Ltd., Tokyo, Japan). Each film sample was placed in phosphoric acid solution (pH 2.0) and then homogenized using an Ultra-Turrax model T25 (IKA-Works, Inc., Wilmington, NC) at 300 rpm for 1.5 min before filtration through a syringe filter (0.45 μm, PTFE, Fisher Scientific, Pittsburgh, PA). A 10 μL aliquot was injected into the column using the HPLC autosampler.

The AA content of AA−WPI films was analyzed using a high-performance liquid chromatography (HPLC) system according to established methods with minor modifications.[15] A Perkin-Elmer HPLC system equipped with a 140-LC pump, an ISS 200 autosampler, and an LC135C diode array detector was used. The detection wavelength was 245 nm. HPLC chromatograph peak areas were calculated using Totalchrom Workstation software (version 6.2.0, Perkin-Elmer). A reverse-phase C18 column, 25 cm × 4.6 mm i.d., 5 μm particles (Supelco, Bellefonte, PA), with a C18 guard column (5 μm, Supelco) was used to separate the AA using by an isocratic method using aqueous phosphoric acid (pH 2.0) as the mobile phase. The mobile phase was prepared from B-pure water brought to pH 2.0 with phosphoric acid and then filtered using 0.45 μm membrane filters (White Nylon, HNWP, 47 mm, Millipore Corp., Billerica, MA) and degassed using helium gas before passing through the column at a flow rate of 1.0 mL/min.

Table 1. Conditions for Film-Casting Drying Process and Storage of the Film Samples Used for Studying Degradation of AA in AA−WPI Films during Storage

film sample[a]	pH of film-forming solution	drying condition[b]	storage condition[c]
pH 2.0-OLC-OLC	2.0	OLC	OLC
pH 2.0-OLC-AC	2.0	OLC	AC
pH 2.0-AC-AC	2.0	AC	AC
pH 3.5-OLC-AC	3.5	OLC	AC
pH 3.5-AC-AC	3.5	AC	AC

[a] OLC means oxygen-limited condition, <3% O_2. AC means aerobic condition, ∼21% O_2. [b] 30 ± 5% RH, 22 ± 3 °C, 48 h. [c] 30 ± 5% RH, 22 ± 3 °C.

A standard calibration curve was obtained by using L-ascorbic acid as an external standard (Sigma-Aldrich, St. Louis, MO). The linearity range was determined from 0.01 to 0.20 mg/mL (R^2 = 0.9970), yielding a peak area range of 225,557−5,730,026 μV*s.

Color Measurement. Samples, placed on a white background standard, were evaluated for color using the tristimulus color analyzer for measuring reflective colors of surfaces, Minolta Chroma meter CR-200 (Minolta, Osaka, Japan) with an 8 mm diameter measuring area, 0° viewing angle, and light source C. Color was recorded using the CIE L^*, a^*, b^* color space. Five random positions were measured for each test sample.

In this color space, L^* indicates lightness and a^* and b^* are the chromaticity coordinates: L^* = 0 indicates black and L^* = 100 indicates diffuse whiteness; a $+a^*$ value indicates redness, and a $-a^*$ value indicates greenness; and a $+b^*$ value indicates yellowness, and a $-b^*$ value indicates blueness. Total color difference (ΔE^*_{ab}) was calculated from eq 1.

$$\Delta E^*_{ab} = \left[(L_{standard}^* - L_{sample}^*)^2 + (a_{standard}^* - a_{sample}^*)^2 - (b_{standard}^* - b_{sample}^*)^2\right]^{1/2} \quad (1)$$

The value of ΔE^*_{ab} was chosen to report the nonenzymatic browning developed in the AA−WPI films during storage, because it is perceptually analogous to human visual perception of color difference. The values of ΔE^*_{ab} can be roughly classified into three different levels to reflect the degrees of color difference perceived by humans. The color difference is hardly perceptible when ΔE^*_{ab} is <3, perceptible but still tolerable/acceptable when ΔE^*_{ab} is between 3 and 6, and usually not acceptable when ΔE^*_{ab} is >6.[16]

Determination of Kinetics Parameters. Rate constants were calculated from the slopes of AA content and ΔE^*_{ab} plotted against storage time using a zero-order kinetics model, which is the most conservative estimate, for each storage temperature. The slope of each line was fitted with the general linear regression model using Minitab 13.31 (Minitab, Inc., State College, PA).

The temperature dependence of the degradation of AA and color change during storage was described by the Arrhenius model[17]

$$k = A\, e^{-E_a/RT} \quad (2)$$

where k is the rate constant or kinetics coefficient (s^{-1}), E_a is the activation energy (kJ mol^{-1}), R is the universal gas constant (8.314 J K^{-1} mol^{-1}), T is the absolute temperature (K), and A is a pre-exponential factor, which is a constant characteristic of the system (s^{-1}). The E_a is the minimum amount of energy a molecule must possess for a reaction to occur.[10] The E_a values were determined by the Arrhenius plot, which is the natural log (ln) of the rate constant as a function of the inverse of the absolute temperature. The slope of the

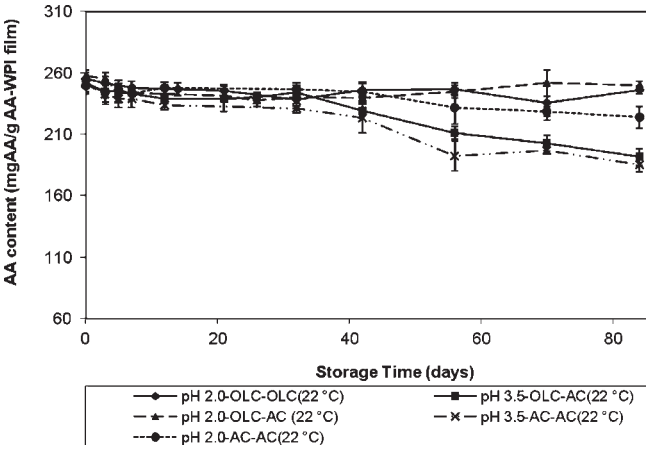

Figure 1. Ascorbic acid (AA) contents in various ascorbic-acid-containing whey protein isolate (AA−WPI) films during storage at 22 °C for 84 days. Error bars shows standard deviation of the significantly different samples at the $p \leq 0.05$ level.

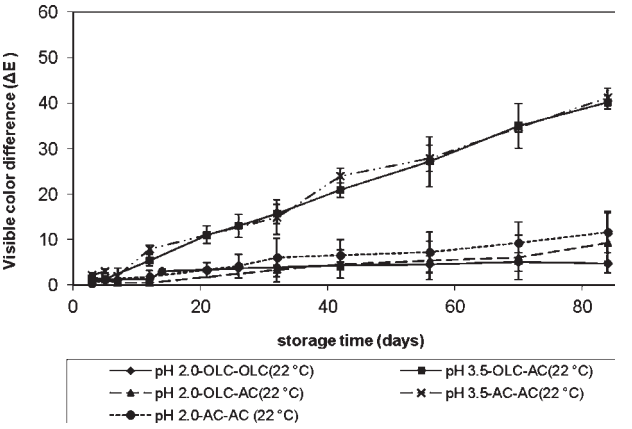

Figure 2. Total color difference (ΔE^*_{ab}) of various ascorbic-acid-containing whey protein isolate (AA−WPI) films during storage at 22 °C for 84 days. Error bars shows standard deviation of the significantly different samples at the $p \leq 0.05$ level.

straight line yields $-E_a/R$.

$$\ln(k) = -\frac{E_a}{R}\frac{1}{T} + \ln(A) \quad (3)$$

The Q_{10} temperature coefficient is a measure of the rate of change of a biological or chemical system as a consequence of increasing the temperature by 10 °C. Q_{10} values were determined from the equation [10]

$$Q_{10} = \exp(10E_a/RT^2) \quad (4)$$

Statistical Analysis. Three replications were done in every analysis. Data were analyzed by one-way analysis of variance (ANOVA), whereas the standard calibration curve of ascorbic acid and rate constant were analyzed by linear regression using Minitab 13.31 at the 5% significance level.

RESULTS AND DISCUSSION

Ascorbic Acid Content. Figure 1 shows the AA contents retained in various AA−WPI films during simulated commercial storage. No significant loss of AA content ($p > 0.05$) was found in any AA−WPI film prepared from pH 2.0 casting solution (pH 2.0-OLC-OLC, pH 2.0-OLC-AC, pH 2.0-AC-AC) and stored at 22 °C for 84 days. There was no significant loss in AA concentration in films prepared from pH 3.5 casting solution (pH 3.5-OLC-AC or pH 3.5-AC-AC) for up to 32 days of storage ($p > 0.05$). More than 80% of AA content of AA−WPI films prepared from pH 3.5 casting solution was retained after 84 days. Therefore, it can be concluded that AA content in AA−WPI films was effectively stabilized by lowering the casting solution to a pH below the pK_{a1} of AA. Oxidative degradation of AA was greatly reduced, and anaerobic degradation and nonenzymatic browning of AA was insignificant.

No significant difference in AA content was found between pH 3.5-OLC-AC and pH 2.0-OLC-AC or between pH 3.5-AC-AC and pH 2.0-AC-AC during storage for up to 56 days ($p > 0.05$). After 56 days, AA contents of pH 2.0 films were significantly higher than those of the corresponding pH 3.5 films ($p < 0.05$). Thus, reduction of the casting solution pH to 2.0 effectively stabilized AA content in AA−WPI better than the pH 3.5 casting solution by reducing the oxidative pathway, anaerobic pathway, and nonenzymatic browning.[12−14]

The different drying methods, drying in AC or in OLC, did not result in different AA concentrations in the films (pH 3.5-OLC-AC vs pH 3.5-AC-AC, pH 2.0-OLC-AC vs pH 2.0-AC-AC). This lack of difference was continuously observed throughout storage (pH 2.0-OLC-OLC vs pH 2.0-OLC-AC). Nonenzymatic browning of AA was found to be insignificant compared to oxidative degradation at these acidic pH values. Thus, neither casting nor storing in an OLC environment was necessary to stabilize AA content in AA−WPI films made from pH 2.0 and 3.5 casting solutions.

Total Color Difference (ΔE^*_{ab}). The effects of casting solution pH, casting and storage environments, and storage time on ΔE^*_{ab} are shown in Figure 2. ΔE^*_{ab} values of all AA−WPI films (Figure 2) increased over storage time. The ΔE^*_{ab} of corresponding blank films without AA was insignificant (data not shown). Although the major cause of AA−WPI film color change was from browning of AA, the increase in ΔE^*_{ab} in all films over time implies other causes of color changes related to WPI films themselves, for example, caramelization or Maillard reaction of trace amounts of lactose and protein in WPI. A significant increase of WPI film yellowing index during 1 year of storage was reported in previous work.[18]

The effectiveness of stabilizing AA−WPI films by lowering the pH of the casting solution to 2.0 over 3.5 can also be demonstrated by the significantly higher ΔE^*_{ab} value of pH 3.5 films (pH 3.5-OLC-AC or pH 3.5-AC-AC) compared to pH 2.0 films (pH 2.0-OLC-OLC, pH 2.0-OLC-AC, or pH 2.0-AC-AC) ($p < 0.05$). This can be ascribed to the fact that there is >16% of AA in the active form (ascorbate monoanion, AH^-) that can undergo oxidative degradation causing brown color at pH 3.5. On the other hand, there is <1% of the O_2-sensitive AH^- at pH 2.0.

The different drying methods, drying in AC or in OLC, did not result in different yellowness of the films during storage at 22 °C ($p > 0.05$). This was observed by comparing the values between pH 3.5-OLC-AC and pH 3.5-AC-AC or between pH 2.0-OLC-AC and pH 2.0-AC-AC. The nonoxidative browning of AA is considered to be minimal compared to oxidative browning.

Kinetics Determination. AA degraded and ΔE^*_{ab} increased with time and storage temperature. In the present study, the most

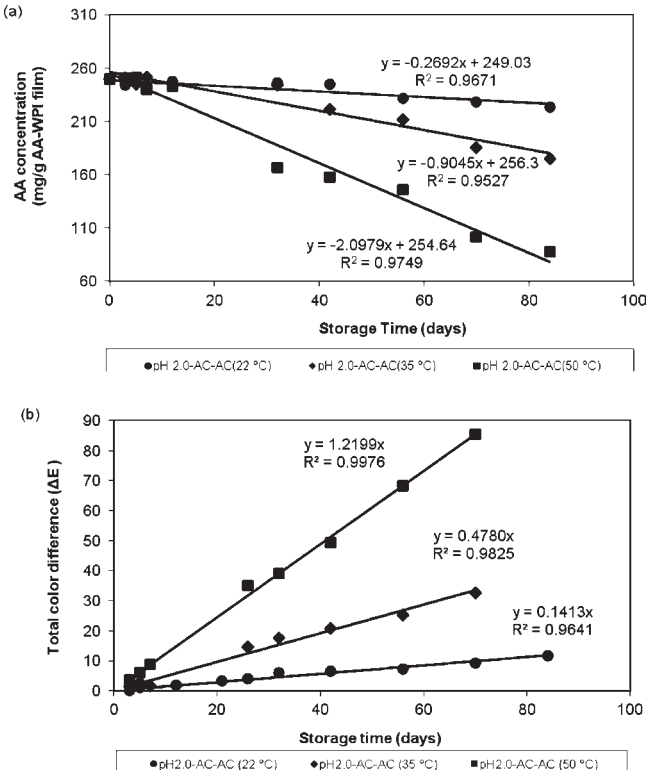

Figure 3. Effect of storage temperature on AA content (a) and total color difference (ΔE^*_{ab}) (b) of pH 2.0-AC-AC film during storage at 22, 35, and 50 °C.

stable AA−WPI film made from pH 2.0 casting solution was chosen for the kinetics studies. A zero-order reaction model produced the best fits to AA degradation and color change during storage of the pH 2.0-AC-AC film at 22, 35, and 50 °C (Figure 3). In all cases, a significant linear regression ($p < 0.05$) with coefficient of determination values (R^2) of >0.95 were obtained. The half-life of AA−WPI film (pH 2.0-AC-AC) cast and stored under normal atmosphere at 22 °C and 30 ± 5% RH was calculated to be 520 days. The excellent stability of AA was achieved as a result of the reduction of the pH of the casting solution. A lower half-life of 36 days of AA-incorporated gellan gum edible film with pH 3.87 stored at 25 °C and 33.3% RH was reported.[19] The higher stability of AA incorporated in WPI films, compared to AA in gellan film, was probably also due to other factors such as the low WPI film oxygen permeability of 200 $cm^3 \cdot \mu m/m^2 \cdot d \cdot kPa$.[1,3] However, the oxygen permeability values of gellan gum films for comparison were not reported.[19]

Storage temperature strongly affected both loss of AA content and change in color (Figure 3) ($p < 0.05$). The pH 2.0-AC-AC films stored at elevated temperatures (35 and 50 °C) had significant loss of AA concentrations compared to films stored at 22 °C ($p < 0.05$). The ΔE^*_{ab} of the pH 2.0-AC-AC films did not change significantly during storage at 22 °C ($p > 0.05$), but increased during storage at 35 and 50 °C ($p < 0.05$).

Although AA degradation is the major cause of film color change, the small increase in ΔE^*_{ab} of blank films (without AA) with corresponding casting pH, stored at corresponding elevated temperatures (data not shown), implies other minor browning mechanism(s), as mentioned earlier. This is consistent with previous observations, as Trezza and Krochta[18] found that E_a values

Table 2. Kinetics Parameters Including Activation Energies (E_a), Q_{10} Values, Arrhenius Pre-exponential Factors (A), and Coefficients of Determination (R^2) for the Increasing ΔE^*_{ab} and Decreasing AA Content of AA−WPI Films

	Q_{10}[a]	E_a (KJ mol^{-1})	A	R^2
AA content	2.25	58.0	5.36×10^9	0.986
ΔE^*_{ab}	2.35	60.9	9.12×10^9	0.992

[a] Q_{10} values were determined at 22 °C using eq 4

of the yellow index of WPI coatings were in the general range of E_a values for nonenzymatic (Maillard) browning reactions of 105−126 kJ mol^{-1},[20] therefore suggesting that Maillard browning was responsible for the color change in WPI coatings.

The Arrhenius equation (eq 2) was used to describe the temperature dependence of the reaction rate constants. Dependence of the rate constants, of both AA loss and color change, on temperature obeyed the Arrhenius relationship ($R^2 > 0.95$) (Table 2). As shown in Table 2, the E_a values of AA degradation and color change were found to be 58 and 61 kJ/mol, respectively. Q_{10} was also determined to express the temperature dependence of chemical reactions. Q_{10} values of AA degradation and color change were found to be 2.25 and 2.35, respectively. These kinetics parameters imply that the degradation of color change of AA−WPI films was slightly more temperature dependent than the degradation of AA during storage.

In conclusion, both the stability of AA and the color change in AA−WPI films were found to be mainly affected by the pH of the casting solution and the storage temperature. The stability of AA−WPI films followed Arrhenius behavior. AA−WPI films were effectively stabilized by lowering the casting solution pH to below the pK_{a1} of AA (4.04 at 25 °C). Oxidative degradation of AA was greatly reduced, and anaerobic degradation was insignificant. The storage stability of AA in AA−WPI film demonstrated by this study suggests that AA−WPI film can maintain antioxidant and oxygen-scavenging capabilities for a reasonably long time before being used to protect foods from oxidation.

AUTHOR INFORMATION

Corresponding Author

*Phone (530) 752-2164. Fax: (530) 752-4759. E-mail: jmkrochta@ucdavis.edu.

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