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ARTICLE

Impact of Protein on Darkening in Yellow Alkaline Noodles

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Darkening in yellow alkaline noodles (YAN) was examined over a 24 h period in noodles made from 4 wheat varieties, including varieties with different levels of polyphenol oxidase (PPO) activity, selected to cover a range of protein levels. Noodles were made in the presence and absence of the PPO inhibitor, tropolone. The darkening was divided into two time periods: 0−4 h and 4−24 h. The first four hours was described by a composite rate equation, and this period was subdivided into two stages. The rate of darkening in the first stage was independent of both protein concentration and PPO activity. The amount of darkening (c), however, was highly dependent on protein concentration during this stage (−tropolone, r=0.902; +tropolone, r=0.905), but independent of PPO activity. The first stage darkening was a zero order reaction where additional protein does not increase the reaction rate, but when the protein supply has been depleted, the reaction stops. The rate of darkening during the first stage ($k'_1 = 5.6 \pm 1.0$) was similar to the rate of change in the protein structure ($k'_1 = 6.5 \pm 1.3$) as measured using the amide II band by infrared spectroscopy. This suggested that the first stage of darkening represents changes in light reflectance and absorbance caused by changes in hydrogen bonding rather than changes in covalent bonding. During the second stage of darkening, both the rate (k'_2) and amount of darkening ($\Delta L^*_{4h\text{-}c}$) were significantly correlated with protein concentration (−tropolone, $r = 0.465$; +tropolone, $r = 0.813$), and in the absence of tropolone the amount of darkening was increased by PPO activity. The amount of darkening (ΔL^*_{24h-4h}) during the second time period (4−24 h) (or third stage) was significantly correlated in the presence of tropolone (r=0.375) and in the absence of tropolone (r=0.428) with protein concentration. However, compared with earlier stages the response of non-PPO darkening during the third stage to change in protein concentration was smaller. Protein oxidation, or more specifically oxidation of tyrosine groups within the protein, appears to be the main mechanism involved in non-PPO darkening in YAN during the second and third stages with glutenin being the main reactant. Albumin and globulin are important substrates for PPO. No differences in darkening were detected in YAN made from the four varieties in the presence of tropolone; however, differences in YAN darkening were observed for the second and third stages due to site and year variation.

KEYWORDS: Yellow alkaline noodle; polyphenol oxidase; non-PPO darkening

INTRODUCTION

Yellow alkaline noodles (YAN) are made by treating wheat flour with alkaline salts. They are usually sold as a fresh product. A bright yellow color is highly desirable while dark or gray colors are not. YAN darken over time, and it is this discoloration which gives consumers an indication of the age of the noodle and decreases YAN marketability. Part of the darkening is caused by the enzyme, polyphenol oxidase (PPO), while the remainder is described as non-PPO darkening. It is possible to limit damage caused by PPO darkening by the selection of wheat varieties with low or zero PPO activity (*1*, *2*). However, it is not known whether it is possible to reduce non-PPO darkening by breeding or other means that are acceptable to the consumer. Previous data suggested that proteins are involved in non-PPO darkening (*3*, *4*) and therefore they warrant further investigation.

YAN darkening can be measured by a change in the CieLab L^* value (**Figure 1**). It can be described using two time periods: 0−4 h and 4−24 h (*4*). The choice of these two time periods was based on the sampling times used by previous authors and due to constraints arising from lack of automation (*4*). The 0 to 4 h time period encompasses two stages of darkening (ΔL^*) that can be defined using the rate equation

$$\Delta L^*_{0\rightarrow 4} = a\,e^{-k'_1 t} + k'_2 t + c \quad (1)$$

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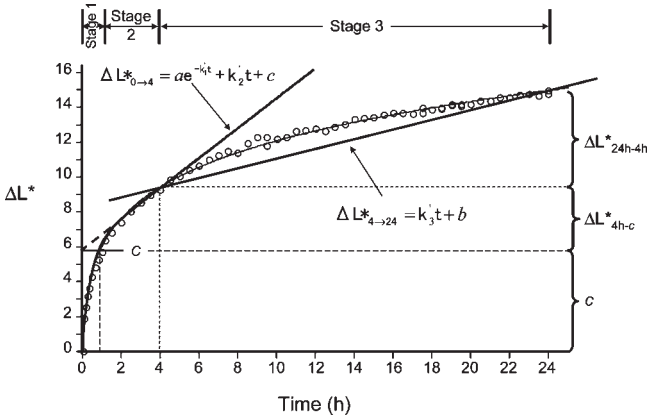

Figure 1. YAN made from 'Tasman' flour indicating the darkening (ΔL^*) over the two time periods, 0−4 h and 4−24 h, with the eqs **1** and **2** fitted. The three stages of darkening and the amount of darkening for each stage are also indicated. (Adapted from ref *4*.)

where t is time (h), k'_1 and k'_2 are rate constants (ΔL^*/h), c is a measure of darkening (ΔL^*) during the first stage and a is a constant (*4*). The amount of darkening during the second stage of darkening is the difference between L^* at 4 h and c (ΔL^*_{4h-c}). The second time period 4−24 h constitutes a third stage of YAN darkening where darkening is considered linear:

$$\Delta L*_{4\rightarrow 24} = k'_3 t + b \quad (2)$$

where k'_3 is the rate constant (ΔL^*/h), t is time (h) and b is constant. The magnitude of this third stage of darkening can be defined as the difference in L^* between 4 h and 24 h (ΔL^*_{24h-4h}).

CieLab L^* value is a function of the diffuse light reflectance (scattering) as well as light absorption. Thus both physical changes and chemical changes can result in a change in the L^* value. It is expected that scattering and absorption are principally a function of the molecules on the noodle surface; however, light may penetrate the surface so the amount of this light reflected and therefore L^* can also be influenced by molecular refraction and dispersion within the noodle. Noodles are in a state that allows for changes both in the distribution of molecules and in the structure of complex molecules such as protein. It is well-known that proteins undergo reorientation in solution due to enhancement of hydrogen bonding both internally (formation of β-sheets) and from adsorption to surfaces (*5*−*7*). Such changes in the orientation of protein molecules and protein structure may lead to a decrease in reflectivity and therefore a decrease in the L^* value. This can be measured by mid-infrared (mid-IR) spectrometry (*6*, *7*).

The objective of this study was to investigate the importance of proteins on YAN darkening. This was performed by comparing the darkening of noodles made from flour derived from four varieties: two wheats with high PPO activity, 'Walkatchem' and 'Janz' (*8*), a wheat with intermediate-PPO activity, 'Krichauff' (*8*), and a wheat with low PPO activity, 'Axe' (*8*), sourced from different sites in South Australia and possessing a range of protein contents. Experimentally, non-PPO and PPO darkening were differentiated by the addition of the PPO inhibitor tropolone which at the concentration used (0.01 mol L^{-1}) totally inhibits PPO in wheat with high PPO activity (*9*, *10*). PPO activity can be determined by the difference between darkening in YAN made with tropolone and darkening in YAN made without tropolone. The mechanism of protein's role in YAN darkening was further investigated by mid-IR spectroscopy and by identifying the protein fractions and possible substrates involved.

Table 1. Range of Protein Concentrations as a Percentage for the Four Varieties Selected

variety	no. of samples	2007	no. of samples	2008
'Axe'	7	11.4−17.6	7	8.6−16.6
'Janz'	6	11.6−18.1	7	8.8−16.6
'Krichauff'	7	12.0−18.9	5	11.2−16.5
'Wyalkatchem'	6	11.1−19.0	7	8.5−17.5

MATERIALS AND METHODS

Chemicals. Unless otherwise stated all chemicals were obtained from (Sigma-Aldrich, St. Louis, MO).

Wheat Varieties and Selection. YAN were made from flour samples of four varieties of wheat (*Triticum aestivum* L.): 'Axe' (low PPO activity; RAC875//Kukri/Excalibar), 'Krichauff' (intermediate PPO activity; Wariquan/Kloka/Pitic63/3/Warimek/Halberd/4/3Ag3 Aroona), 'Janz' (high PPO activity; 3Ag3/4*Condor//Cook) and 'Wyalkatchem' (high PPO activity; Machete/W84.129*504), sourced over two seasons, 2007 and 2008, from 7 sites selected each year from a possible 26 sites in South Australia to give a broad range of protein content (**Table 1**). Wyalkatchem and Krichauff are classified as Australian Premium White, and Janz and Axe as Australian Hard. The levels of PPO activity were according to Mares (*8*). Wheat was obtained from the National Variety Trials (Australian Crop Accreditation System (ACAS) Limited, Australia), which conduct trials representing the breadth of the wheat production area in Australia including a range of soil types and climatic conditions. The protein contents were determined by the National Variety Trials using near-infrared spectroscopy.

For standards, 'Kamilaroi' (*T. durum* Desf. cv. Kamilaroi), a durum wheat cultivar with near-zero PPO activity (*4*), and 'Tasman' (*T. aestivum* L. cv. Tasman), a bread wheat with high PPO activity (*4*), sourced from field trials conducted by the University of Adelaide, were used. The protein levels for 'Kamilaroi' and 'Tasman' were 14.2% and 10.5%, respectively.

Flour Milling and Noodle Sheet Preparation. Noodles were prepared by standard methods (*2*, *4*). Grain was conditioned overnight to 15% moisture and milled on a Quadrumat Junior mill (Brabender GMBH & Co. KG, Duisburg, Germany). The pollard stream was resieved on a shaker and recovered flour added to the combined streams from the mill. Flour, 10 g, and 3.6 mL of 2% sodium carbonate solution were mixed into dough in a cylindrical mixing bowl using a drill press with a modified mixing paddle. The dough was mixed for 105 s with intermittent pauses to clean dough adhering to the paddle. The dough was rolled into a ball and made into a noodle sheet using a domestic pasta maker (Atlas 150, Marcato S.p.A., Campodarego, Italy). The noodle sheet was then placed in a resealable plastic bag to prevent drying and make noodle handling easier. CIE-Lab color measurements were made using a Dataflash 100 (Datacolor International, Lawrenceville, NJ) reflectance spectrophotometer. The raw noodles were made and stored at room temperature (22 ± 1 °C). Noodle L^* values were measured at 2, 5, 10, 15, 20, 30, 40, 50 min and at 1, 1.5, 2, 2.5, 3, 3.5, 4, and 24 h. The data for the first 4 h was fitted with eq **1** and the data from 4 h and 24 h fitted with eq **2** using WinCurveFit (*11*). To inhibit PPO activity, tropolone was added at a concentration of 0.01 mol L^{-1} in the bicarbonate solution (i.e., 4.4 mg/10 g noodle) (*8*, *9*).

Statistical Analyses. The graphs' standard errors and correlation coefficients were estimated using WinCurvefit (*11*). Correlation table and Tukey−Kramer means comparisons were performed using JMP Version 5.01 (*12*).

Protein Fractionation and Assay. The four varieties obtained from the NVT trials from all the sites and both years were assayed for protein content. The flours were separated using a modified Osborne (*13*) fractionation to obtain 5 fractions. The first fraction (albumin fraction) was obtained by extracting flour (20 mg) with 1 mL of water incubated for 30 min at 60 °C with intermittent mixing. The samples were centrifuged for 10 min at 13 250*g*. The extraction was repeated with 0.1 mol L^{-1} sodium chloride (globulin fraction), 15% ethanol (gliadin fraction), 50% propanol (gliadin/unpolymerized glutenin fraction) and 50% propanol/0.1% sodium dodecyl sulfate/0.02% dithiothreitol (polymerized glutenin fraction). The supernatant (10 μL) was pipetted into the microplate well and 200 μL of Bradford reagent (Bio-Rad Protein Assay; Bio-Rad Laboratories Inc.,

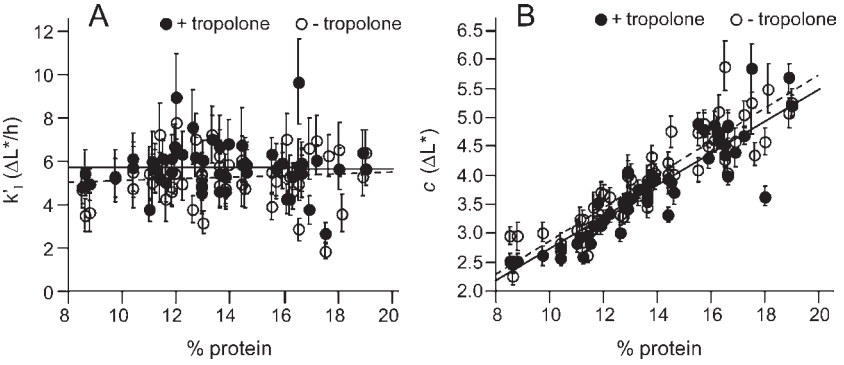

Figure 2. Relationship between (**A**) stage 1 kinetics (k'_1) and protein concentration and (**B**) between stage 1 darkening (c) and protein concentration in the presence and absence of tropolone. Each point represents the average of four measurements of YAN made from the four wheat varieties sourced from the NVT Trials for a single site for a single year. The lines fitted in **B** were $y = 0.286(\pm 0.003)x$ ($r = 0.902$) in the absence of tropolone (dashed line) and $y = 0.274(\pm 0.004)x$ ($r = 0.906$) in the presence of tropolone (solid line).

Hercules, CA) added. The plates were incubated at room temperature for 5 min, and the absorbance was measured at 595 nm.

Effect of Protein Fractions on Darkening. To identify the active component for non-PPO darkening, different protein fractions were added to YAN. 'Kamilaroi' flour was separated using the modified Osborne (*13*) fractionation. Approximately 100 g of flour was extracted with 750 mL of water at 60 °C for 30 min, yielding the albumin fraction. This was centrifuged at 1500*g* and the flour re-extracted with 500 mL of 0.15 mol L^{-1} NaCl (final concentration, 0.1 mol L^{-1} NaCl) at 60 °C for 30 min giving the globulin fraction. This was centrifuged again and extracted at 60 °C for 30 min with 500 mL of 22.5% ethanol (final concentration, 15% ethanol) yielding the gliadin fraction. Finally 500 mL of 75% propan-1-ol (final concentration, 50% propanol) was added, re-extracted again at 60 °C for 30 min and then centrifuged with the supernatant providing the gliadin/unpolymerized gluteinin fraction. The albumin and globulin fractions were lyophilized while the gliadin and gliadin/unpolymerized gluteinin fractions were dried by rotary evaporation at 45 °C and reduced pressure. Propan-1-ol was added to the gliadin fraction to prevent foaming and to form an azeotrope with water to aid evaporation. The yields for the albumin, globulin, gliadin and gliadin/unpolymerized glutenin fractions were 6.7 g, 5.0 g, 2.9 g and 3.6 g, respectively.

To determine which fractions are important for YAN darkening, noodles were made using 0.5 g of each dried extract added to 9.5 g of 'Tasman' flour and rates determined in the presence and absence of tropolone. The extracted flour, which contains the nonextractable protein, was compared with the 'Kamilaroi' control. SDS−PAGE was conducted using the 15% ethanol and 50% propanol extracts to analyze the proteins present.

Substrates/Reactants and Transition Metals for YAN Darkening. Amino acid and peptide substrates/reactants were added to noodle to examine their possible role in YAN darkening. YAN were made using Tasman flour with or without 0.01 mol L^{-1} tropolone (*9*, *10*). The substrates/reactants, tyrosine, peptide (GQQGYYPTS; GenScript Corp., Piscataway, NJ) and tryptophan, were added at a rate of 0.001 mol L^{-1}, 0.005 mol L^{-1} and 0.01 mol L^{-1}, respectively. Tyrosine and tryptophan were selected as aromatic amino acids likely to contribute to non-PPO darkening, and the peptide was chosen because of its similarity to peptide fragments found in glutenins and to the consensus peptide GYYPTSPQQ; however, the tyrosine groups are located more centrally in the peptide. Tyrosine and peptide were added at a rate to give a similar amount of darkening in YAN made from Tasman flour with tropolone added. Ferrous sulfate and manganese sulfate were added at the rate of 0.001 mol L^{-1} to noodles made from 'Kamilaroi' to observe their impact on non-PPO darkening.

Mid-Infrared (Mid-IR) Spectroscopy. Proteins are composed of amino acids linked by amide bonds which are also known as peptide bonds. The mid-IR frequencies were used to examine changes in the protein quaternary structure by measuring the amide I band (1580−1720 cm^{-1}; 80% C═O stretch, 10% C−N bend and 10% N−H bend) and amide II (1480−1580 cm^{-1}; 60% N−H bend and 40% C−N stretch) (*6*, *7*). The amide III band (1430−1480 cm^{-1}) was small and variable. The spectrum was obtained using a Perkin-Elmer (Waltham, MA) Spectrum One FT-IR spectrometer equipped with a Pike Technologies (Madison, WI) diffuse reflectance auto sampler. The data was transferred to GRAMS/32 (*14*) software, and the areas of the amide bands were determined manually.

Protein Electrophoresis. SDS−PAGE was used to determine the composition of the protein fractions that were added to YAN. Approximately 20 mg of the dried 15% ethanol and 50% propanol extracts were resolubilized at 60 °C for 120 min with intermittent vortexing in 200 μL of 50% (v/v) propan-1-ol:80 mmol L^{-1} Tris-HCl (pH 8.0) containing 1% (w/v) dithiothreitol (DTT). This was followed by the addition of 200 μL of sample buffer (0.02% bromophenol blue; 80 mmol L^{-1} Tris-HCl (pH 8.0); 69 mmol L^{-1} SDS) and heated a further 15 min at 60 °C. Samples were stored at −20 °C prior to electrophoresis. SDS−PAGE was conducted according to the method of Singh et al. (*15*) with minor modifications, using a SE600 vertical electrophoresis unit (Hoefer, San Francisco, CA), with 16 × 18 cm glass plates separated by a 1 mm spacer. The discontinuous polyacrylamide gel system of Singh et al. (*15*) was modified to employ a 3% stacking gel and a 8−12% gradient acrylamide separating gel with 1.5% cross-linker concentration (bisacrylamide:acrylamide). Gels were loaded with 10 μL of sample, and electrophoresis was carried out at approximately 10 °C and 40 mA/gel for approximately 3.5 h. Gels were stained overnight in Coomassie brilliant blue R-250 (*16*) and destained in water for 3 h, aided by the addition of Kimwipe tissues (Kimberley-Clark Australia Pty. Ltd., Milsons Point, NSW, Australia).

RESULTS AND DISCUSSION

First Stage YAN Darkening. The rate of darkening (k'_1) and the amount of darkening (c) during the first stage of YAN darkening were independent. There was no correlation between k'_1 and protein concentration (**Figure 2A**). By contrast, the amount of darkening that occurs during the first stage (c) is significantly correlated with protein concentration with regression lines fitted through the origin (control, $r = 0.902$; tropolone, $r = 0.906$; **Figure 2B**). This agrees with eq **1** that describes a zero order reaction where additional protein (reactant) does not increase the reaction rate but when the protein (limiting reactant) is exhausted, the reaction stops. The addition of tropolone had no significant effect on the amount of darkening (c), and therefore PPO activity was not important during the first stage of rapid darkening. These results strongly indicate that it is possible to predict the early amount of non-PPO darkening (c) from the protein concentration.

Change in Refractivity/Reflectivity as a Mechanism for Early YAN Darkening. A process that occurs at a similar rate to the first stage of non-PPO darkening (k'_1) is the reorientation of protein in solution caused by enhanced hydrogen bonding, both internally and to surfaces (*5*, *7*). These changes in protein structure may lead to a decrease in the L^* value by altering the reflectivity of visible

light at the noodle surface. Generally the amide I band has been used to study protein secondary structure by mid-IR spectrometry (*7*); however, water makes a large spectral contribution to the amide I band (*7*) and therefore shows a lack of sensitivity in matrices with the presence of high concentrations of water as in YAN. In YAN it was found that the amide II band was more responsive than the amide I band to changes during stage 1 noodle darkening. Measurement of changes in the amide II band by mid-IR confirmed that this process occurs at a virtually identical rate as the early rapid darkening (**Table 2**, **Figure 3**). After this early rapid increase in the area of the amide II band there is little further change in the area of the amide II band and no correlation between stage 2 darkening and the amide II band area. This is consistent with the suggestion that protein reorientation determines the amount of stage 1 darkening (*c*).

Second Stage YAN Darkening. During the second stage the rate of darkening k'_2 and the amount of darkening (ΔL^*_{4h-c}) in both the presence and absence of the PPO inhibitor, tropolone, are significantly correlated with the protein concentration (k'_2 tropolone, $r = 0.796$; control, $r = 0.446$; ΔL^*_{4h-c} tropolone, $r = 0.814$; control, $r = 0.435$; **Figure 4**). The rate (k'_2) and amount of darkening (ΔL^*_{4h-c}) during the second stage of darkening are highly correlated (tropolone, $r = 0.980$; control, $r = 0.992$), which is consistent with the observation that k'_2 is first order with respect to darkening. In the absence of tropolone, an increase in the amount of darkening was related to the PPO activity.

Table 2. Rates of Noodle Darkening (ΔL^*) Compared with the Rates of Change in Amide II Band Area in Noodles Made from 'Tasman' Flour in the Presence and Absence of Tropolone during Stages 1 (k'_1) and 2 (k'_2) Estimated Using Eq 2

	k'_1		k'_2	
treatment	ΔL^*	amide II area	ΔL^*	amide II area
−tropolone	3.53 ± 0.77	3.90 ± 0.80	1.18 ± 0.08	0.06 ± 0.03
+tropolone	4.07 ± 0.82	6.32 ± 1.40	0.61 ± 0.05	0.15 ± 0.45

Third Stage YAN Darkening. Protein concentration had a significant effect ($p = 0.05$) on the amount darkening (ΔL^*_{24h-4h}) in the presence ($r = 0.376$) and absence ($r = 0.413$) of tropolone during stage 3 darkening (**Figure 5**). Protein only describes a small amount of variation in darkening, and furthermore in the presence of tropolone there is only a small increase in the darkening with increasing protein, suggesting that selection of varieties for low non-PPO darkening is best performed using stage 3 darkening in the presence of tropolone. In the absence of tropolone, PPO activity explains the increase in the amount of darkening.

Modified Osborne (*13*) Protein Fractions and YAN Darkening. In the absence of tropolone there was no correlation between the YAN darkening at either stage 2 or 3 and the protein concentration as determined by the Bradford reagent of the protein fractions obtained by the modified Osborne method (*13*) (**Table 3**). In noodles made with tropolone, the gliadin, gliadin/unpolymerized glutenin and polymerized glutenin fractions were correlated with darkening during stage 2 but not darkening during stage 3 (**Table 2**), suggesting one or more of their fractions

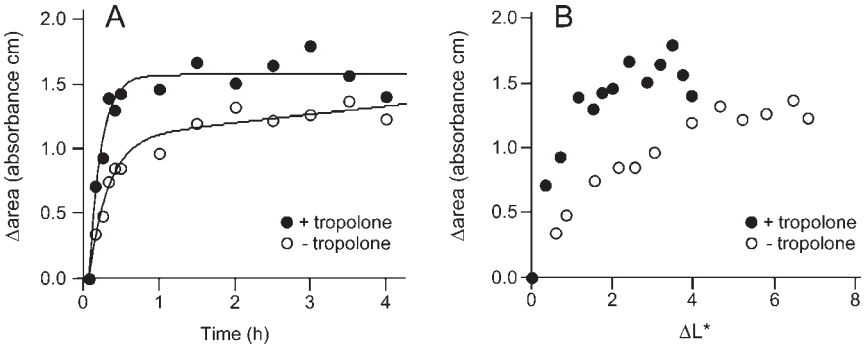

Figure 3. Changes in protein quaternary structure measured by changes in the area of the amide II band. (**A**) Change in the area of the amide II band with time. Each point represents the average of two measurements. (**B**) Correlation between the area of the amide II band and the darkening in 'Tasman' with and without tropolone.

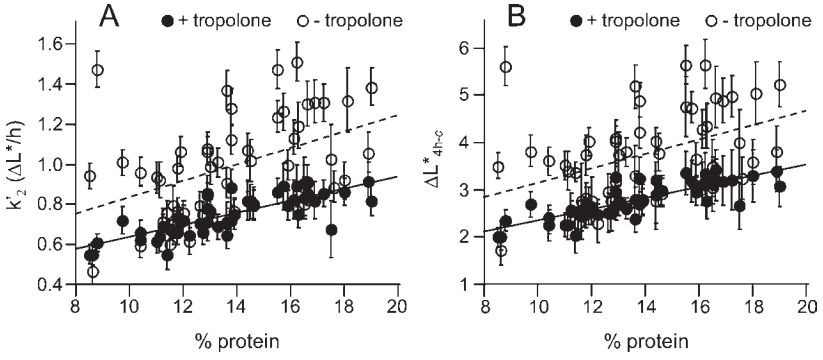

Figure 4. Relationship between (**A**) stage 2 kinetics (k'_2) and protein concentration (%) and (**B**) between stage 2 darkening (ΔL^*_{4h-c}) and protein concentration (%) in the presence of tropolone and absence of tropolone. The lines fitted were with tropolone (solid line), $y = 0.03(\pm 0.01)x + 0.33(\pm 0.05)$, $r = 0.796$, and without tropolone (dashed line), $y = 0.04(\pm 0.01)x + 0.43(\pm 0.16)$, $r = 0.446$. (**B**) Correlation between stage 2 darkening (ΔL^*_{4h-c}) and % protein with tropolone and without tropolone. The lines fitted were with tropolone (solid line), $y = 0.12(\pm 0.01)x + 1.17(\pm 0.17)$, $r = 0.813$, and without tropolone (dashed line), $y = 0.15(\pm 0.04)x + 1.62(\pm 0.63)$, $r = 0.435$. Each point represents the average of four measurements of YAN made from the four wheat varieties sourced from the NVT Trials for a single site for a single year.

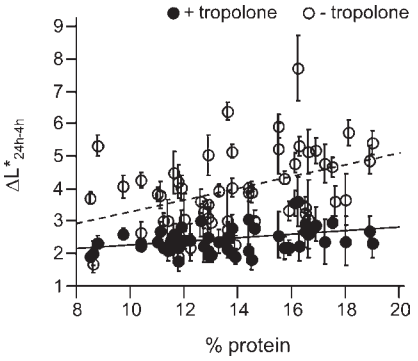

Figure 5. Relationship between stage 3 darkening (ΔL^*_{24h-4h}) and % protein with and without tropolone. Each point represents the average of four measurements of YAN made from the four wheat varieties sourced from the NVT Trials for a single site for a single year. The lines fitted were $y = 0.06(\pm 0.02)x + 1.67(\pm 0.28)$, $r = 0.376$, with tropolone (solid line) and $y = 0.18(\pm 0.06)x + 1.49(\pm 0.78)$, $r = 0.413$, without tropolone (dashed line).

Table 3. Correlation (r) between YAN Darkening (ΔL^*) over 24 h in the Presence and Absence of Tropolone for the Stage 2 and Stage 3 Darkening and the Concentration of Protein Determined by Bradford Reagent in Different Fractions Obtained Using the Modified Osborne Method (*13*)[a]

treatment	stage	albumin	globulin	gliadin	gli/UP glu[b]	P glu[c]
−tropolone	2	−0.041	0.045	0.129	0.248	0.223
	3	−0.049	−0.097	0.020	0.212	0.159
+tropolone	2	0.214	0.245	0.646a[d]	0.551a	0.541a
	3	0.167	−0.237	0.064	0.137	0.103
PPO	2	−0.142	−0.060	−0.148	0.026	0.003
	3	−0.118	−0.017	−0.003	0.183	0.137

[a] The four varieties 'Axe', 'Krichauff', 'Janz' and 'Wyalkatchem' obtained from the 14 NVT trial sites for 2007 and 2008 were used. [b] gli/UP glu = gliadin/unpolymerized glutenin. [c] P glu = polymerized glutenin. [d] a, significant at the 0.001 level.

are involved in non-PPO darkening during stage 2 darkening. There was no correlation between PPO darkening and any protein fractions, and therefore it was not possible to identify a protein fraction responsible for PPO darkening from these data.

When individual fractions were isolated and added to noodles made from 'Tasman' flour, there was only non-PPO darkening and no PPO darkening during the first stage of darkening (**Table 4**). The difference in the amount of stage 1 darkening compared with the control with no protein supplement ($\Delta L^*_{c(\text{treatment-control})}$) reflected the amount of protein added to each fraction. Using the combined second and third stage darkening, ΔL^*_{24h-c}, the protein fractions with the greatest PPO activity were the albumin and globulin fractions while the non-PPO activity was associated with the gliadin/unpolymerized glutenin fraction (**Table 4**). If the two stages are considered separately, then it appears that more PPO darkening occurs during stage 2 than stage 3 and PPO oxidized substrates in the albumin fraction more rapidly than substrates in the globulin fraction. The albumin fraction contains small molecular weight material, such as peptides, in addition to protein, and therefore these may be oxidized faster than substrates in the globulin fraction. Non-PPO darkening was more complex for stages 2 and 3, with the albumin and gliadin fractions showing both negative and positive values, and the globulin fraction had negative values. Only the gliadin/unpolymerized glutenin fraction had positive values for both stages of non-PPO darkening. The extracted 'Kamilaroi' flour precipitate still showed darkening during all three stages indicating not all protein was extracted.

Oxidation of Protein as a Possible Mechanism for YAN Darkening. The close association between protein content during the middle stage of non-PPO darkening suggests that a possible mechanism for darkening is the oxidation of aromatic amino acids, such as tyrosine and tryptophan, within the protein. It is well-known that transition metals enhance oxidation. Addition of 0.001 mol L^{-1} transition metal ions such as iron and manganese to 'Kamilaroi', wheat with negligible PPO activity (*4*), has no effect on darkening during stage 1 but enhances darkening during both stages 2 and 3, although more in stage 2 than 3, which is consistent with a possible oxidation mechanism for non-PPO darkening (**Table 5**).

Non-PPO activity is associated with the 50% propanol fraction (**Table 4**). These extracts contained a high concentration of gliadins and unpolymerized glutenins. Glutenins and gliadins are a possible source of oxidizable tyrosine as they contain the consensus peptide GYYPTSPQQ. When tyrosine and the tyrosine containing peptide, GQQGYYPTS (where Y = tyrosine), were added to noodles, they acted as substrates for PPO and reactants for non-PPO darkening (**Table 6**). Tryptophan is a very poor reactant and is unlikely to be a major contributor to YAN darkening. Tyrosine is the more effective substrate for both PPO and non-PPO because it gives a greater difference in L^* between the treatment and the control ($\Delta L^*_{24h\text{-control}}$) per mol L^{-1} added. These reactants/substrates contribute more to darkening during stages 2 and 3 than stage 1, indicating the protein oxidation is slow compared with the initial darkening reaction.

The oxidation of wheat protein tyrosine may lead to the formation of dityrosine or isodityrosine (*17*). It is also conceivable that oxidation of the GYY or GYYP portion of a protein yields a chromophore analogous to green fluorescent protein (*18*). Alternatively, hydroxyl radicals that form as a result of oxidation can lead to the formation of dihydroxyphenylalanine (*19*). However, to obtain the melanin pigment associated with YAN darkening, it is still necessary for subsequent polymerization of one, some, or all of these chromophores. The lack of pH sensitivity for non-PPO darkening, observed previously, suggests the mechanism for oxidation and/or polymerization involves free radicals (*4*), and there is no evidence for enzyme involvement (*4*). During the third stage, the rate limiting step may involve the formation/diffusion of a suitable oxidant. Diffusion of an oxidant would follow an Arrhenius type mechanism as was observed previously for stage 3 (*4*). One possibility is that milling damage exposes the tyrosine groups or alternatively tyrosine groups become exposed during the protein reconformation that occurs in the first stage of darkening. The observed changes in rate over time may reflect different degrees of exposure/sensitivity of tyrosine groups, and eventually diffusion of an oxidant becomes rate limiting.

Varietal, Site and Year Differences. There are two possible measurements that can be considered when studying and selecting varieties with low non-PPO darkening, namely, stage 2 darkening (ΔL^*_{4h-c}) and stage 3 darkening (ΔL^*_{24h-4h}). When the varieties were compared, there were no significant differences between varieties for non-PPO darkening (**Table 7**). There were significant differences between YAN darkening in varieties made without tropolone and therefore PPO darkening. The differences in PPO darkening indicate that PPO activity is greatest in 'Janz' > 'Wyalkatchem' > 'Krichauff' > 'Axe'. While actual levels of PPO activity varied between sites, the ranking remained the same.

There were no significant site differences ($p = 0.05$) observed in the darkening of the noodles made without tropolone, but there were significant differences in sites observed in the darkening of noodles with tropolone added (non-PPO darkening) for stages 2 and 3 (**Table 8**). Stage 2 non-PPO darkening was significantly correlated at the 5% level with the protein concentration

Table 4. Comparison of the Darkening of YAN Made from 'Tasman' (High PPO Activity) Flour Made with Different Protein Extracts from 'Kamilaroi' (Zero PPO Activity) Flour in the Presence and Absence of Tropolone Where c Represents the Darkening during the First Stage, ΔL^*_{4h-c} the Darkening during the Second Stage and ΔL^*_{24h-4h} the Darkening during the Third Stage and ΔL^*_{24h-c} Is the Amount of Darkening during the Stages 2 and 3 Combined

treatment	c	$\Delta L^*_{c\ (\text{treatment-control})}$	ΔL^*_{4h-c}	$\Delta L^*_{4h-c\ (\text{treatment-control})}$	ΔL^*_{24h-4h}	$\Delta L^*_{24h-4h\ (\text{treatment-control})}$	$\Delta L^*_{24h-c\ (\text{treatment-control})}$
				'Tasman'			
				−Tropolone			
none	3.49 ± 0.19	0	5.53 ± 0.78	0	5.18 ± 0.87	0	0
albumin	4.06 ± 0.28	0.57	5.72 ± 0.42	0.19	6.00 ± 0.32	0.82	1.01
globulin	4.32 ± 0.24	0.83	6.03 ± 0.33	0.50	5.75 ± 0.16	0.57	1.07
gli/UP glu[a]	3.99 0.32	0.50	5.39 ± 0.40	−0.14	5.17 ± 0.16	−0.01	−0.15
P glu[b]	4.04 ± 0.23	0.55	5.49 ± 0.17	−0.04	5.91 ± 0.56	0.73	0.69
				+Tropolone			
none	3.24 ± 0.14	0	2.60 ± 0.26	0	2.29 ± 0.21	0	0
albumin	4.02 ± 0.25	0.78	2.09 ± 0.36	−0.51	2.76 ± 0.64	0.47	−0.04
globulin	3.86 ± 0.23	0.62	2.25 ± 0.34	−0.35	2.19 ± 0.20	−0.20	−0.45
gli/UP glu	3.64 ± 0.14	0.40	2.84 ± 0.46	0.24	1.71 ± 0.42	−0.58	−0.34
P glu	3.76 ± 0.20	0.52	2.94 ± 0.25	0.34	2.74 ± 0.14	0.45	0.79
				PPO			
none	0.25 ± 0.33	0	2.93 ± 1.04	0	2.89 ± 1.08	0	0
albumin	0.04 ± 0.53	−0.19	3.63 ± 0.78	0.70	3.24 ± 0.96	0.35	1.05
globulin	0.46 ± 0.47	0.21	3.78 ± 0.67	0.85	3.56 ± 0.36	0.67	1.52
gli/UP glu	0.35 ± 0.46	0.10	2.55 ± 0.86	−0.38	3.46 ± 0.58	0.57	0.19
P glu	0.28 ± 0.43	0.03	2.55 ± 0.42	−0.38	3.17 ± 0.70	0.28	−0.10
				'Kamilaroi'			
none	1.09 ± 0.15	0	2.24 ± 0.40	0	2.97 ± 0.60	0	0
extracted flour	1.67 ± 0.89	0.58	1.72 ± 0.36	−0.52	1.14 ± 0.29	−1.83	−2.41

[a] gli/UP glu = gliadin/unpolymerized glutenin. [b] P glu = polymerized glutenin.

Table 5. Rates of Amount Darkening (ΔL^*) in Noodles Made from 'Kamilaroi' Flour with 0.001 mol L^{-1} Transition Metal Sulfate Added Compared with the Control Where c Represents the Darkening during the First Stage, ΔL^*_{4h-c} the Darkening during the Second Stage and ΔL^*_{24h-4h} the Darkening during the Third Stage

treatment	c	$\Delta L^*_{c\ (\text{treatment-control})}$	ΔL^*_{4h-c}	$\Delta L^*_{4h-c\ (\text{treatment-control})}$	ΔL^*_{24h-4h}	$\Delta L^*_{24h-4h\ (\text{treatment-control})}$
control	2.65 ± 0.12	0	2.05 ± 0.31		2.90 ± 0.38	0
$MnSO_4$	2.65 ± 0.24	0	2.86 ± 0.56	0.81	3.26 ± 0.19	0.36
$FeSO_4$	2.69 ± 0.15	0.04	3.27 ± 0.97	1.22	3.25 ± 0.19	0.35

Table 6. Comparison of the Darkening of YAN Made from 'Tasman' Flour and with Different Substrates in the Presence and Absence of Tropolone Where c Represents the Darkening during the First Stage, ΔL^*_{4h-c} the Darkening during the Second Stage and ΔL^*_{24h-4h} the Darkening during the Third Stage[a]

treatment	c (ΔL^*)	ΔL^*_{4h-c}	ΔL^*_{24h-4h}	$\Delta L^*_{24h\text{-control}}$	$\Delta L^*_{24h\text{-control}}$/mol L^{-1} substrate added
			−Tropolone		
control	3.49 ± 0.19	5.53 ± 0.78	5.18 ± 0.87	0	
peptide	2.50 ± 0.10	9.81 ± 0.98	20.29 ± 1.73	18.40	3680
tyrosine	6.21 ± 0.85	8.52 ± 1.25	8.95 ± 0.91	9.58	9580
tryptophan	3.59 ± 0.23	4.20 ± 0.33	5.48 ± 0.29	−0.83	−83
			+Tropolone		
control	3.46 ± 0.15	2.77 ± 0.23	2.71 ± 0.15	0	
peptide	3.17 ± 0.14	3.16 ± 0.32	3.97 ± 0.37	1.36	272
tyrosine	3.80 ± 0.21	3.11 ± 0.31	3.15 ± 0.23	1.12	1120
tryptophan	3.64 ± 0.17	2.65 ± 0.43	2.77 ± 0.51	0.12	12

[a] The substrates tyrosine, peptide (GQQGYYPTS) and tryptophan were added at a rate of 0.001 mol L^{-1}, 0.005 mol L^{-1} and 0.01 mol L^{-1}, respectively.

(r = 0.951); however, stage 3 non-PPO darkening was not significantly correlated at the 5% level with protein concentration (r = 0.415). Variation during stage 3 darkening observed in both years may be due to environmental variation in protein composition due to temperature (*20*, *21*) and nutrient levels (*21*−*24*).

There were significant differences (p = 0.05) between the years for stage 3 darkening in noodles made with and without tropolone (**Table 9**). There was greater stage 3 non-PPO darkening in the year with high protein levels suggesting a strong seasonal link between non-PPO darkening and protein.

Table 7. Variety Mean Comparisons of YAN Made from the Four Wheat Varieties Sourced from the NVT Trials Where ΔL^*_{4h-c} Represents the Darkening during the Second Stage and ΔL^*_{24h-4h} the Darkening during the Third Stage[a]

		−tropolone		+tropolone		PPO	
variety	protein	ΔL^*_{4h-c}	ΔL^*_{24h-4h}	ΔL^*_{4h-c}	ΔL^*_{24h-4h}	ΔL^*_{4h-c}	ΔL^*_{24h-4h}
				2007[b]			
Krichauff	15.0 A	3.60 BC	4.01 BC	2.92 A	2.80 A	0.68 BC	1.21 BC
Janz	14.9 A	4.88 A	5.83 A	2.95 A	2.67 A	1.93 A	3.16 A
Wyalkatchem	14.4 A	4.41 AB	4.76 AB	2.90 A	2.68 A	1.51 AB	2.09 AB
Axe	14.1 A	3.00 C	3. 07 C	2.68 A	2.50 A	0.28 C	0.57 C
				2008[b]			
Krichauff	14.2 A	3.14 BC	3.36 BC	3.02 A	2.26 A	0.12 B	1.10 B
Janz	13.1 A	4.26 A	4.44 A	2.76 A	2.24 A	1.50 A	2.20 A
Wyalkatchem	12.7 A	3.98 AB	4.03 AB	2.60 A	2.29 A	1.38 A	1.74 AB
Axe	12.3 A	2.58 C	2.40 C	2.61 A	2.22 A	−0.03 B	0.18 C

[a] 'Janz' and 'Wyalkatchem' are wheat varieties with high PPO activity, 'Krichauff' has intermediate PPO activity and 'Axe' has low PPO activity. Different letters indicate significance at the 0.05 probability level. [b] Year.

Table 8. Site Mean Comparisons of YAN Made from the Four Wheat Varieties Sourced from the NVT Trials Where ΔL^*_{4h-c} Represents the Darkening during the Second Stage and ΔL^*_{24h-4h} the Darkening during the Third Stage[a]

		−tropolone		+tropolone		PPO	
site	protein	ΔL^*_{4h-c}	ΔL^*_{24h-4h}	ΔL^*_{4h-c}	ΔL^*_{24h-4h}	ΔL^*_{4h-c}	ΔL^*_{24h-4h}
				2007[b]			
Kimba	18.4 A	4.33 A	4.91 A	3.24 A	2.53 AB	1.09 A	2.38 A
Wanbi	17.2 A	4.13 A	4.21 A	3.23 A	2.65 AB	0.90 A	1.56 A
Streaky Bay	15.6 B	4.29 A	5.17 A	3.14 AB	3.33 A	1.15 A	1.84 A
Rudall	14.3 BC	4.46 A	4.54 A	3.07 ABC	2.73 AB	1.39 A	1.81 A
Spalding	13.1 CD	3.95 A	4.67 A	2.62 BCD	2.33 B	1.33 A	2.34 A
Geranium	12.1 DE	3.09 A	3.29 A	2.56 CD	2.76 AB	0.53 A	0.53 A
Paskeville	11.5 E	3.08 A	3.60 A	2.36 D	2.46 B	0.72 A	1.14 A
				2008[b]			
Warramboo	16.8 A	3.97 A	4.08 A	3.11 A	2.79 A	0.86 A	1.29 A
Pinaroo	15.5 B	3.88 A	3.81 A	2.92 AB	2.13 B	0.96 A	1.68 A
Nangari	14.1 C	3.38 A	3.64 A	2.92 AB	2.11 B	0.46 A	1.53 A
Wanbi	12.7 D	3.47 A	2.89 A	2.80 ABC	1.95 B	0.67 A	0.94 A
Paskeville	11.4 DE	3.03 A	3.39 A	2.53 ABC	2.29 B	0.50 A	1.10 A
Rudall	10.2 EF	3.23 A	3.67 A	2.46 ABC	2.40 AB	0.77 A	1.27 A
Cummins	8.6 F	3.62 A	3.57 A	2.13 C	2.10 B	1.49 A	1.47 A

[a] Different letters indicate significance at the 0.05 probability level. [b] Year.

Table 9. Year Mean Comparisons of YAN Made from the Four Wheat Varieties Sourced from the NVT Trials Where ΔL^*_{4h-c} Represents the Darkening during the Second Stage and ΔL^*_{24h-4h} the Darkening during the Third Stage[a]

		−tropolone		+tropolone		PPO	
year	protein	ΔL^*_{4h-c}	ΔL^*_{24h-4h}	ΔL^*_{4h-c}	ΔL^*_{24h-4h}	ΔL^*_{4h-c}	ΔL^*_{24h-4h}
2007	14.6 A	3.92 A	4.35 A	2.86 A	2.67 A	1.04 A	1.67 A
2008	13.0 B	3.52 A	3.57 B	2.73 A	2.25 B	0.79 A	1.32 A

[a] Different letters indicate significance at the 0.05 probability level.

These data indicate that while PPO activity is independent of protein concentration, non-PPO darkening is not. Although it should be possible to select varieties with low non-PPO darkening by using stage 3 darkening in YAN made with tropolone, the site and year comparisons suggest this is difficult due to the confounding effects of protein concentration and composition. The four wheat varieties were selected as representative of different genetic backgrounds in the Australian breeding germplasm and therefore likely to show variation in non-PPO darkening if such variation exists. No differences were observed; however, it would be worthwhile to extend the scope of the genetic survey to include a much broader range of genotypes.

Darkening and YAN. These data confirm that the use of varieties with low PPO will reduce YAN darkening. Furthermore, in order to minimize non-PPO darkening the only currently available option would appear to be use of flour with the minimum acceptable protein levels. The initial darkening (stage 1) appears to be caused by changes in hydrogen bonding patterns leading changes in structure of proteins rather than changes in covalent bonding, and little can be done to reduce this darkening other than reduce the protein levels or change the protein

structure by using treatments such as cooking. The later stages (stages 2 and 3) of non-PPO darkening appear to be the result of protein oxidation. A greater understanding of the mechanism(s) of protein oxidation will help develop alternative methods for reducing YAN darkening.

While protein concentration has little effect on PPO darkening, the albumin and globulin fractions contain substrates for PPO darkening. The lack of activity in the gliadin fraction compared to the activity observed for the gliadin/unpolymerized glutenin fraction suggests the low molecular weight glutenins and possibly the high molecular weight glutenins are important substrates/reactants for non-PPO darkening.

The selection of varieties with low non-PPO darkening should be made using stage 3 darkening (ΔL^*_{24h-4h}) in noodles made with tropolone sourcing the wheat from a single site during a single year as a selection criterion. Site and year comparisons are difficult due to the effects of protein concentration and composition.

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