



Accelerated shelf-life testing of quality loss for a commercial hydrolysed hen egg white powder

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ARTICLE INFO

Article history:

Received 10 February 2012

Received in revised form 23 April 2012

Accepted 2 May 2012

Available online 11 May 2012

Keywords:

Hydrolysed hen egg white powder

Accelerated shelf-life testing

Moisture content

Water activity

Quality loss

Maillard reaction

Physicochemical properties

ABSTRACT

In recent years, due to the specific health benefits associated with bioactive peptides and the reduction of protein allergenicity by enzymatic hydrolysis, the utilisation of protein hydrolysates in functional foods and beverages for both protein supplementation and clinical use has significantly increased. However, few studies have explored the moisture-induced effects on food protein hydrolysates, and the resulting changes in the structure and texture of the food matrix as well as the loss in functional properties of bioactive peptides during storage. The main purpose of this study is to determine the influence of water activity (a_w) on the storage quality of a commercial spray-dried hydrolysed hen egg white powder (HEW). During storage at 45 °C for two months at different a_w s (0.05–0.79), the selected physicochemical properties of the HEW samples were analysed. Overall, the effect of a_w on the colour change of HEW at 45 °C for one month was similar to that of HEW after four months at 23 °C due to the presence of a small amount of glucose in HEW. Several structural changes occurred at a_w s from 0.43 to 0.79 including agglomeration, stickiness and collapse. Kinetic analysis showed a first-order hyperbolic model fit for the change in the L^* value, the total colour difference (ΔE^*) and the fluorescence intensity (FI). There was a high correlation between colour change and fluorescence, as expected for the Maillard reaction. The reduction in the remaining free amino groups was about 5% at a_w 0.50 and 6% at a_w 0.79 after one month storage. In summary, during storage, the Maillard reaction and/or its resulting products could decrease the nutritional value and the quality of HEW.

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1. Introduction

Since 2002, the annual production of hen egg powders in the USA has been about 126,421 metric tons in which about one third is dried egg white (DEW) (USDA, 2012). In the food industry, dried egg products are being used extensively as an ideal ingredient in bakery foods, bakery mixes, mayonnaise and salad dressings, confections, ice cream, pastas, and many convenience foods (Bergquist, 1995). This is due to not only the nutritional and functional properties of dried egg products, but also several advantages compared with those of liquid egg products: (1) transport and storage of dried egg products is less costly and requires less space; (2) at low moisture content, dried egg products are less susceptible to microbial growth; and (3) the uniformity and easy dosage of dried egg products (American Egg Board, 2004, 2006; Bergquist, 1995).

In recent years, due to the specific health benefits associated with the bioactive peptides and the reduction of protein allergenicity by enzymatic hydrolysis (Kitts & Weiler, 2003), the utilisation of protein hydrolysates in functional foods and beverages for both

protein supplementation and clinical use has significantly increased (UBIC-Consulting, 2011). Between 2005 and 2010, the global production of protein hydrolysates increased by about 32% (Dairymark.com, 2010). It has been reported that the enzymatic hydrolysis of egg white with pepsin resulted in the production of peptides with free radical-scavenging capacity and lipid peroxidation inhibition ability (Davalos, Miguel, Bartolome, & Lopez-Fandino, 2004). Some of these peptides also had *in vitro* angiotensin converting enzyme (ACE) inhibitory activity (Miguel, Aleixandre, Ramos, & Lopez-Fandino, 2006; Miguel, Recio, Gomez-Ruiz, Ramos, & Lopez-Fandino, 2004) and vasodilator properties (Garcia-Redondo, Roque, Miguel, Lopez-Fandino, & Salices, 2010; Miguel, Alvarez, Lopez-Fandino, Alonso, & Salices, 2007; Miguel et al., 2007). The short-term administration of hydrolysed hen egg white (HEW) decreased arterial blood pressure in spontaneously hypertensive rats (SHRs) (Miguel, Lopez-Fandino, Ramos, & Aleixandre, 2005) and attenuated the symptoms of inflammatory bowel disease (IBD) using a porcine model (Lee et al., 2009). The long-term intake of HEW attenuated the development of hypertension (Miguel, Lopez-Fandino, Ramos, & Aleixandre, 2006) and prevented or reversed abnormalities associated with the metabolic syndrome and its complications in SHRs (Manso et al., 2008).

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From our previous study (Rao & Labuza, 2012), after short-term storage at 23 °C, the effect of moisture content on physicochemical properties (colour and hardness changes) of HEW was more severe than those of DEW. These changes were mainly due to the difference of the inherent characteristics related to the two egg white powders, which included the reducing sugar content (Maillard reaction), noncovalent (hydrophilic and hydrophobic) bonding, and covalent (disulphide) interactions. The main purpose of this study is to further determine the influence of water activity (a_w) on the storage quality (fluorescent Maillard compounds and free amino group content) of a commercial spray-dried HEW powder through accelerated shelf-life testing (ASLT).

2. Materials and methods

2.1. Sample preparation

Two spray-dried hen egg white powders, Dried Egg Whites (DEW, H227) and Hydrolysed Egg White (HEW, EP-1 #400) were obtained from Debel Food Products, LLC (Elizabeth, NJ, USA) and Henningsen Foods, Inc. (Omaha, NE, USA), respectively. Both samples were kept at –20 °C until tested. The partial process information and selected physicochemical properties of both products were summarised in our previous study (Rao & Labuza, 2012). For the determination of quality loss, the static desiccator method was used. Briefly, quantitative amounts of HEW were weighed into glass vials and placed into eight plastic desiccators containing either Drierite desiccant or saturated salt slurries (LiCl, MgCl₂, K₂CO₃, Mg(NO₃)₂, CoCl₂, NaCl and KCl) at a_w s from 0.05 to 0.79 (Greenspan, 1977; Labuza, Kaanane, & Chen, 1985) at 45 °C. The samples were stored in these sealed desiccators for two months and sampled periodically.

2.2. Capillary electrophoresis (CE)

After receiving both commercial products, the molecular weight (MW) distribution of DEW and HEW was measured using a P/ACE™ MDQ CE system (Beckman Coulter, Inc., Brea, CA, USA) equipped with a UV detector (220 nm). Separation was obtained using a 50-μm bare fused-silica capillary of 30.2 cm with a 20.2 cm effective length from the inlet to the detection window. All CE-grade reagents were obtained as a part of the Proteom-eLab™ SDS-MW Analysis Kit (Beckman Coulter) that was designed for the separation of protein–sodium dodecyl sulphate (SDS) complexes using a replaceable gel matrix. The separating gel was formulated to provide an effective sieving range of approximately 10 to 225 kDa. The SDS-MW size standard (from 10 to 225 kDa, Beckman Coulter) was used to calibrate the gel to estimate the protein MW distribution of the sample. A 10-kDa protein (Beckman Coulter) was used as a mobility marker. The mobility of all protein samples were calculated relative to this mobility marker allowing for more accurate size estimation. A capillary conditioning method was run before analysing each sample. It consisted of a basic rinse (0.1 N NaOH, 10 min, 20 psi), followed by an acidic rinse (0.1 N HCl, 5 min, 20 psi), a water rinse (CE-grade H₂O, 2 min, 20 psi) and finally an SDS gel separation buffer rinse (10 min, 70 psi). The voltage equilibration (15 kV for 10 min, 5-min ramping time) was then applied to the filled SDS gel. The total protein concentration of each sample was 1 mg/ml after the addition of the SDS-MW sample buffer with about 5% (ml/ml) 2-mercaptoethanol (2-ME). The sample vials were capped tightly and heated in a water bath at 100 °C for 3 min. The vials were placed at room temperature to cool at least 5 min before injection. Each sample was injected into the gel-filled capillary at 5 kV for 20 s. The separation was performed at 15 kV for 30 min with reverse polarity in filled SDS gel. All CE

steps were carried out at room temperature. Analysis was performed with the 32 Karat® software (version 8.0, Beckman Coulter).

2.3. Colour analysis

During storage, the colour of the samples was analysed at room temperature using the Minolta Chroma Meter CR-200 (Minolta Camera Co., Osaka, Japan) at designated time intervals. Before analysis, the metre was calibrated with the white CR-200 calibration plate (Minolta) and the colour standards (white, pink, green, yellow, blue, and grey) from Hunter Associates Laboratory, Inc. (Fairfax, VA, USA). The colour was measured using an absolute measuring mode following the manufacturer's instruction and calculated automatically using the CIE 1976 $L^*a^*b^*$ colour space system (International Commission on Illumination, 2008). The three coordinates represent the lightness of the colour ($L^* = 0$ yields black and $L^* = 100$ indicates diffuse white), its position between red/magenta and green (a^* , negative values indicate green while positive values indicate magenta) and its position between yellow and blue (b^* , negative values indicate blue and positive values indicate yellow). The total colour difference (ΔE^*) was calculated using the following equation. The standard (control) used for each a_w level was the HEW sample stored at 45 °C for one day at the same a_w level.

$$\Delta E^* = \sqrt{(L^*_{\text{sample}} - L^*_{\text{standard}})^2 + (a^*_{\text{sample}} - a^*_{\text{standard}})^2 + (b^*_{\text{sample}} - b^*_{\text{standard}})^2}$$

2.4. Determination of fluorescent Maillard compounds

During storage at 45 °C, the presence of fluorescent compounds in the samples was measured at designated time intervals. Each sample (75 mg of protein, dry basis) was added into 2.5 ml distilled and deionized water and mixed thoroughly using a vortex mixer. The mixture was then mixed with pronase solution (0.667 ml, 20 U/ml in 50 mM Tris–HCl, pH 7.2) and incubated for 50 min on a shaker. Pronase from *Streptomyces griseus* was from Roche Diagnostics Co. (Indianapolis, IN, USA). After centrifuging at 13,000g for 15 min, the supernatant of the enzymatic hydrolysates was used to determine the fluorescence intensity (FI). Simultaneously, a blank (control), the original HEW sample stored at –20 °C, was prepared using the same procedure. All steps were carried out at room temperature.

After sample preparation, the FI of the samples was determined according to the methods of Morales and van Boekel (1997) and Palombo, Gertler, and Saguy (1984) with modifications. Briefly, one part (90 μl) of the supernatant was added into 900 μl of PBS (20 mM phosphate buffer containing 15 mM NaCl (PBS), pH 7.0). The solution (200 μl) was then added into the well of a white opaque 96-well microplate (Thermo Fisher Scientific Inc., Rockford, IL, USA) and the FI was measured at an excitation of $\lambda_{\text{ex}} = 360$ nm and reading at $\lambda_{\text{em}} = 460$ nm using a Synergy HT Multi-Mode microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). After subtracting the FI of the blank, all data were corrected to the FI of 5 ppm quinine sulphate using a standard quinine sulphate solution (100 ppm quinine sulphate in 50 mM sulphuric acid), which was diluted in PBS. The result was expressed as the ratio of the FI per gram of protein (%FI/g) divided by the FI of 5 ppm quinine sulphate.

2.5. o-Phthalaldehyde (OPA) method

During one month of storage at 45 °C at three different a_w s (0.05, 0.53 and 0.79), the effect of increased a_w on the loss of free amino groups of HEW was measured using the OPA method (Goodno,

Swaisgood, & Catignani, 1981) with modifications. The OPA method is based on a fluorescence-inducing reaction for amino acid analysis. In the presence of 2-ME (reducing agent), OPA reacts with amino groups on the proteins or free amino acids in an alkaline medium by giving rise to strongly fluorescing compounds (Roth, 1971). For each 100 ml of the OPA reagent, two components, 80 mg OPA (dissolved in 1 ml absolute methanol) and 200 μ l of 2-ME, were diluted to volume with CB-SDS (50 mM carbonate buffer (CB) containing 1% SDS (g/ml), pH 9.6). This OPA reagent was protected from direct light, and used within 2 h. Each sample was diluted in CB-SDS and incubated for 3 h on a shaker at 100 rpm. The final protein concentration was 0.5 mg/ml. Each sample (100 μ l/well) was added into a 96-well clear flat bottom polystyrene microplate (Corning Inc., Corning, NY, USA). The blank was the CB-SDS buffer without HEW (100 μ l/well). The OPA reagent (100 μ l/well) was then added into each well using a Matrix electronic 8 channel pipette (Thermo Fisher Scientific). Right before measurement, the microplate was shaken briefly. The absorbance was read using a microplate reader (BioTek Instruments) at 340 nm. In order to obtain optimal results, the incubation time of each sample after adding the OPA reagent was set to 1.5 min. All the steps were performed at room temperature. The remaining free amino groups in each sample were calculated using the following equation:

$$\% \text{ Free amino groups} = \frac{\text{treated } A_{340} - \text{blank } A_{340}}{\text{original } A_{340} - \text{blank } A_{340}} \times 100$$

2.6. Reaction kinetic analysis

The principles of chemical kinetics, which involve the study of the rates of chemical reactions, were used to study the food quality degradation rate of the HEW samples (Labuza & Kamman, 1983). In general, the rate of food quality change can be expressed as a function of composition (including the reactant concentrations and a_w) and environmental factors (including temperature and relative humidity) (Taoukis, Labuza, & Saguy, 1997). In this study, three dynamic parameters (Y) of HEW (L^* , ΔE^* and FI) were analysed

using a first-order hyperbolic model. The exponential forms are expressed in the following equations:

$$L^* : \Gamma = \frac{Y - Y_{\min}}{Y_0 - Y_{\min}} = e^{-kt}$$

$$\Delta E^* \text{ and FI} : \Gamma = \frac{Y - Y_0}{Y_{\max} - Y_0} = 1 - e^{-kt}$$

where t is the storage time; k is the reaction rate constant; Y_0 is the Y value at $t = 0$; and $Y_{\max}$ and $Y_{\min}$ are the Y values at infinite times. Additionally, the half time ($t_{1/2}$) of the reaction (the time for the quality index Y to increase to half between Y_0 and $Y_{\max}$ or decrease to half between Y_0 and $Y_{\min}$) was also calculated using the equation: $t_{1/2} = \ln 2/k$. In this study, Y_0 of both ΔE^* and FI at each a_w level was zero after the subtraction of the control value. This model assumes that over time one reactant is used up so a hyperbolic pattern of amount vs. time occurs.

Additionally, the Q_A plots for the rate constants of the three dynamic parameters (L^* , ΔE^* and FI) were plotted on a semi-log plot as a function of a_w , respectively. From the slope (b) of the uptrend straight line, the Q_A value was calculated using the following equation (Bell, 2007):

$$Q_A = e^{0.1b}$$

2.7. Statistical analysis

Each sample condition was tested at least in duplicate at each time. To compare the change of the dynamic parameters, one-way ANOVA with Tukey's multiple comparison test was performed. The Pearson correlation between FI and colour change (L^* and ΔE^*) was also measured. Additionally, the parameters of reaction kinetics were calculated. $P < 0.05$ was considered to be statistically significant. The software, GraphPad Prism for Windows (version 5.04, GraphPad Software, La Jolla, CA, USA), was used to analyse the data.

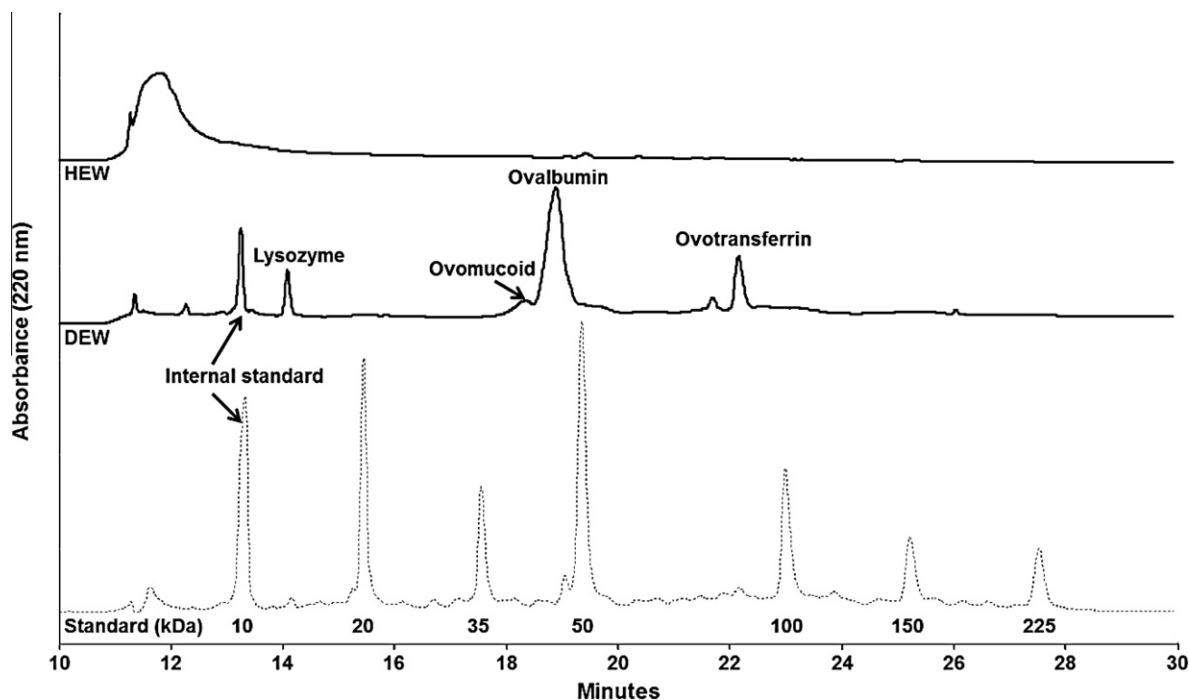


Fig. 1. Electropherograms of two commercial spray-dried hen egg white powders using capillary electrophoresis (CE). DEW: dried egg white; HEW: hydrolysed egg white.

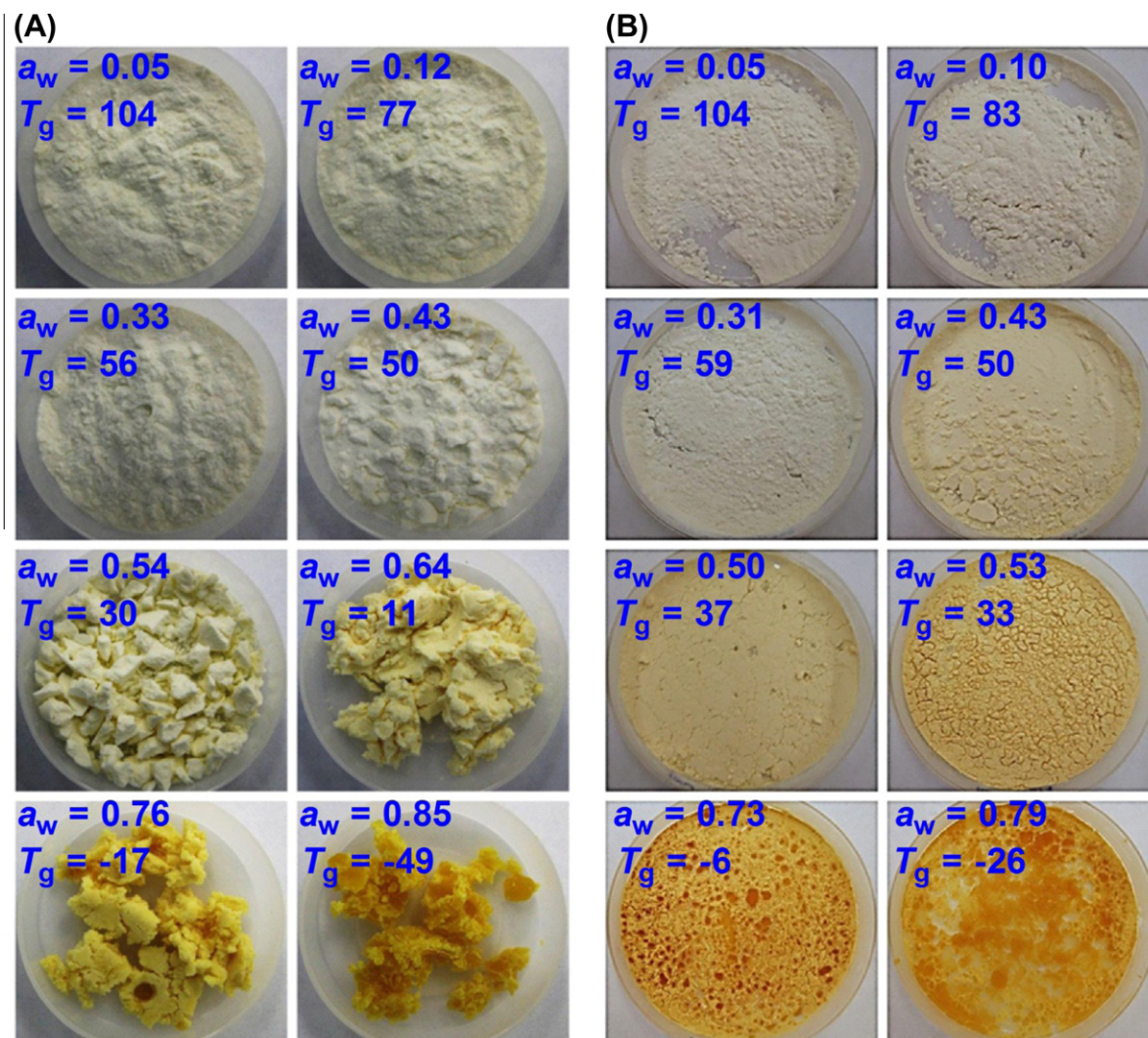


Fig. 2. Images of colour changes of HEW at different a_w s (A) after four months of storage at 23 °C and (B) after one month of storage at 45 °C. The glass transition temperature (T_g) of HEW at different a_w s was calculated using the Gordon–Taylor equation (Rao and Labuza, 2012). *Note:* (A) was reprinted from the study of Rao and Labuza (2012) with permission from Elsevier Ltd. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Results and discussion

3.1. Electropherograms of DEW and HEW

The electropherograms of DEW and HEW using CE are shown in Fig. 1. Overall, no typical egg white protein peaks were detected in the HEW sample as expected. Only one major broad peak, whose MW was smaller than 10 kDa, was observed (Fig. 1). This indicates that the major egg white proteins had been enzymatically hydrolysed into fairly small peptides and/or free amino acids, while for DEW, the four major egg white protein (lysozyme, ovomucoid, ovalbumin and ovotransferrin) peaks were observed (Fig. 1).

3.2. Colour and fluorescence analyses

After one month of storage at 45 °C, the effect of a_w on the colour change of HEW was similar to that of HEW after four months of storage at 23 °C (Fig. 2) as was found by Rao and Labuza (2012). One of the reactions related to colour change in dry systems containing amines and reducing sugar is non-enzymatic browning (NEB or Maillard reaction). The reaction in HEW is likely due to the presence of a small amount of glucose (reducing sugar,

$\leq 0.07\%$). Although the glucose content was very small, it will react with both lysine and the N-terminal groups of the vast amount of small HEW peptides (Rao & Labuza, 2012).

As seen in Fig. 3A, the L^* value of HEW decreased more rapidly as a function of increased storage a_w at 45 °C. This quantitates the visual darkening of HEW seen during storage (Fig. 2). From Fig. 3A, after about two months of storage, the L^* value decreased only slightly, but not significantly, when the samples were stored at a_w s between 0.05 and 0.31. However, when the a_w was greater than 0.31 (from 0.43 to 0.79), the L^* value decreased significantly ($P < 0.05$) compared to those at lower a_w s (from 0.05 to 0.31) and to a greater extent as the storage a_w was raised. This pattern as a function of a_w fits very well into the concept of Labuza, Tannenbaum, and Karel (1970), i.e. below the GAB (Guggenheim–Anderson–de Boer) monolayer moisture value (m_0), molecular mobility is reduced so reaction rates are minimal to almost zero, while above the GAB m_0 , reaction rates increase with increased storage a_w .

It is also noted that within one day of storage at 45 °C, the L^* values of the HEW samples stored at a_w s from 0.50 to 0.79 decreased significantly compared with those of the samples stored at a_w s from 0.05 to 0.31 ($P < 0.05$, Fig. 3A). The difference between $a_w = 0.05$ and 0.79 was about 11% within one day of storage

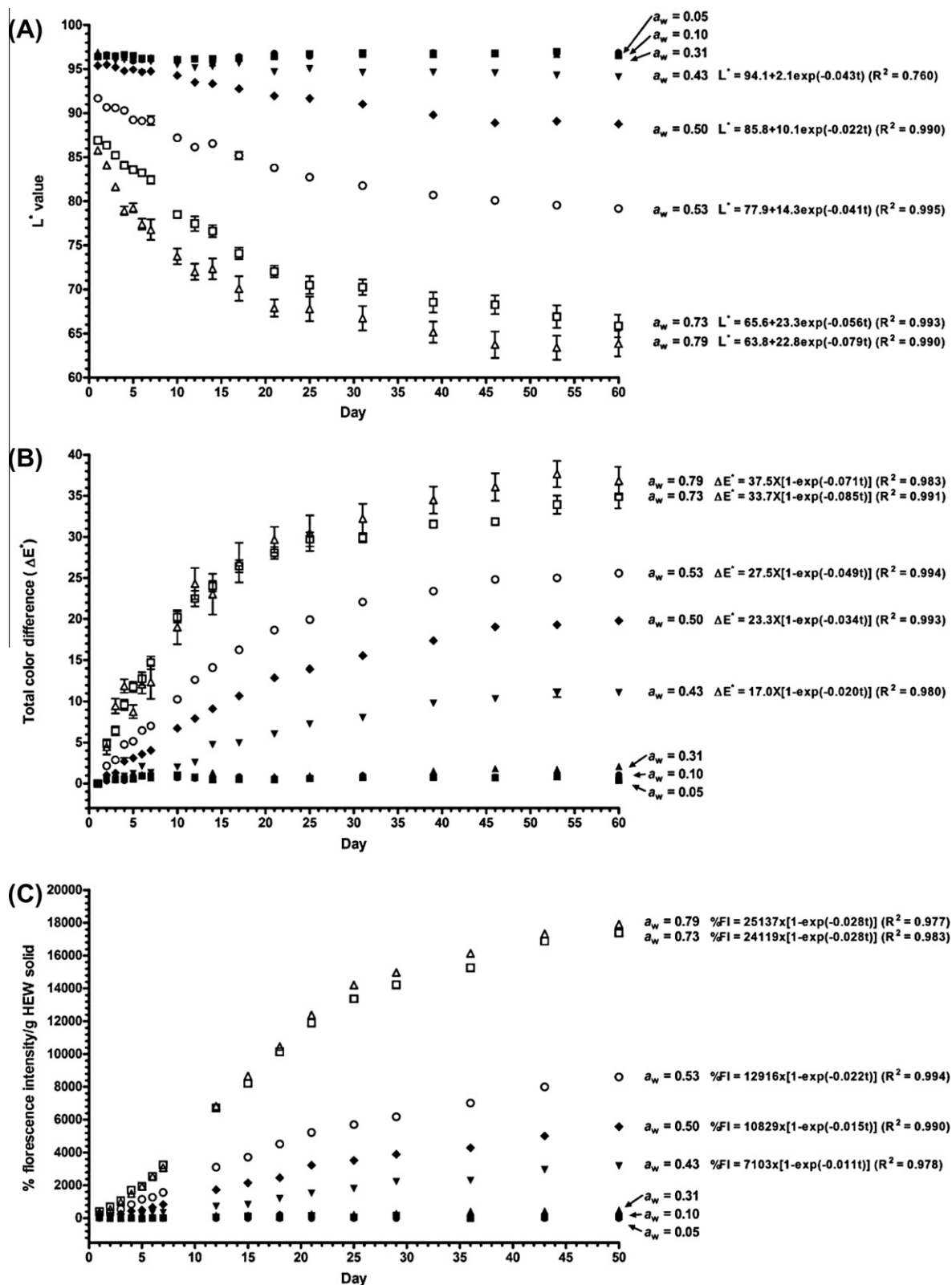


Fig. 3. Effect of a_w on (A) the L^* value, (B) the total colour difference (ΔE^*) and (C) the fluorescence intensity (FI) of HEW during storage at 45 °C. The exponential form of the three dynamic parameters (L^* , ΔE^* and FI) was analysed using a first-order hyperbolic model. The value R^2 quantifies goodness of fit.

(Fig. 3A). There could be two major reasons for this. Firstly, it might be due to a change in the light reflection (physical effect) with higher water content. It was observed that the colour of the HEW powder changed immediately from white to light yellow when

several drops of water were added to the powder at room temperature. Secondly, it could be due to the Maillard reaction between amines and glucose. With greater a_w , more reactants dissolved and their mobility further increased, resulting in faster reaction

Table 1Kinetic analysis of apparent first-order hyperbolic model fit for the change in the L^* value as a function of water activity (a_w) for HEW stored at 45 °C.

Apparent first-order model	a_w				
	0.43	0.50	0.53	0.73	0.79
<i>Best-fit values[#]</i>					
$L^*_{\max}$	96.2 ^a	95.9 ^a	92.2 ^b	88.9 ^c	86.7 ^d
$L^*_{\min}$	94.1 ^a	85.8 ^b	77.9 ^c	65.6 ^d	63.8 ^d
$k \times 10^2$ (day ⁻¹)	4.3 ^a	2.2 ^{ab}	4.1 ^{ac}	5.6 ^{ad}	7.9 ^{ae}
$t_{1/2}$ (day)	16.0 ^a	31.4 ^{ab}	16.8 ^{ac}	12.4 ^{ad}	8.8 ^{ae}
<i>95% CI</i>					
$L^*_{\max}$	95.7–96.7	95.6–96.2	91.8–92.6	88.0–89.9	85.4–87.9
$L^*_{\min}$	93.2–95.1	83.8–87.8	77.1–78.8	64.5–66.8	62.9–64.8
$k \times 10^2$ (day ⁻¹)	0.0–9.4	1.4–3.0	3.5–4.7	4.8–6.4	6.7–9.1
$t_{1/2}$ (day)	≥7.4	23.3–48.1	14.7–19.8	10.8–14.5	7.6–10.3
<i>Goodness of fit</i>					
R^2	0.760	0.990	0.995	0.993	0.990

[#] Values in the same row with different letters are significantly different ($P < 0.05$).**Table 2**Kinetic analysis of apparent first-order hyperbolic model fit for the change in the total colour difference (ΔE^*) as a function of water activity (a_w) for HEW stored at 45 °C.

Apparent first-order model	a_w				
	0.43	0.50	0.53	0.73	0.79
<i>Best-fit values[#]</i>					
$\Delta E^*_{\max}$	17.0 ^a	23.3 ^a	27.5 ^b	33.7 ^c	37.5 ^d
$k \times 10^2$ (day ⁻¹)	2.0 ^a	3.4 ^b	4.9 ^c	8.5 ^d	7.1 ^d
$t_{1/2}$ (day)	35.2 ^a	20.1 ^b	14.0 ^c	8.2 ^d	9.8 ^d
<i>95% CI</i>					
$\Delta E^*_{\max}$	12.4–21.6	21.5–25.2	26.2–28.7	32.5–34.9	35.4–39.6
$k \times 10^2$ (day ⁻¹)	1.2–2.8	2.9–4.0	4.4–5.5	7.6–9.3	6.0–8.1
$t_{1/2}$ (day)	25.0–59.2	17.5–23.6	12.7–15.6	7.4–9.1	8.6–11.5
<i>Goodness of fit</i>					
R^2	0.980	0.993	0.994	0.991	0.983

[#] Values in the same row with different letters are significantly different ($P < 0.05$).**Table 3**Kinetic analysis of apparent first-order hyperbolic model fit for the change in the fluorescence intensity (FI) as a function of water activity (a_w) for HEW stored at 45 °C.

Apparent first-order model	a_w				
	0.43	0.50	0.53	0.73	0.79
<i>Best-fit values[#]</i>					
$FI_{\max} \times 10^{-3}$	7.1 ^a	10.8 ^{ab}	12.9 ^b	24.1 ^c	25.1 ^c
$k \times 10^2$ (day ⁻¹)	1.1 ^a	1.5 ^{ab}	2.2 ^b	2.8 ^b	2.8 ^b
$t_{1/2}$ (day)	60.7 ^a	47.3 ^{ab}	30.9 ^b	24.5 ^b	24.6 ^b
<i>95% CI</i>					
$FI_{\max} \times 10^{-3}$	5.2–9.0	7.0–14.7	10.8–15.1	18.8–29.5	18.6–31.7
$k \times 10^2$ (day ⁻¹)	1.1–1.2	0.8–2.1	1.7–2.8	1.8–3.8	1.6–4.0
$t_{1/2}$ (day)	56.8–65.1	32.4–87.8	24.8–41.0	18.1–38.1	17.4–42.2
<i>Goodness of fit</i>					
R^2	0.978	0.990	0.994	0.983	0.977

[#] Values in the same row with different letters are significantly different ($P < 0.05$).

rates (Rao & Labuza, 2012). It has been reported that the FI value is significantly correlated to both the total protein content of the sample and any fluorescent Maillard compounds, which are mainly linked to the protein backbone (Delgado-Andrade, Rufian-Henares, & Morales, 2006). However, compared with the FI value of the sample stored at an a_w of 0.05, after one day of storage at 45 °C (Fig. 3C), there was no significant difference in fluorescence except for the samples stored at higher a_w s (0.73 and 0.79). This means that during the first day of storage at an a_w of 0.50, although the colour of HEW darkened significantly, the amount of fluorescent Maillard compounds did not increase significantly compared with those of the samples stored at an a_w of 0.05. This initial colour change, therefore, must be due to a change in the physical

properties of the protein particles, i.e. in the way they interact with incident light when wetted. Furthermore, the a_w of the HEW sample, especially at $a_w > 0.50$, would not be fully equilibrated at 45 °C during the early stages of storage, which depends on both the inherent properties of the sample and the storage conditions (sample size, temperature and relative humidity). Therefore, before reaching the moisture-equilibrated condition in each HEW sample, the physical effect of water on light reflection might contribute more to the decrease of the L^* value compared with the chemical effects such as the Maillard reaction. To minimise the effect of light reflection on the total colour difference (ΔE^*), the standard (control) used for each a_w level was the HEW sample stored at 45 °C for one day at the same a_w level.

Table 4

Pearson correlation coefficients (r , $P < 0.05$) between the fluorescence intensity (FI) and the two dynamic parameters related to colour change (the L^* value and the total colour difference (ΔE^*)).

Colour change parameters	a_w				
	0.43	0.50	0.53	0.73	0.79
L^*					
r	–0.688	–0.993	–0.993	–0.996	–0.938
R^2 #	0.474	0.992	0.986	0.992	0.881
ΔE^*					
r	0.989	0.993	0.991	0.951	0.947
R^2	0.979	0.986	0.983	0.905	0.897

The value R^2 quantifies goodness of fit.

The two other dynamic parameters, ΔE^* and FI, increased as a function of a_w during storage at 45 °C (Fig. 3B and C). ΔE^* is a single dynamic parameter which takes into account the differences between the three colour coordinates (L^* , a^* and b^*) of the sample as compared to a standard (International Commission on Illumination, 2008). Simultaneously with NEB, Amadori products could form cross-links between adjacent proteins or with other amino groups, giving rise to polymeric aggregates, so-called advanced glycation end products (AGEs) (Morales, Romero, & JimenezPerez, 1996). The FI ($\lambda_{\text{ex}} = 340\text{--}370$ nm and $\lambda_{\text{em}} = 420\text{--}440$ nm) of AGE compounds has been widely used as a marker of AGE-modified protein levels (Contreras-Calderon, Guerra-Hernandez, & Garcia-Villanova, 2009; Ferrer, Alegria, Farre, Clemente, & Calvo, 2005; Morales & Jimenez-Perez, 2001; Morales & van Boekel, 1997). From Fig. 3B and C, after about two months of storage, for the samples stored at a_w s from 0.05 to 0.31, the values of both ΔE^* and FI increased only slightly, but not significantly. However, when the storage a_w was from 0.43 to 0.79, both values increased significantly ($P < 0.05$) compared with those at lower storage a_w s (from 0.05 to 0.31).

Overall, for storage a_w s from 0.43 to 0.79, firstly, through reaction kinetics analysis, the apparent first-order hyperbolic model fit very well for the change in the three dynamic parameters (L^* , ΔE^* and FI) as a function of a_w (Tables 1–3); secondly, there was a

significant Pearson correlation ($P < 0.05$) between FI and colour change (L^* and ΔE^* , Table 4). The value of the two best-fit values (L^*_{max} and L^*_{min}) of L^* decreased at higher a_w , while the best-fit value (Y_{max}) of both ΔE^* and FI increased (Tables 1–3). For the L^* value, the best-fit value of k increased as a function of increased storage a_w , while that of $t_{1/2}$ decreased except for the sample stored at a_w 0.43 (Table 1). For both ΔE^* and FI, $\ln k$ increased with storage a_w at about the same Q_A value (Fig. 4). The Q_A value of the three dynamic parameters (L^* , ΔE^* and FI) is shown in Fig. 4 and represents the increase in reaction rate (decrease in $t_{1/2}$) due to a 0.1-unit increase in a_w . The Q_A values fit in the expected range of about 1.5–2.5 (Labuza et al., 1970). However, as expected, above a storage a_w of about 0.65, the reaction rates peaked and then decreased (Fig. 4 and Tables 2 and 3), indicating that additional moisture sorption caused dilution of the reactants (Labuza et al., 1970).

At 45 °C, for storage a_w in the range of 0.05 and 0.31, the HEW powder system is below the glass transition temperature (T_g) as would be the same for storage at 23 °C. Thus there were no visible colour and physical structural changes as shown in Fig. 2. However, when the storage a_w was greater than 0.43, the T_g as noted in Fig. 2 is below the storage temperature of 45 °C, several structural changes occurred, as expected, including agglomeration, stickiness and collapse (flow under the force of gravity, Fig. 2) (Labuza & Labuza, 2004). These colour and physical changes in HEW are closely related to the inherent properties of the sample at the molecular level and the relevant mechanisms have been elaborated in our previous study (Rao & Labuza, 2012).

3.3. Quality loss of HEW

After one month of storage at 45 °C, compared with that of the original sample, the remaining free amino groups at a_w s of 0.50 and 0.79 were reduced by about 5% and 6%, respectively, while there was no significant change in the sample at an a_w of 0.05 (Fig. 5). Several studies have shown that when ovalbumin was stored with different reducing sugars (1:1, g/g) in a solid system at 50 °C and 65% relative humidity, due to the Maillard reaction, the remaining free amino groups of ovalbumin were less than

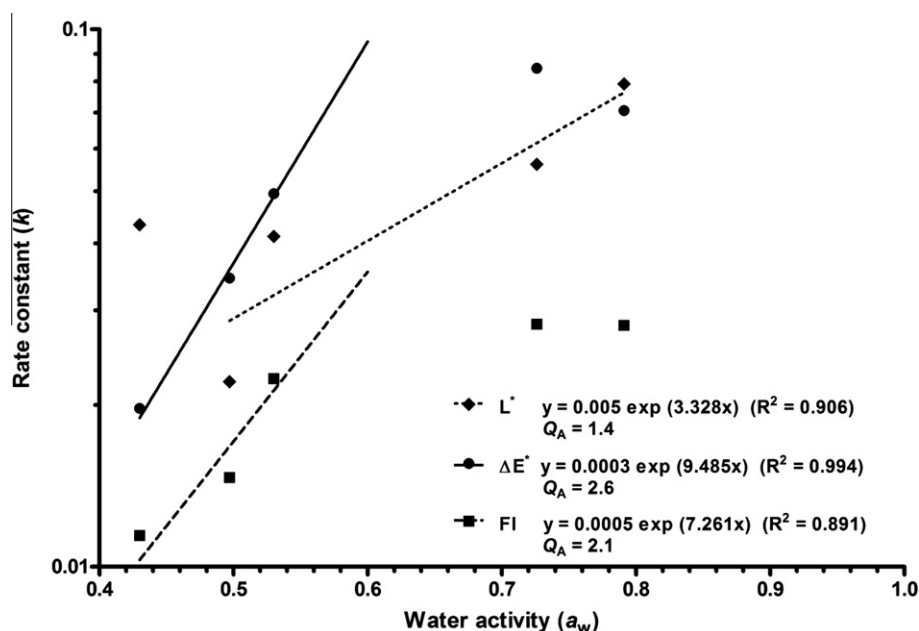


Fig. 4. Effect of a_w on the reaction rates (k) of the L^* value, the total colour difference (ΔE^*) and the fluorescence intensity (FI) during storage at 45 °C. The uptrend line indicates the exponential relationship between the reaction rate and a_w up to a maximum. The Q_A of each parameter is indicated. The value R^2 quantifies goodness of fit.

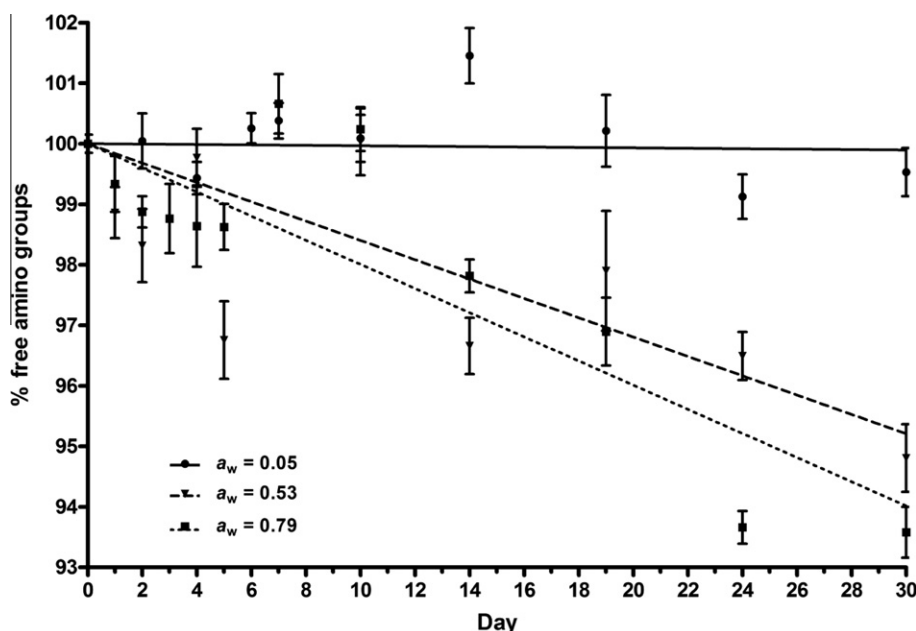


Fig. 5. Effect of a_w on the free amino groups of HEW during one month of storage at 45 °C. Three lines, solid ($a_w = 0.05$), dashed ($a_w = 0.53$) and dotted ($a_w = 0.79$), indicate the downtrend of the remaining free amino groups of HEW stored at three different a_w s.

30% of the original within one month (Kato, Matsuda, Kato, & Nakamura, 1988, 1989; Kato, Matsuda, Kato, Watanabe, & Nakamura, 1986). Additionally, in these ovalbumin-sugar systems, the free amino group content decreased with an increase in the protein-sugar ratio (Kato et al., 1986). In our sample system, the protein-sugar ratio was only about 1:0.07 and thus the loss was much lower than in the studies noted previously. Additionally, after enzymatic hydrolysis (degree of hydrolysis: 7–14%), HEW contains a large amount of small peptides and/or even free amino acids (Fig. 1) (Rao & Labuza, 2012). Therefore, the remaining free amino groups in the HEW samples are still high (>90%, Fig. 5) after one month of storage at 45 °C.

In the food industry, the two important functions of protein hydrolysates are use as a plasticizer to reduce the hardness of products because of their T_g s below room temperature and secondly for their beneficial biological activities. However, the amount of some special bioactive peptides and/or essential amino acids might decrease or even disappear during storage due to the Maillard reaction (Perez-Locas & Yaylayan, 2010). Interestingly, this bioactivity or nutritional loss might not be associated with NEB. It was reported that almost no browning was found for ovalbumin samples even after more than 80% of the free amino groups had been glycosylated with maltose, cellobiose or lactose (Kato et al., 1989). On the other hand, from *in vivo* studies, it was shown that the consumption of a diet rich in Maillard reaction products (MRPs) negatively affected protein digestibility and reduced iron bioavailability in adolescent males (Delgado-Andrade et al., 2006; Garcia, Seiquer, Delgado-Andrade, Galdo, & Navarro, 2009). Another study showed that MRPs reduced phosphorus digestibility in male adolescent mice (Delgado-Andrade, Seiquer, Garcia, Galdo, & Navarro, 2011). Therefore, prevention or reducing such biological activity or nutritional losses in food products during storage is a critical need, especially for intermediate-moisture foods (IMF) whose a_w is from 0.55 to 0.85, such as high protein nutrition bars and protein rich energy bars.

From our previous findings obtained with Raman spectroscopy (Rao & Labuza, 2012), the number of intermolecular disulphide bonds of HEW decreased when the a_w was slightly greater than about 0.33 at 23 °C, i.e. when the moisture content was larger than

its GAB m_0 . Since GAB m_0 decreases with increasing temperature, as per the Clausius–Clapeyron equation, we estimate that it is about 0.25 for the HEW powder system at 45 °C (Labuza et al., 1985). The negative effect on the secondary structure of the hydrolysed molecule should not be visible until the intermolecular interactions occurred to a larger extent in the HEW system at higher storage a_w (Fig. 2). This quality loss might be related to the redox potential between the reactants in the sample system. Redox potential is a measure of the tendency of a chemical species to acquire electrons and thereby be reduced. The more negative the redox potential, the stronger the reductant and the weaker the oxidant. In general, the disulphide redox potentials in proteins vary from –122 to –270 mV (Winther, Ostergaard, Henriksen, & Hansen, 2001). During the early stages of the Maillard reaction, highly reactive intermediates, reductones, can be formed (Hodge, 1953; Rizzi, 2003). For these Maillard products, if the redox potential is greater than that of the disulphide bond, they have the ability to cleave the intermolecular disulphide bonds and affect the storage stability of the products. This assumption however needs to be experimentally confirmed.

4. Conclusions

A HEW powder system was used to study the effect of increased storage a_w in the range of 0.05–0.79 on physical and chemical quality changes at an accelerated temperature (45 °C). Overall, when the storage a_w was higher than that of the GAB m_0 of HEW, several quality losses occurred during storage. Firstly, structural changes occurred including agglomeration, stickiness and collapse in the HEW system when the storage temperature was greater than the T_g . Secondly, kinetic analysis showed a first-order hyperbolic model fit for the change in three storage quality parameters (L^* , ΔE^* and FI). Both ΔE^* and FI increased as a function of increased a_w , while the colour darkened (L^* value) in the same fashion with increased storage a_w . Thirdly, the reduction in the remaining free amino groups was only about 5% at $a_w = 0.50$ and 6% at $a_w = 0.79$ after one month of storage at 45 °C. This small change is likely due to the relatively small amount of glucose ($\leq 0.07\%$) found in HEW. Furthermore, when the storage a_w was higher than that of the

GAB m_0 of HEW, the Maillard reaction and/or its resulting products would also have a negative effect on the secondary structure of proteins and/or peptides. This situation will be more complicated in IMF, such as high protein nutrition bars and energy bars, which contain more ingredients (fat, plasticizer and added sugar). In conclusion, significant quality loss was found for the HEW powder system at $a_w > 0.31$. The presence of residual reducing sugar in HEW, even though very low, caused some of these changes and thus efforts to reduce the critical amount during manufacturing of the powder should be undertaken. In addition, the use of sweeteners with high amounts of reducing sugar, such as high fructose corn syrup, should be eliminated in any IMF containing protein hydrolysates such as HEW. Since HEW has a significantly lower T_g as moisture increases, it acts as a useful plasticizer. Thus other plasticizers, such as sugar alcohols, may not be needed to soften the texture. If used, the industry should seek out those which do not have residual reducing sugars.

Acknowledgements

This project was supported by the American Egg Board (Grant No. DUNS555917996). The authors would like to thank Debel Food Products, LLC and Henningsen Foods, Inc. for providing the spray-dried hen egg white powders.

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