Structural Changes of Sinapic Acid During Alkali-Induced Air Oxidation and the Development of Colored Substances

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ABSTRACT: Structural changes of sinapic acid were induced by air oxidation in aqueous solutions at pH 7–10 and followed by spectral and high-performance liquid chromatographic (HPLC) analysis. Color properties of the sinapic acid solutions were determined by taking the transmittance spectra, calculating the Commission Internationale de l'Eclairage (CIE) 1931 tristimulus values, and converting to Hunter L a b values. Reaction rate constants for sinapic acid were determined by a kinetic study based on the quantitative results from HPLC analysis. These reactions were first order with respect to sinapic acid and fit the appropriate equation with a coefficient of $R^2 > 0.97$. Sinapic acid was converted to thomasidioic acid with reaction rate constants (k) of 8.54×10^{-6}, 2.51×10^{-5}, and 4.87×10^{-5} s^{-1} in phosphate-boric acid buffers of pH 7, 8.5, and 10, respectively. Similar reactions in ammonium bicarbonate buffers were more than 10 times faster. With time, thomasidioic acid further converted to 2,6-dimethoxy-*p*-benzoquinone and 6-hydroxy-5,7-dimethoxy-2-naphthoic acid. Air oxidation of sinapic acid aqueous solutions caused darkening of the color for the system, with the 2,6-dimethoxy-*p*-benzoquinone as a major color contributor.

Paper no. J9024 in *JAOCS 76,* 757–764 (June 1999).

KEY WORDS: Canola phenolics, chromatographic analysis, color, 2,6-dimethoxy-*p*-benzoquinone, 6-hydroxy-5,7-dimethoxy-2-naphthoic acid, reaction rate constant, sinapic acid, thomasidioic acid.

With an annual global production of more than 27 million metric tons (1), canola/rapeseed represents one of the most important oilseeds in the world (2). There is an interest in preparing a food-grade protein from canola meal (3–5), especially because the amino acid content of canola meal is well balanced (6,7). However, the utilization of canola/rapeseed protein in human foods has been limited by the presence of antinutritional factors such as glucosinolates, phytates, and phenolics (8). Among them, phenolics have been the subject of many studies owing to their contributions to the dark color, bitter taste, and the astringency of rapeseed/canola meal (9) as well as their detrimental effect on the gelation property of canola protein (10). Canola phenolics are present in free, esterified, and bound forms (11). Canola flour contains from 91 to 93.5% of their phenolic acid in the esterified form (9). Sinapine, the choline ester of sinapic acid, is the major esterified phenolic acid (12–14), whereas sinapic acid represents a high proportion of the free phenolic acid and 99% of the phenolic acids released from hydrolysis of the esters in the flour (15).

One of the major problems limiting the utilization of canola protein is the dark-colored meal remaining after oil extraction (16). During protein isolation, conditions of high pH produce a darker protein isolate than do conditions of low pH (17). Although the observations of a dark meal or protein isolate have been frequently reported (16,17) and more or less associated with phenolics (8,9), detailed information regarding the reactions responsible for the development of colored substances is not available. Such information, however, should indicate the mechanisms by which the colored substances develop and, therefore, is important to the oilseed processors.

Although no relationship has ever been reported between the structural changes of the phenolics and color properties of canola/rapeseed protein, several researchers have observed structural changes of sinapic acid in air-saturated basic conditions (18–21). Rubino *et al*. (19) reported the formation of thomasidioic acid showing that the conversion was oxygen dependent (20). Charlton and Lee (18) reported the formation of 2,6-dimethoxy-*p*-benzoquinone and 6-hydroxy-5,7-dimethoxy-2-naphthoic acid from sinapic acid *via* thomasidioic acid at pH 13 in the presence of oxygen. With high-performance liquid chromatography (HPLC) analysis, Bouchereau *et al*. (21) detected new compounds derived from rapeseed phenolics in the methanol extract of rapeseed flour, although the compounds were not identified. In a study looking at the effect of processing conditions on phenolic content, treatments, especially heating, have been shown to decrease the sinapine content and increase the lignan amount (22). Although the oxidation of sinapic acid under alkaline conditions is known to lead to thomasidioic acid, 2,6-dimethoxy-*p*-benzoquinone and 6-hydroxy-5,7-dimethoxy-2-naphthoic acid, the color properties of these oxidation products have not been determined. Reaction rate constants for these conversions under different pH conditions also have not been determined,

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which should provide useful information when considering protein isolation under alkaline conditions.

As part of a series of investigations looking at the contributions of phenolics to the color property of canola protein, structural changes of sinapic acid induced by air oxidation at pH 7–10 were followed by spectral and HPLC analysis for 10 d. Reaction rate constants were determined at pH values of 7, 8.5, and 10. Color properties of solutions were also determined in order to evaluate the color contributions of the new substances to the system.

MATERIALS AND METHODS

Sources of materials. Sinapic acid (3,5-dimethoxy-4-hydroxycinnamic acid) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Acetic acid and sodium hydroxide used for HPLC were verified American Chemical Society (A.C.S.) grade and purchased from Fisher Scientific Co. (Nepean, Ontario, Canada). Other chemicals used for HPLC were HPLC-grade. All other chemicals, unless stated otherwise, were verified A.C.S grade and purchased from Fisher Scientific Co.

Thomasidioic acid was prepared according to the procedure outlined by Lee (23) and Rubino *et al*. (19), which involved the oxidation of sinapic acid in an ammonium bicarbonate buffer (pH 8.5). The crude product was recrystallized from acetone several times to yield thomasidioic acid as a colorless solid (24). This purified thomasidioic acid was used as a standard for identifying thomasidioic acid formed from sinapic acid. 2,6-Dimethoxy-*p*-benzoquinone was prepared according to the method outlined by Lee (23), which involved air oxidation of 2,6-dimethoxyphenol in aqueous acetic acid solution in the presence of chromium trioxide. 6-Hydroxy-5,7-dimethoxy-2-naphthoic acid was also prepared using the method of Lee (23) by air oxidation of sinapic acid in strongly basic solution (pH 13). 2,6-Dimethoxy-*p*-benzoquinone is reported to be yellow, whereas 6-hydroxy-5,7-dimethoxy-2-naphthoic acid crystallizes as pale tan needles from MeOH/H_2O (25).

Sample preparation. Phosphate-boric acid buffers were prepared according to Britton and Robinson (26). Ammonium bicarbonate buffers (0.12 M) were prepared according to Lee (23) and adjusted to pH 8.5 and 10 using ammonium hydroxide. Experiments in this buffer system were conducted to complement the phosphate-boric acid system. Results in phosphate-boric acid buffers constituted the main body of work in this paper.

A sinapic acid solution (200 μg/mL) was prepared with deionized water. Three samples of 4 mL each in phosphate-boric acid buffer of pH 7, 8.5, and 10 (two samples at pH 8.5 and 10 in ammonium bicarbonate buffers), respectively, were prepared by combining 2 mL of the sinapic acid solution with 2 mL of each buffer solution of different pH values so that 100 μg/mL (0.446 mmol) sinapic acid solutions with different pH values were obtained. These solutions were stirred in air at room temperature (22°C). Oxygen is needed for the conversion of sinapic acid to thomasidioic acid (20) and the conversion of thomasidioic acid to 2,6-dimethoxy-*p*-benzoquinone and 6-hydroxy-5,7-dimethoxy-2-naphthoic acid (18). Water vapor loss was 1% per day under the test condition. This loss was ignored during the kinetic study. The pH values of these solutions were checked before each analysis was made. Only the pH value of the phosphate-boric acid solution at pH 10 had a slight deviation (decreased) and was readjusted by adding sodium hydroxide. Spectral and HPLC analyses were carried out for 10 d. For HPLC, a sample size of 1 μL was used for the kinetic study whereas 25 μL was injected during the identification of the *p*-benzoquinone and the 2-naphthoic acid using a 25-μL sample loop. Three samples of pH 7, 8.5, and 10 for ultraviolet (UV) spectral analysis were prepared by a 20-fold dilution of the sinapic acid solution (200 μg/mL) with phosphate-boric acid buffers of different pH values and stirred in air at room temperature (22°C). For the spectral and HPLC analyses of the the standard thomasidioic acid, 2,6-dimethoxy-*p*-benzoquinone and 6-hydroxy-5,7-dimethoxy-2-naphthoic acid, these compounds were dissolved directly in the appropriate buffers just before the measurement.

HPLC analysis. Chromatographic equipment consisted of two Waters (Milford, MA) pumps (models 501 and 510) and an automated gradient controller model 680, a Shimadzu (Kyoto, Japan) SPD-6A UV spectrophotometric detector, and a Hewlett-Packard (Avondale, PA) model HP3396II integrator. A reverse-phase C18 column (Supelcosyl, 3-μm particle size, 33 × 4.6 mm i.d.; Supelco, Bellefonte, PA) was used. Buffer A was a 0.05 M acetate buffer prepared by a 1:100 dilution of a stock pH 4.7 acetate buffer. The stock buffer was prepared by adjusting 5 M acetic acid to pH 4.7 with solid sodium hydroxide (27). Buffer A was filtered through a 0.45 μm filter. Buffer B was 100% HPLC-grade methanol. The column was maintained at 37°C and run at a constant flow rate of 1.4 mL/min.

The initial elution solvent was 15% methanol and 85% buffer A. After a 12-min isocratic flow, a 2-min linear gradient was used to change the solvent composition to 100% methanol. This composition was maintained for 2 min, after which another 2-min linear gradient returned the solvent to its original composition.

Peaks were identified with standard sinapic acid, thomasidioic acid, 2,6-dimethoxy-*p*-benzoquinone, and 6-hydroxy-5,7-dimethoxy-2-naphthoic acid; and the HPLC response (peak area) was calibrated using standardized solutions of the above compounds.

Kinetic study. Reaction rate constants (k) were obtained by a kinetic study based on the time-dependent change in concentration of sinapic acid as determined from the peak areas of the HPLC chromatograms.

For each reaction at pH 7, 8.5, and 10, the concentration of sinapic acid was determined about 10 times by HPLC during the course of the reaction. Each of the three groups of time-dependent reactant concentrations at different pH values was fitted to a first-order reaction equation,

$$\ln = \frac{c_o}{c} = kt \quad [1]$$

and a second-order equation,

$$\frac{1}{c} - \frac{1}{c_o} = kt \quad [2]$$

where c_o and c were the initial concentration (mol/L) and the concentration at time t, t is the reaction time (s), and k is the reaction rate constant; s^{-1} in the first-order equation, and L mol^{-1} s^{-1} in the second-order equation.

If the reaction is first order, the plot of ln (1/c) vs. t should give a straight line. Similarly, if the reaction is second order, the plot of 1/c vs. t should give a straight line. A linear regression was conducted for each group of sinapic acid concentrations. R^2 (R = correlation coefficient) was used to judge the linear relationship between the independent value t and the dependent values ln (1/c) or 1/c and therefore to determine which equation better fit the data (28). The equation better able to fit the data was used to calculate the reaction rate constant k.

Spectral analysis and color determination. Spectral analysis and color determination have been described elsewhere (29).

TABLE 1
Concentration of Sinapic Acid, Thomasidioic Acid, 2,6-Dimethoxy-*p*-benzoquinone, and 6-Hydroxy-5,7-dimethoxy-2-naphthoic Acid as a Function of Time During Alkaline Oxidation of a 0.446 mmol/L Sinapic Acid Solution in Phosphate-Boric Acid Buffers of pH 7, 8.5, and 10

pH 7			pH 8.5			pH 10		
Time (h)	Conc. (mmol/L) SA[a]	TA	Time (h)	Conc. (mmol/L) SA[a]	TA	Time (h)	Conc. (mmol/L) SA[a]	TA
0	0.446	0	0	0.446	0	0	0.446	0
5	0.349	N.D.	1	0.351	0.014	0.5	0.374	0.026
10	0.316	0.017	3	0.310	0.033	2	0.284	0.069
16	0.259	0.037	5	0.251	0.076	4	0.216	0.113
23	0.225	0.060	7	0.229	0.077	5	0.176	0.140
27	0.198	0.066	11	0.179	0.088	7	0.113	0.165
34	0.142	0.074	16	0.092	0.112	10	0.045	0.174
51	0.085	0.090	20	0.063	0.145	15	0.030	0.180
55	0.090	0.094	24	0.045	0.168	20	0.012	0.189
132	0.007	0.143	26	0.041	0.171	24	0.007	0.196
169	0.001	0.184	169	N.D.	0.136	168	N.D.	0.041
240	N.D.	0.141	241	N.D.	0.078	241	N.D.	0.003
	BQ	NA		BQ	NA		BQ	NA
133	N.D.	N.D.	26	N.D.	N.D.	24	N.D.	N.D.
169	0.008	0.017	169	0.011	0.072	168	0.044	0.132
240	0.013	0.030	240	0.044	0.125	241	0.043	0.144

[a]Reaction rate constant k value as in Table 2. SA, sinapic acid; TA, thomasidioic acid; BQ, 2,6-dimethoxy-*p*-benzoquinone; NA, 6-hydroxy-5,7-dimethoxy-2-naphthoic acid; N.D., not detected.

RESULTS AND DISCUSSION

Structural changes. (i) HPLC analysis. The concentrations for sinapic acid, thomasidioic acid, 2,6-dimethoxy-*p*-benzoquinone, and 6-hydroxy-5,7-dimethoxy-2-naphthoic acid as a function of time during air oxidation of three 0.466 mmol/L sinapic acid in phosphate-boric acid buffer solutions of pH 7, 8.5, and 10 are given in Table 1. Virtually all the sinapic acid was converted to thomasidioic acid during the alkali-induced air oxidation at pH 7, 8.5, or 10. With the reaction at pH 7, approximately 50% conversion to thomasidioic acid occurred after 23 h and 99.8% after 169 h, resulting in the production of 0.184 mmol/L of thomasidioic acid. However, between 169 and 240 h the thomasidioic acid concentration decreased to 0.141 mmol/L while those of the 2,6-dimethoxy-*p*-benzoquinone and 6-hydroxy-5,7-dimethoxy-2-naphthoic acid increased from 0.008 and 0.017 mmol/L to 0.013 and 0.030 mmol/L, respectively. At pH 8.5, there was 91% conversion to thomasidioic acid after 26 h, while at pH 10, there was 90% conversion to thomasidioic acid after only 10 h. The conversion was nearly complete (98.4%) within 24 h. The concentrations of thomasidioic acid peaked at 0.171 mmol/L after 26 h at pH 8.5 and at 0.196 mmol/L after 24 h at pH 10. At both pH levels, increased oxidation (up to 241 h) resulted in decreases in thomasidioic acid concentration that were accompanied by increases in the levels of 2,6-dimethoxy-*p*-benzoquinone and 6-hydroxy-5,7-dimethoxy-2-naphthoic acid. Structural changes of sinapic acid were apparently similar for all three pH conditions, but the conversions were slower at lower pH.

The air oxidative conversion of sinapic acid to 2,6-dimethoxy-*p*-benzoquinone and 6-hydroxy-5,7-dimethoxy-2-naphthoic acid *via* thomasidioic acid at high pH has been previously reported (18). In this study, these two oxidation products were found in samples at all three pH values (7, 8.5, and 10) after 169 h. The fact that the concentrations of 2,6-dimethoxy-*p*-benzoquinone and 6-hydroxy-5,7-dimethoxy-2-naphthoic acid increased while that of thomasidioic acid decreased was consistent with the known oxidation of thomasidioic acid to 6-hydroxy-5,7-dimethoxy-2-naphthoic acid (18).

The amounts of 2,6-dimethoxy-*p*-benzoquinone and 6-hydroxy-5,7-dimethoxy-2-naphthoic acid were determined to be 0.013 and 0.030, 0.044 and 0.125, and 0.043 and 0.144 mmol/L, a mole ratio of 1:2.3, 1:2.8, and 1:3.3 for pH 7, 8.5, and 10, respectively, after air oxidation for approximately 240 h. This ratio is less than the theoretical mole ratio of 1:1 that would result if equal number of moles of the two compounds were produced as the only reaction products from thomasidioic acid.

Similar reactions were found in ammonium bicarbonate buffers at pH 8.5 and pH 10, but the reaction rates were more than 10 times faster.

(ii) UV spectrum. The UV spectra of 10 µg/mL sinapic acid solutions after air oxidation for 0 and 24 h are shown in Figure 1A and B. The UV spectra of 10 µg/mL standard thomasidioic acid solutions of different pH values are given in Figure 1C. At zero time, increasing pH caused a strong bathochromic shift, the shift of the maximum absorbance toward higher wavelengths, relative to the spectrum recorded at

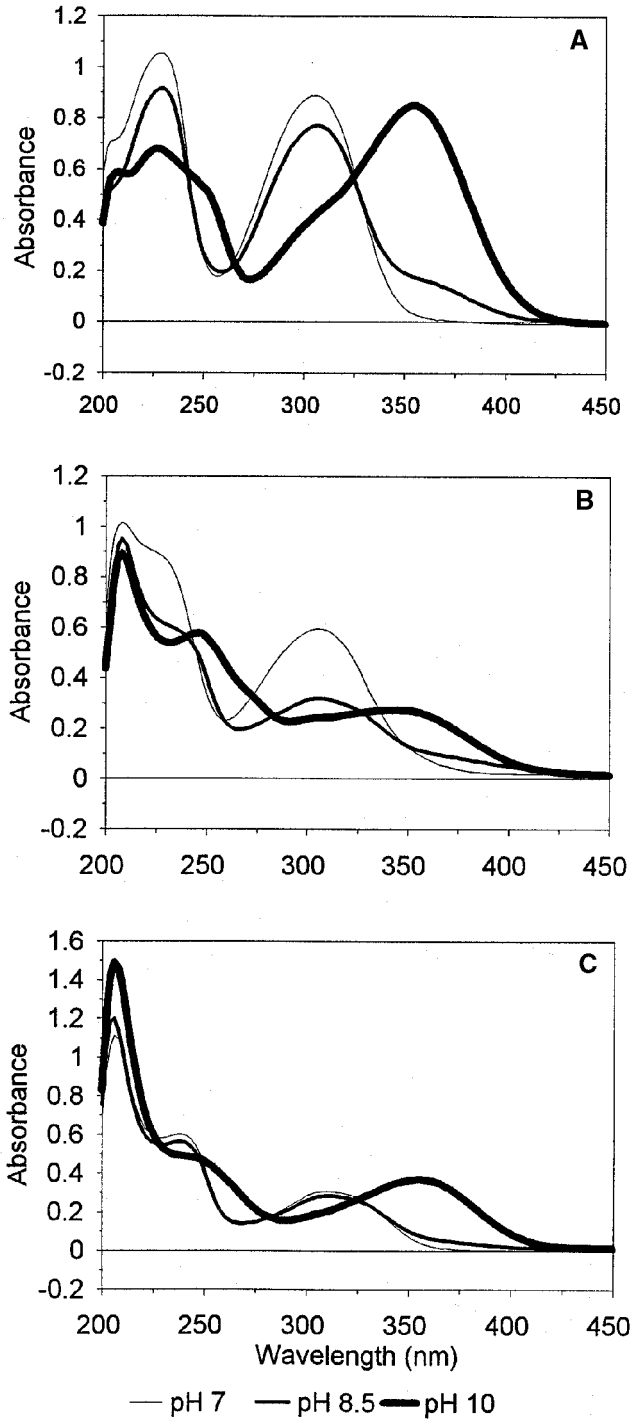
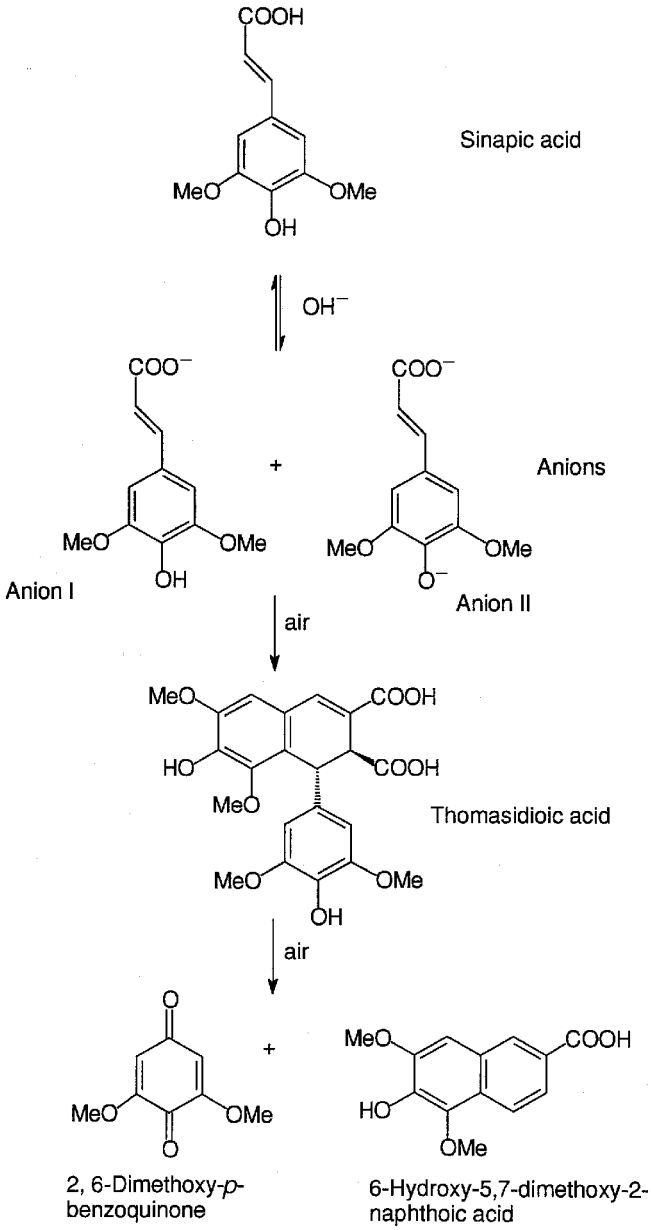


FIG. 1. Ultraviolet spectra of a 10 μg/mL phosphate-boric acid buffer solution of pH 7, 8.5, and 10 containing (A) sinapic acid after 0 h, (B) sinapic acid after 24 h, and (C) standard thomasidioic acid.

pH 7 (30). The effect of alkaline conditions on the absorption properties of phenolic compounds has been reported by several researchers (31,32). Large bathochromic shifts were noted in most cases under basic conditions (32). The pK_a values of the carboxylic and hydroxy groups of sinapic acid are 4.47 and 9.21, respectively (33). Therefore, at pH 7 only the carboxylic group is ionized (anion I). At pH 10, both the carboxylic and the hydroxy groups are ionized (anion II) (Scheme 1). The strongest bathochromic shifts occur on ionization of the phenolic hydroxyl. After 24 h (Fig. 1B), interpretation of the UV spectrum found that the absorbances of sinapic acid of different anionic forms (anion I, 305 nm, and anion II, 355 nm) had decreased as expected. The UV absorbances of the solutions at pH 8.5 and 10 in fact were similar to the spectra of the oxidized product, thomasidioic acid (Fig. 1B,C). Unlike the situations at pH 8.5 and 10, the spectrum obtained after air oxidation at pH 7 for 24 h did not resemble that of the standard thomasidioic acid, since only 50% sinapic acid was converted to thomasidioic acid.

Kinetic study of the disappearance of sinapic acid. The correlation coefficients (R^2) for the first-order equations were 0.98, 0.99, and 0.99, respectively, for the reactions in phos-



SCHEME 1

TABLE 2
First-Order Reaction Equations, Reaction Rate Constants (k), Half-Lives ($t_{1/2}$), and Correlation Coefficients (R_2) for the Reactions of Sinapic Acid in Solutions of pH 7, 8.5, and 10

pH	Equation	k (s^{-1})	$t_{1/2}$ (h)[a]	R^2
Phosphate-boric acid buffer				
7	$\ln (c_o/c) = 0.0375 + 8.54 \times 10^{-6}\ t$	8.54×10^{-6}	22.50	0.98
8.5	$\ln (c_o/c) = 0.075 + 2.51 \times 10^{-5}\ t$	2.51×10^{-5}	7.66	0.99
10	$\ln (c_o/c) = 0.0828 + 4.87 \times 10^{-5}\ t$	4.87×10^{-5}	3.95	0.99
Ammonium bicarbonate buffer				
8.5	$\ln (c_o/c) = 0.0878 + 4.30 \times 10^{-4}\ t$	4.30×10^{-4}	0.45	0.99
10	$\ln (c_o/c) = -0.1765 + 6.18 \times 10^{-4}\ t$	6.18×10^{-4}	0.31	0.97

[a]The half-life ($t_{1/2}$) for the reactions was found as $t_{1/2} = 0.693/k$, where k is the reaction rate constant.

phate-boric acid buffers of pH 7, 8.5, and 10, whereas R^2 for the second-order equations were 0.93, 0.65, and 0.85, respectively (Table 2). The first-order equations were therefore used to calculate the reaction rate constant (k) values.

The reaction equations, reaction constants (k), and half-lives ($t_{1/2}$) for both the phosphate-boric acid buffer and the ammonium bicarbonate buffer are also summarized in Table 2. In phosphate-boric acid buffers, the reaction rate constants are 8.54×10^{-6}, 2.51×10^{-5}, and 4.87×10^{-5} s^{-1} for reactions at pH 7, 8.5, and 10, respectively. The reaction at pH 10 was about twice as fast as the reaction at pH 8.5 and about five times as fast as the reaction at pH 7. These differences can also be seen from the half-lives ($t_{1/2}$) of the reactions, which were 22.50, 7.66, and 3.95 h for pH 7, 8.5, and 10, respectively. The reactions in ammonium bicarbonate buffers were more than 10 times faster than those in the phosphate-boric acid buffer, with reaction rate constants (k) of 4.30×10^{-4} and 6.18×10^{-4} s^{-1} at pH 8.5 and 10, respectively. This demonstrates the possible effect of different media on the reaction. Reaction orders in ammonium bicarbonate buffers, however, were the same as those in phosphate-boric acid buffers.

Color changes in relation to structural changes. (i) Color intensity, Hunter L a b values. The effects of time on the Hunter L a b values of the reaction mixtures (phosphate-boric acid buffers) at pH 7, 8.5, and 10 are shown in Table 3. Initially the color of the reaction mixture at pH 7 was almost unchanged compared to the control. However, this color intensity steadily increased with time as seen by a decrease in the L value and an increase in the magnitude of both a and b values within 169 h. For reactions at pH 8.5 and 10, yellow intensity (b) was higher than that of the control at the beginning of the reaction. This yellow color increased during the initial stage of the reaction (18 h), decreased slightly at 28 h, but became most intense by 169 h. At this time, both 2,6-dimethoxy-*p*-benzoquinone and 6-hydroxy-5,7-dimethoxy-2-naphthoic acid were found to be present. The initial high-intensity yellow of the solution could be attributed to the absorption of the sinapic acid phenolate anion (anion II), as seen from the UV spectra (Fig.1A). This color increased and then faded slightly during the course of the conversion of sinapic acid to thomasidioic acid. According to the HPLC results, 92% (pH 8.5) and 99% (pH 10) of sinapic acid was converted to thomasidioic acid within 28 h. Therefore, yellow intensity at this time should reflect the color of the phenolate anions of thomasidioic acid. Moreover, the conversion to thomasidioic acid from sinapic acid may involve the formation of some intermediates, which may affect the color. It seemed apparent that at least one intermediate between sinapic acid and thomasidioic acid must account for the color increase at 18 h. Since 2,6-dimethoxy-*p*-benzoquinone and 6-hydroxy-5,7-dimethoxy-2-naphthoic acid were present when the color darkening was greatest at the end of the reaction, these substances could be major contributors to color of the system.

Color profiles in ammonium bicarbonate buffers at pH 8.5 and 10 were found similar to those found in phosphate-boric acid buffers

(ii) Color property of sinapic acid and thomasidioic acid in basic conditions. The color properties of sinapic acid and thomasidioic acid in phosphate-boric buffers were determined

TABLE 3
Effect of Time on the Hunter L a b Values of 0.446 mmol/L Sinapic Acid Solutions During Oxidations in Phosphate-Boric Acid Buffers of pH 7, 8.5, and 10[a]

	Hunter color value		
Time (h)	L	a	b
Control[b]	99.77 ± 0.15	−5.55 ± 0.31	5.47 ± 0.25
pH 7			
0	99.18 ± 0.71	−5.22 ± 0.17	5.85 ± 0.48
18	96.84 ± 0.54	−5.92 ± 0.37	12.52 ± 0.52
28	96.33 ± 0.38	−4.47 ± 0.29	15.53 ± 0.06
169	88.68 ± 0.91	−7.72 ± 0.39	19.46 ± 0.58
pH 8.5			
0	98.40 ± 0.53	−5.80 ± 0.17	7.70 ± 0.53
18	92.32 ± 1.16	−6.46 ± 0.36	14.23 ± 0.57
28	91.00 ± 2.38	−8.27 ± 1.45	12.84 ± 1.16
169	88.40 ± 1.28	−5.69 ± 0.45	19.08 ± 0.39
pH 10			
0	99.43 ± 0.81	−7.90 ± 0.61	11.47 ± 0.59
18	93.49 ± 1.32	−6.49 ± 0.36	12.49 ± 0.5
28	97.00 ± 1.1	−6.25 ± 0.27	11.69 ± 1.1
169	86.97 ± 0.91	−2.42 ± 0.33	17.9 ± 0.98

[a]Mean of three replicates ± SD.
[b]Control: sinapic acid in deionized water with a natural pH of 4.3 at zero time.

based on visible light transmittance spectra. A comparison between transmittance spectra of equal weight concentrations of sinapic acid and thomasidioic acid at different pH values is shown in Figure 2. Both compounds showed little color at pH 7 and a remarkable decrease in percentage transmittance at low wavelengths as pH increased from 7 to 10. These agreed with the UV spectra where a large absorption band was found for both compounds in the visible region. However, the transmittance decreases for thomasidioic acid at pH 8.5 and 10 were less pronounced than those for sinapic acid, indicating that thomasidioic acid was less colored than sinapic acid at pH 8.5 and 10.

As the color properties of sinapic acid and thomasidioic acid are pH dependent, the color determined using basic conditions will be different from those determined at neutral or acidic conditions. Similarly, during protein isolation from canola meal with basic extraction and acidic precipitation, the colored sinapic or thomasidioic anions at basic conditions can become colorless during acidic precipitation provided no other interactions between protein and phenolics occur. However, the secondary oxidation products such as the 2,6-dimethoxy-*p*-benzoquinone should remain as colored substances if these substances are present.

*(iii) Spectral properties of 6-hydroxy-5,7-dimethoxy-2-naphthoic acid and 2,6-dimethoxy-*p*-benzoquinone.* UV and light transmittance spectra of the 6-hydroxy-5,7-dimethoxy-2-naphthoic acid at different pH values are shown in Figure 3. At pH 7, the 2-naphthoic acid showed two major peaks at around 250 and 300 nm (Fig. 3A). Higher pH caused a significant shift of the peaks toward higher wavelengths (around 255 and 330 nm at pH 10). However, these shifts did not cause a large change in the visual transmittance spectra (Fig. 3B). At pH 7 the 2-naphthoic acid showed little color. The color intensity only had a slight increase as pH increased from 7 to 10, indicating it was not a significant color contributor even under basic conditions. 2-Naphthoic acid occurs as pale tan needles (25).

A comparison of UV and light transmittance spectra of the 2,6-dimethoxy-*p*-benzoquinone is shown in Figure 4. In the UV spectra (Fig. 4A), 2,6-dimethoxy-*p*-benzoquinone showed a maximal absorbance at 280 nm in contrast to the two maxima of sinapic acid at 230 and 325 nm. Although sinapic acid showed no absorbance at wavelengths above 380 nm, *p*-benzoquinone showed some absorbance over this region, indicative of a colored substance. In the light transmittance spectra (Fig. 4B), 2,6-dimethoxy-*p*-benzoquinone

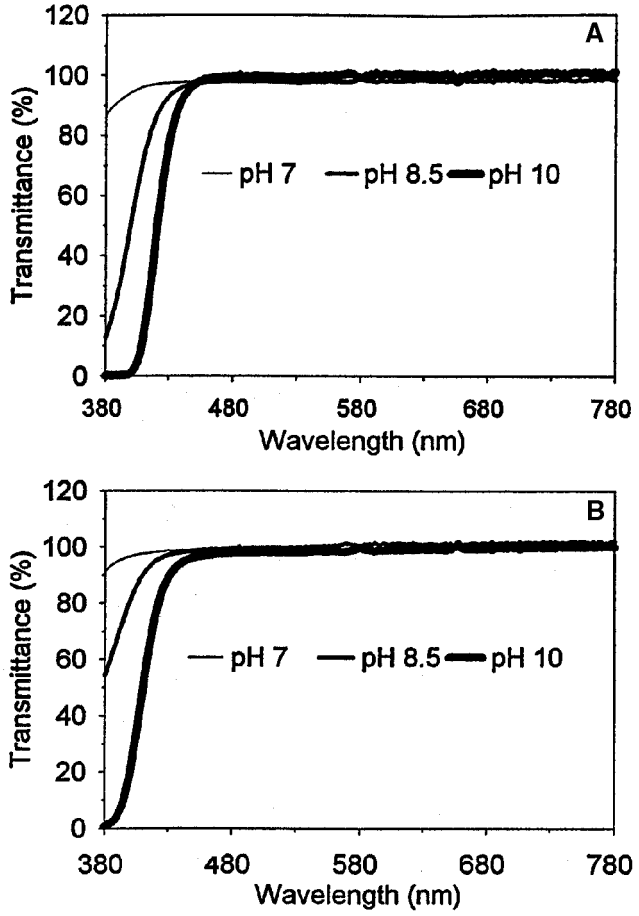


FIG. 2. Transmittance spectra of a 100 µg/mL phosphate-boric buffer solution of pH 7, 8.5, and 10 containing (A) sinapic acid and (B) thomasidioic acid.

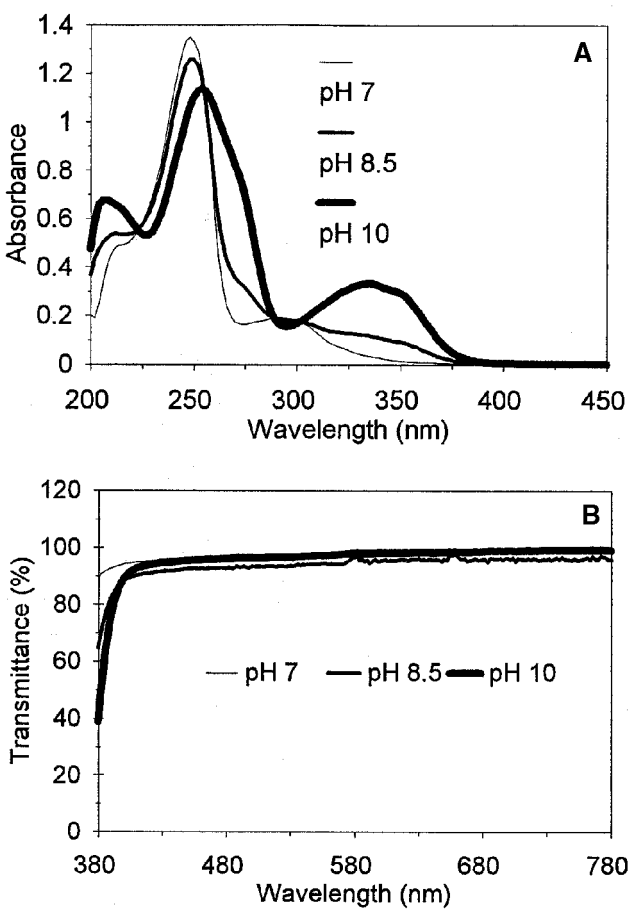


FIG. 3. Ultraviolet absorbance (A) (solution diluted 10 times) and visual light transmittance (B) spectra of a 100 µg/mL 6-hydroxy-5,7-dimethoxy-2-naphthoic acid.

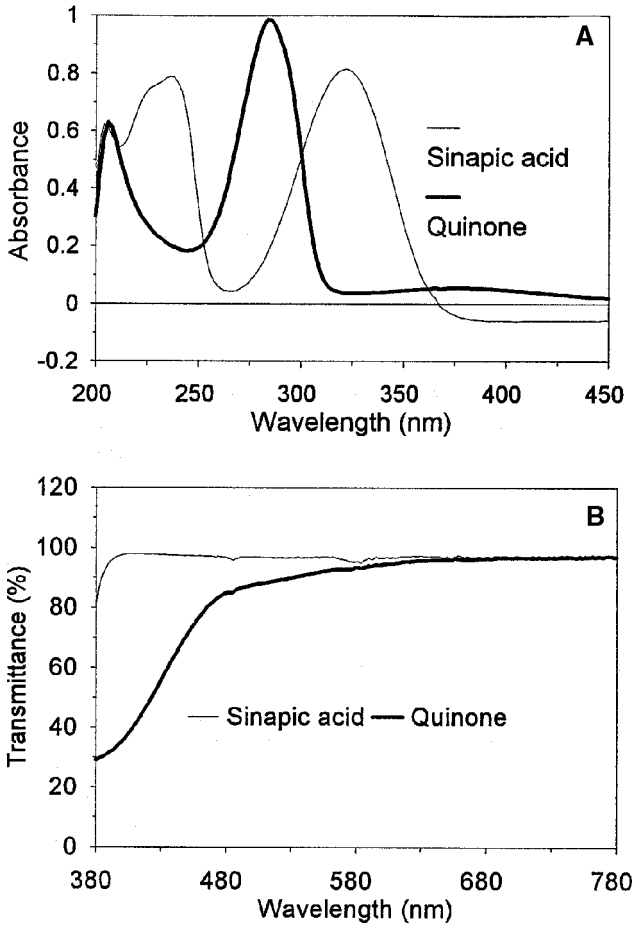


FIG. 4. Ultraviolet absorbance (A) (solution diluted 10–20 times) and visual light transmittance (B) spectra of a 100 μg/mL 2,6-dimethoxy-*p*-benzoquinone and a 100 μg/mL sinapic acid (all in methanol).

showed a noticeable low transmittance in the wavelength range from 380 to 480 nm. This is consistent with the yellow appearance of that substance (25). This also indicated that *p*-benzoquinone is a strong contributor to the color of the system. HPLC analysis found other undetermined colored substances were produced during the reaction.

Mechanisms of the reactions. Results from this research showed that after 28 h there was 92% (pH 8.5) and 99% (pH 10) disappearance of sinapic acid in phosphate-boric acid buffers (0.446 mmol/L). Bathochromic shifts at the beginning of the reaction were caused by the formation of phenolate anions, which could be responsible for the initial colors of the mixtures (31–33). The color increased and then slightly decreased in the first 28 h at pH 8.5 and pH 10 during the course of the conversion of sinapic acid to thomasidioic acid. Similar color changes were found in reactions in ammonium bicarbonate buffers.

Lee (23) determined that the dimerization of sinapyl radicals was the most likely pathway for oxidation of sinapic acid. The dimerization product, a bisquinone methide, would then partially tautomerize to form a monoquinone methide and then cyclize to form thomasidioic acid. The mechanism for the conversion of sinapic acid to the 2,6-dimethoxy-*p*-benzoquinone and the 6-hydroxy-5,7-dimethoxy-2-naphthoic acid was determined to involve the secondary oxidation of the first formed thomasidioic acid (18). If this is the case, then the two intermediates, the bisquinone methide and the monoquinone methide, may also affect the color profile of the system. These intermediates may account for the color development during the conversion of sinapic acid to thomasidioic acid.

With time, thomasidioic acid gradually oxidized to form the *p*-benzoquinone and the 2-naphthoic acid, which in turn increased the yellow intensity, with the *p*-benzoquinone being the major color contributor. This is also the case for the reaction at pH 7. The steady increase in color intensity is consistent with air oxidation of sinapic acid to form thomasidioic acid and further oxidation to the *p*-benzoquinone and the 2-naphthoic acid. The darkening of color at pH 7 is particularly noteworthy since the solution had a neutral pH value and there was no significant sign of color changes at the onset of the reaction, unlike the more basic pH conditions, which produced an immediate yellow color. A reaction scheme showing the conversion of sinapic acid to thomasidioic acid, and further oxidation to 2,6-dimethoxy-*p*-benzoquinone and 6-hydroxy-5,7-dimethoxy-2-naphthoic acid is given in Scheme 1.

ACKNOWLEDGMENT

Financial support of this research by the Natural Sciences and Engineering Research Council of Canada and the award of the J.W. Barlow Graduate Fellowship to R. Cai are gratefully acknowledged.

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[Received October 2, 1998; accepted March 7, 1999]