Reaction between Albumen and 3,3′,5,5′-Tetramethylbenzidine as a Method to Evaluate Egg Freshness

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The 3,3′,5,5′-tetramethylbenzidine colorimetric test is proposed as a method for the evaluation of shell egg freshness. A simplification of the original method was tested on albumen, and the modified method was used to study the development of albumen−tetramethylbenzidine reaction in eggs stored at three temperatures for different times. The stability of the reagent solution and the repeatability of the analytical method were also studied. The reaction mechanism showed the predominant role of iron ions on the colorimetric response, and the secondary role of copper ions added to the reaction mix in order to enhance the response. The response coefficient of variation, due to the natural variability in eggs laid by hens of two breeds and different ages, was 10.3%, which is similar to the value obtained for furosine, but lower than those previously observed for other egg freshness indices such as Haugh Unit and air-cell height.

Keywords: *Albumen; iron; egg freshness; 3,3′,5,5′-tetramethylbenzidine*

INTRODUCTION

The main criterion for shell egg grading is freshness; however, the only quantitative parameter for egg freshness evaluation considered by European Union regulations (*1*) is air cell height, whereas U.S. grading requirements also consider thick albumen height, expressed as Haugh Units (*2*). The soundness of these two indices for freshness evaluation is questionable because air cell height depends on egg weight (*3*, *4*), and the Haugh Unit depends on laying-hen age (*3*). Moreover, the accuracy of the equation used to compute Haugh Units is doubtful (*5*−*7*). Some studies to find alternative egg freshness indices were carried out (*8*−*10*), but the methods proposed are time-consuming and require specific and expensive equipment (HPLC or LRNMR), making them unsuitable for routine control analyses. Hence, the necessity of a rapid, simple, and reliable alternative method.

Monsey and Jones (*11*) applied a test for blood estimation, based on the 3,3′,5,5′-tetramethylbenzidine (TMB) reaction, to incubator fertile eggs as a possible means of detecting incubator rejects. The authors noticed that by adding copper to whole egg a positive response was also obtained with blood-free eggs. They observed an increase in the reaction product absorbance during storage of infertile eggs at 10, 20, 25, and 37 °C, but no further studies were performed to test the accuracy of the method, to possibly propose it as a means for determining shell egg freshness.

The aim of the present work was to study and simplify the TMB reaction method reported by Monsey and Jones (*11*) in order to evaluate its suitability as a shell egg freshness index.

MATERIALS AND METHODS

Eggs. Grade A-extra eggs (*12*) from commercial channels, as well as newly laid eggs obtained directly from the producer and available in the laboratory within 24 h of laying, were used in this research. The eggs from the producer were laid by hens of known breed and age (weeks from hatching). Following are the different egg lots used: (a) eggs laid by Isa-Brown Warren hens (57 and 64 weeks old), to study the TMB reaction as a function of egg storage time; (b) eggs laid by Hy-Line Brown Plus (42 weeks old), to assess the repeatability of the method; (c) grade A-extra commercial eggs, to evaluate the reagent stability during storage of the TMB solution; (d) eggs laid by Hy-Line Brown Plus (46, 47, 54, and 76 weeks old) and Isa-Brown Warren hens (40, 54, 70, and 82 weeks old), to study the natural variability of the TMB reaction; the 70, 76, and 82 week-old hens underwent molting; and (e) eggs laid by Hy-Line Brown Plus hens (46 weeks old), to study the TMB reaction mechanism in albumen.

Sample Preparation. The measurements were performed on bulked albumen from six eggs. Shelling and accurate separation of albumen from yolk were done manually. The albumens were homogenized using a Sörvall Omni-Mixer (model 17106, Dupont de Nemours & Co, Newtown, CT) at 3000 rpm for 30 s.

TMB Original Method. The TMB−whole egg reaction method proposed by Monsey and Jones (*11*) was applied to albumen: 10 g of homogenized sample with 20 mL of chloroform were vigorously shaken in a 50-mL centrifuge tube for 2 min using a magnetic stirrer. After centrifugation at 4300 rpm (2067*g*) for 15 min using a Centrikon T-42K centrifuge (Kontron Instruments, Milan, Italy), the top layer was withdrawn. To 2 mL of this aqueous extract, 0.1 mL of 40 mmol/L $CuSO_4 \cdot 5H_2O$ solution was added and mixed vigorously in a 5-mL plastic tube using an IKA shaker (IKA Works Inc., Wilmington, NC, model MS1). A 1-mL portion of this mixture was added to 1 mL of TMB (Merck, Darmstadt, Germany) solution (0.2% w/v in 10% acetic acid v/v), placed in a 20-mL stoppered tube, and shaken using the IKA shaker. After 2 min rest, 1 mL of H_2O_2 (1.2% v/v) was added and the contents of the tube were mixed with the IKA shaker. After 5 min rest, 10 mL of acetic acid (10% v/v) was added and mixed. After a further 55 min rest, sample absorbance was measured in 10-mm cells at 370, 450, and 650 nm against water as the blank, and scanned between 325 and 700 nm in an UVIDEC-610 spectrophotometer (Japan Spectroscopic Co, Ltd., Tokyo, Japan). The results are the average of two measurements.

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TMB Modified Method. The original Monsey and Jones method was modified and optimized for albumen by eliminating the chloroform extraction step and by diluting 1 mL of albumen with 1 mL of distilled water. Then, 0.1 mL of 40 mmol/L $CuSO_4{\cdot}5H_2O$ solution was added to the diluted albumen and the test was continued as described in the original method. The results are the average of two measurements.

The repeatability of the TMB modified method was assessed by performing 12 replicate measurements on the same albumen sample from eggs stored at 20 °C for 10 days. The results are expressed in terms of standard deviation (SD) and coefficient of variation (CV, %).

To evaluate the stability of the reagent (0.2% TMB in 10% acetic acid), several TMB solutions aged at 5 °C for different times up to 21 days were used to analyze three albumen samples of 1, 15, and 21-days-old commercial eggs, using the TMB modified method.

Aging of Eggs. The original Monsey and Jones (*11*) TMB reaction test was applied to albumen from two lots of eggs stored at 5, 20, 30, and 38 °C up to 88, 59, 35, and 18 days, respectively. The TMB modified method was also used to analyze one of the two egg lots. The TableCurve 2D (Jandel Scientific, San Rafael, CA) software was used to obtain the isothermal curves representing the best fitting of the experimental points.

Natural Variability. The influence of hen breed and age on the TMB modified method was studied by analyzing six bulked albumens of 1-day-old eggs laid by four groups each of Hy-Line Plus and Isa-Brown Warren hens of different ages. Results are the average of duplicate analysis.

TMB Reaction Mechanism. To study the TMB–albumen reaction mechanism, the copper sulfate solution (0.1 mL) used with this method was substituted with an equal volume of ferrous sulfate solution at various concentrations (from 0 to 4 mmol/L) following the modified method, and analyzing two albumen samples from fresh eggs (stored 6 days at 5 °C) laid by Hy-Line Plus hens (46 weeks old). In the results, iron concentrations are expressed with respect to the total volume of the diluted albumen (2 mL) plus the iron solution (0.1 mL).

To understand the role of copper in the reaction mechanism, the TMB method was performed on two model solutions, modifying the concentrations of the 0.1 mL copper sulfate solution used in the reaction (0, 2, 10, 20, and 40 mmol/L). The model solutions were prepared by mixing 1 mL of 4 mg/mL ovotransferrin solution, 0.96 mL of carbonate–phosphate–citrate buffer pH 8.5 (0.1 M Na_2CO_3, 0.1 M sodium citrate, 0.2 M Na_2HPO_4, and 0.1 M NaH_2PO_4), and 0.04 mL of 2 or 20 mmol/L $FeCl_3$. In the results, copper and iron concentrations are expressed with respect to the total volume of the model solution (2 mL) plus the copper reagent (0.1 mL).

The Microsoft Excel97 (Microsoft Co., Redmond, WA) software was used to obtain the linear regression of the experimental points.

RESULTS AND DISCUSSION

TMB Reaction Method. The method proposed by Monsey and Jones (*11*) for whole egg was initially applied to the albumen of shell eggs stored at different temperatures. As an example, the reaction product absorption spectra for the albumen of fresh and stored eggs are shown in Figure 1. The albumen of stored eggs shows three absorption peaks at 370, 450, and 650 nm, as observed by Monsey and Jones (*11*) for whole egg, but the albumen of fresh eggs does not show any peak. The correlations between the absorbance values found at the three wavelengths for the albumen of eggs were computed ($n = 52$). The absorbances at 370 nm were highly correlated ($p < 0.001$) with the absorbances measured at both 450 nm ($r = 0.99$) and at 650 nm ($r = 1.00$). Because of the correlation observed, the three wavelengths may be considered equivalent. However, the 370 nm wavelength was analytically preferred, because at this wavelength higher absorbance values are detected, as shown in Figure 1.

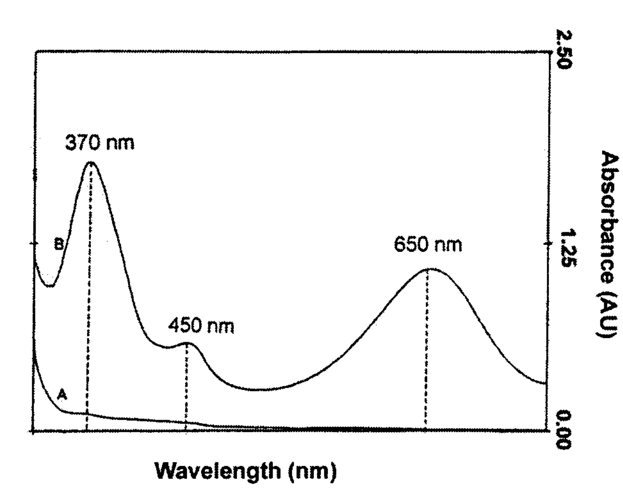

Figure 1. Typical absorbance spectra of the reaction product between TMB and albumen of fresh eggs (A) and eggs stored 35 days at 20 °C (B).

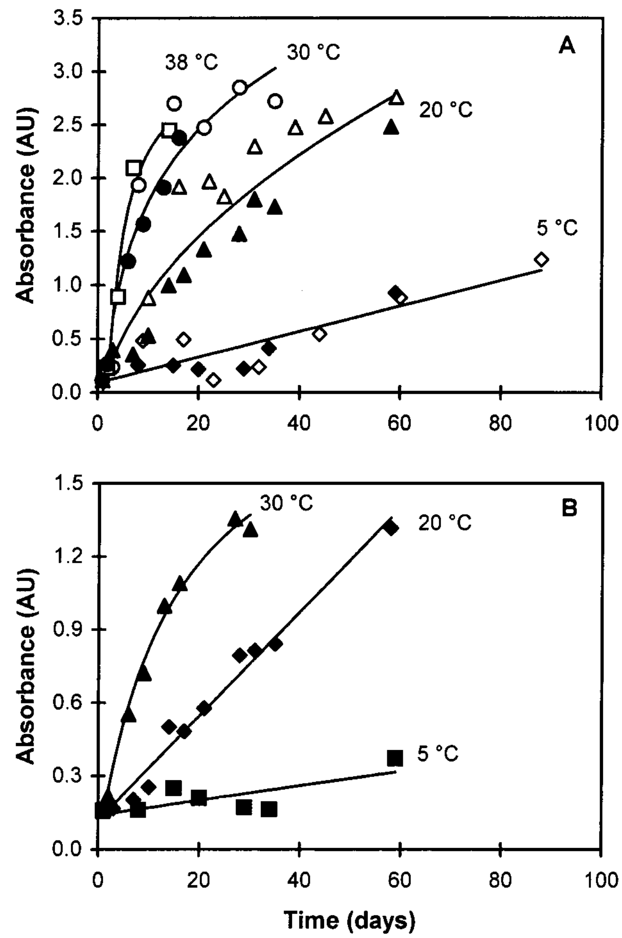

Figure 2. Absorbance at 370 nm of the reaction product between TMB and albumen during storage at different temperatures of eggs from 64- (open symbols) and 57- (solid symbols) week-old Isa-Brown Warren hens, following the Monsey and Jones original method (A), and the modified method (B).

Figure 2, panel A presents the absorbance values of the TMB–albumen reaction at 370 nm as a function of egg storage time. The absorbance increase observed as a function of time and temperature of storage suggests that the method could be used for describing shell egg freshness.

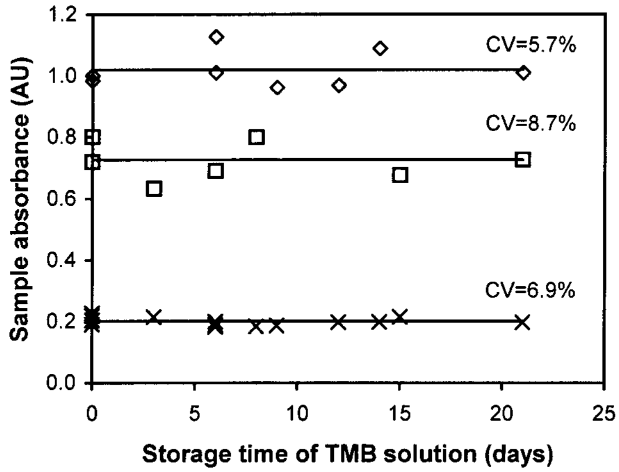

Figure 3. Absorbance at 370 nm of the reaction product between TMB and albumen of 1 day old (×), 15 day old (□) and 21 day old (◇) commercial eggs, as a function of the TMB solution storage time at 5 °C, following the modified method.

Because of the low lipid level in albumen, a simplification of the method might be possible. To test this hypothesis, several experiments were performed. Initially, the chloroform extraction and centrifugation steps were eliminated by directly using 2 mL of homogenized albumen. The reaction product, however, was turbid and the spectrum did not show the absorption maxima typical of the reaction. Filtration of this product did not improve the spectrum; on the contrary, part of the coloring substance was retained on the filter. When applied to albumen diluted 1:1 with distilled water, the method instead gave a spectrum similar to that obtained with the original method.

This modified method was used with the albumen of eggs stored at different temperatures, obtaining the curves presented in Figure 2B. The trends are similar to those obtained with the original method (Figure 2A) but show lower absorbance values (below 1.5 AU). These values bring the absorption within the linearity range of the instrument. A significant correlation ($n = 24$, $p < 0.001$, $r = 0.98$) between absorbance values obtained with the simplified and the original method was observed. Moreover, the modified method is faster and easier.

The repeatability of the modified TMB reaction method, expressed in terms of mean ± SD, and CV, was 0.380 ± 0.029 AU at 370 nm, corresponding to a CV of 7.6% ($n = 12$).

It was observed that the color of the reagent solution (0.2% TMB w/v in 10% acetic acid v/v) varied from pale to deep blue depending on the preparation and storage conditions (type of mixing, time, and temperature). The influence of the TMB solution color and age on the results was investigated. Figure 3 shows the absorbance values of the reaction product between TMB and albumen from eggs of three different ages, as a function of the reagent solution storage time at 5 °C up to 21 days. Although the TMB solutions used for the analysis had different absorbance values (ranging from 0.066 to 0.462 AU at 370 nm), the average variability of the reaction product absorbance obtained for albumen samples (CV= 7.1%) was similar to that found for the method repeatability. This result demonstrates that the TMB reagent can be used for at least 21 days after preparation, regardless of its appearance. Longer times remain to be tested.

Table 1. TMB−Albumen Reaction Product Absorbance at 370 nm of Fresh Eggs Laid by Hens of Different Breed and Age

breed	hen age (week)	absorbance (AU)	SD[b] (AU)
Isa-Brown Warren	40	0.186	0.008
Hy-Line Brown plus	46	0.206	0.008
Hy-Line Brown plus	47	0.165	0.003
Isa-Brown Warren	54	0.177	0.017
Hy-Line Brown plus	54	0.158	0.008
Isa-Brown Warren	70[a]	0.201	0.010
Hy-Line Brown plus	76[a]	0.210	0.008
Isa-Brown Warren	82[a]	0.198	0.007
	mean	0.188	
	SD[b]	0.019	
	CV[c]	10.3	

[a] Hens underwent molting. [b] SD, standard deviation. [c] CV, coefficient of variation (%).

Natural Variability. Table 1 shows the TMB−albumen reaction absorbance of fresh eggs laid by hens of several ages and two breeds. No trend related to hen age was observed. The coefficient of variation due to natural variability was 10.3%, slightly higher than that observed for method repeatability.

TMB Reaction Mechanism. TMB was found to be a valid substitute for benzidine in the classical method used for measuring hemoglobin in urine and plasma (*13*). The chemical reaction leads to the formation of hematin, which catalyzes the conversion of hydrogen peroxide to water and oxygen. In this conversion, benzidine is oxidized to a chromogenic product and the intensity of the resulting color is used as an index of hemoglobin concentration (*14*). Monsey and Jones (*11*), while applying the method to incubated fertile eggs, noted that the addition of copper gave a positive reaction even in blood-free eggs, suggesting the method as a possible means of determining the storage history of shell eggs.

Iron ions, present in albumen bound to ovotransferrin (*15*), might play a role in the TMB reaction of eggs similar to that played in the TMB analysis of hemoglobin. To verify this hypothesis, the TMB modified method was applied to an albumen sample of fresh eggs added with iron at different concentrations, but without copper addition (Figure 4): the absorbance increased linearly with iron concentration. This result suggests that the absorbance trends observed in Figure 2 can be related to an iron increase in albumen, probably as a consequence of iron entering the albumen from the yolk during the storage of eggs. Actually, Capozzi (*10*) reported an increase of iron content from 1 to 3 mg/kg in albumen after three weeks of egg storage at room temperature.

Because the Monsey and Jones method considers a copper addition for the development of the TMB reaction in blood-free eggs, an experiment to investigate the role of copper was carried out. The TMB method was performed on model solutions containing two different iron levels, varying the concentration of copper sulfate solution used with this method. As shown in Figure 5, the absorbance increased linearly as a function of copper concentration. This suggests that the TMB method response in eggs is the sum of both the absorbances due to the copper added and to the iron present in the albumen. The method response is approximately 6 times higher for iron, as can be inferred from Figure 5. The role of copper in the TMB method applied to albumen is probably to develop a detectable absorbance, also in

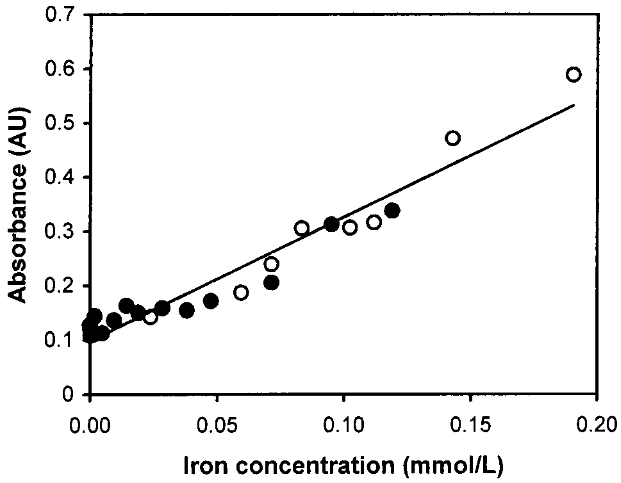

Figure 4. Absorbance at 370 nm of the reaction product between TMB and albumen of fresh eggs (stored 6 days at 5 °C), as a function of iron concentration, following the modified method, but with no copper addition. Open and solid symbols indicate two different egg samples.

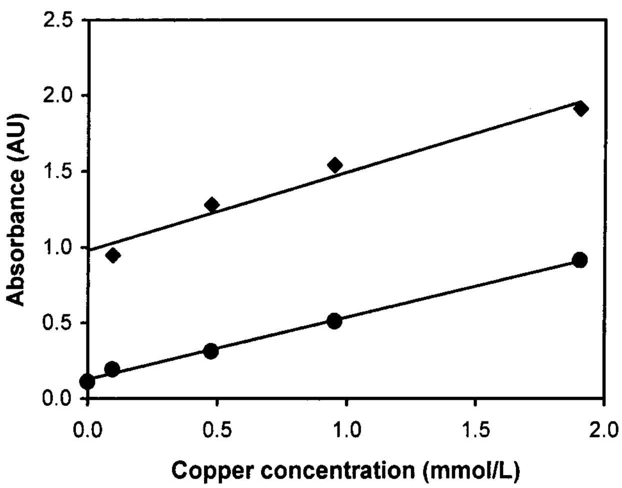

Figure 5. Absorbance at 370 nm of the reaction product between TMB and the model solutions containing two iron levels (◆, 0.4 mmol/L; ●, 0.04 mmol/L), as a function of copper concentration.

the presence of very small iron concentrations, as found in fresh egg albumen. No passage of copper through the vitelline membrane during egg storage should be expected because copper concentration in albumen (3 mg/kg) is higher than that in yolk (1.9 mg/kg) (*16*). On the other hand, the gradient of iron concentration between yolk (57 mg/kg) and albumen (2 mg/kg, *16*) should lead to an increase of iron in the latter. Because the response of the TMB method in the model solutions is much higher for iron than for copper, the trends of Figure 2 can be attributed to the variation of iron level in albumen during egg aging.

CONCLUSIONS

The TMB modified method applied to albumen is a quantitative and simple test for egg freshness evaluation, showing a natural variability of 10.3% CV. This value is lower than those obtained by Rossi et al. (*17*) for the two other rapid methods of Haugh Unit (CV = 12%) and air cell height (CV = 15%), analyzing only three lots of fresh eggs (laid by hens of three different ages but of the same breed). Natural variability is similar to that observed for furosine (CV = 9.7%), measured in the albumen of eggs laid by four different groups of hens (*9*).

Therefore, it is possible to conclude that the TMB modified method is a valid index for the assessment of shell egg freshness and a rapid alternative to the furosine test.

ABBREVIATIONS USED

CV, coefficient of variation; HPLC, high-performance liquid chromatography; LRNMR, low-resolution nuclear magnetic resonance; p, probability; r, correlation coefficient; SD, standard deviation; TMB, 3,3′,5,5′-tetramethyl benzidine.

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