Structural Changes of Sinapic Acid and Sinapine Bisulfate During Autoclaving with Respect to the Development of Colored Substances

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ABSTRACT: Structural changes in sinapic acid during autoclaving were studied using spectral analysis, thin-layer chromatography, high-performance liquid chromatography, nuclear magnetic resonance (NMR), and mass spectroscopy. Color properties of sinapic acid and its derivatives were studied by determining the transmittance spectrum, calculating the Commission Internationale de l'Eclairage 1931 tristimulus values and converting to Hunter L a b values. It was found that the colorless sinapic acid aqueous solution (100 μg/mL) turned yellow after 15 min in an autoclave at 121°C and 0.1 MPa. Filtering the yellow aqueous solution through a 0.45-μm filter removed a brown solid consisting of at least three undetermined colored substances and left a yellow liquid. A newly developed yellow substance, syringaldehyde, was identified in the liquid phase by comparing the NMR and mass spectra of the unknown with those of authentic syringaldehyde. Thomasidioic acid was also found in the liquid phase. Under the same autoclaving conditions, sinapine bisulfate showed no evidence of any structural or color changes.

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KEY WORDS: Autoclaving, canola phenolics, chromatographic analysis, color, sinapic acid, sinapine bisulfate, syringaldehyde.

Canola provides an excellent edible oil source (1) and is one of the most important oilseeds in the world (2,3). Canola protein has a well-balanced amino acid content and a favorable protein efficiency ratio (4,5). Therefore, interest in preparing food-grade protein from canola meal has been increasing (6–8). Unfortunately, the use of canola protein is limited by the presence of some antinutritional compounds such as glucosinolates, phytates and phenolics (9). Phenolics have significant effects on the visual attributes, flavor characteristics, nutritional, and even functional properties of oilseed products (9–12). Color and some other effects are believed to be developed during oilseed processing (11). Commercial defatting of canola is currently accomplished almost exclusively by prepress and solvent extraction systems, with flaking and cooking of the whole seed before pressing in expellers (2,13,14). The severe moist-heat treatment, in the cooker and desolventizer unit, darkens the meal color and denatures the protein (11). The resulting commercial canola meals are less than ideal for food use partly because of the dark color and bitter flavor associated with products produced from phenolics during processing. Determination of the reactions that would occur involving these phenolics during processing is the key to further research on this problem.

In contrast to the effect of pH on the color properties of oilseed phenolics (15–17), which generally occurs during protein isolation (18), the effects of heat and pressure are closely related to the steps during oilseed extraction, such as preheating, cooking, pressing, and desolventization (2,3,13). During these steps, heat and pressure are normally required. In a study on sunflower seed phenolics, heating was found to decrease the content of simple phenolics (19). Heat treatments were also shown to result in a decrease in the content of sinapine bisulfate and an increase in lignan content in rapeseed (20). Heat and pressure seemed to have significant effects on the color and structure of phenolics. However, the exact nature of these changes has rarely been reported, partly because of the complexity of the systems. In addition, there appeared to be no reports on the coloration of canola phenolics during autoclaving.

Sinapic acid and sinapine bisulfate are the focus of this paper since they are the major simple and esterified phenolics in canola (9,21–24). The present study reports the structural changes and coloration of sinapic acid and sinapine bisulfate during autoclaving with a focus on the development of colored substances. The experiments were performed with pure phenolic solutions to ensure that the information obtained reflected the substances of interest.

MATERIALS AND METHODS

Sources of materials. Sinapic acid (3,5-dimethoxy-4-hydroxycinnamic acid) and syringaldehyde (4-hydroxy-3,5-dimethoxybenzaldehyde) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Syringaldehyde is a dull yellow solid (Sigma-Aldrich Canada Ltd., material safety data sheet, 1997). Aldrich silica gel (230–400 mesh, 60 Å) was used for column chromatography. Sinapine was isolated from

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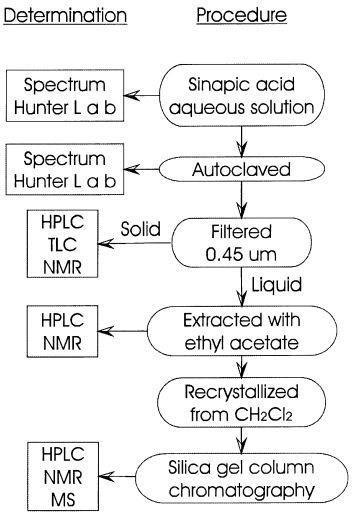
Sinapis alba certified seed from Tilney Mustard crop as sinapine bisulfate according to the method outlined by Clandinin (25). Sinapine bisulfate was used in the place of sinapine for assessing the autoclaving effect. Acetic acid and sodium hydroxide used for high-performance liquid chromatography (HPLC) were verified American Chemical Society (ACS)-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 ACS-grade and purchased from Fisher Scientific Co.

Thomasidioic acid was prepared according to Ahmed *et al.* (26) and Rubino *et al.* (27) with some modifications. Sinapic acid (20 mg) was dissolved in 0.4 mL methanol. This solution was added to an aqueous $FeCl_3$ solution (40 mg/1.6 mL). The solution was aerated for a few seconds. The violet-red precipitate was collected and treated with concentrated H_2SO_4 for 30 s. After diluting with an equal amount of water, the resulting mixture was extracted with ethyl acetate (3 times–5 mL each), dried with $MgSO_4$, and evaporated under vacuum. The residue was dissolved in methanol, activated carbon was added, and the mixture was then filtered through a 0.45-µm filter. The methanol was evaporated under vacuum. The resulting thomasidioic acid was used as a standard for the identification of new compounds formed during autoclaving. Thomasidioic acid is a colorless solid (26).

Procedure for determination of structural changes and coloration of sinapic acid. The procedure for the determination of color and structural changes of sinapic acid is shown in Scheme 1. A 100 µg/mL solution was prepared by dissolving 200 mg of sinapic acid in 2000 mL distilled water in a 3000-mL beaker. About 10 mL of this solution was kept as a control sample. In determining the effect of autoclaving time on the color properties of the solution, three samples of 10 mL each were taken from the above solution and autoclaved for 15, 30, and 45 min, respectively. The rest of the solution was autoclaved for 30 min. Autoclaving was conducted with an AMSCO Eagle 3000 Series Sterilizer (American Sterilizer Company, Horsham, PA). Time, temperature, and pressure were controlled automatically. A gravity model was used for the autoclaving of the samples, where no liquid water was added during autoclaving. Temperature and pressure were routinely set to 121°C (250°F) and 0.1 MPa (15 psi). Spectral analysis of the samples was conducted both before and after autoclaving to determine the ultraviolet (UV) spectra and Hunter L a b values. After autoclaving, the solution was filtered through a 0.45-µm filter, giving a brown solid phase and a yellow liquid phase.



SCHEME 1

Analysis of the brown solid. The solid phase was recovered from the filter paper by washing with ethyl acetate, drying with $MgSO_4$, and evaporating under vacuum to remove solvent. HPLC analysis and thin-layer chromatography (TLC; 100% ethyl acetate) were conducted on the recovered solid samples. Three fractions from the TLC at R_f 0.95, 0.91, and 0.72 were recovered from the silica gel by extracting with methanol, filtering through a 0.45-µm filter to remove silica gel, and evaporating under vacuum to remove the solvent. A nuclear magnetic resonance (NMR) spectrum analysis was performed for each of these fractions.

Analysis of the yellow liquid. The yellow liquid phase from the filtration was analyzed immediately following the autoclaving and filtration using HPLC analysis. A 10-mL sample was kept as a control for later HPLC analysis. The substances in the rest of the liquid phase were recovered by extracting the solution with ethyl acetate. The organic phases were combined, dried with $MgSO_4$, and evaporated under vacuum to remove the solvent. After recording its ^1H NMR spectrum, the residue, a red solid, was recrystallized from CH_2Cl_2. The crystals, which were mainly sinapic acid (TLC, NMR), were discarded, while the liquid CH_2Cl_2 solution was chromatographed through a 300 × 2 cm silica gel column using ethyl acetate as eluant. The elution was run under slight air pressure and 20 fractions were collected. These fractions were evaporated to dryness under a stream of warm air. The fourth fraction, a dull orange solid, was the major fraction. This solid was analyzed using NMR spectroscopy, mass spectroscopy, and HPLC.

Preparation and treatment of sinapine bisulfate solution. A 100 µg/mL sinapine bisulfate solution was prepared by dissolving 5 mg of sinapine bisulfate in 50 mL distilled water. While 10 mL of this solution was kept as control, the rest was autoclaved for 45 min at the same conditions, as for sinapic acid. Spectral and HPLC analysis were performed for the control and the autoclaved sample.

Spectral analysis and color determination. Spectral analyses were performed with a Hewlett-Packard 8452 diode array spectrophotometer (Hewlett-Packard, Palo Alto, CA) with MS-DOS UV-visible (VIS) software looking at both UV (200–350 nm) and VIS spectra (380–780 nm). The same samples were used for both spectral and HPLC analysis. UV spectra were recorded as curves of absorbance as a function of wavelength. VIS spectra were recorded as percentage of

light transmittance as a function of wavelength. The color of a solution was determined by taking the transmittance data at the wavelength range from 380 to 780 nm, calculating the Commission Internationale de l'Eclairage (CIE) 1931 tristimulus values, X, Y, and Z, and mathematically converting to Hunter L a and b values (28).

The calculation of the CIE 1931 tristimulus values, X, Y, and Z from the above transmittance data was based on a weight-ordinate method with a wavelength interval $\Delta\lambda = 10$ (28). This calculation was done with a computer spreadsheet program (Quattro Pro, Version 7, Corel Corporation Limited, Canada). The CIE standard illuminant D_{65} was used as the illuminant in the calculation. Since these X, Y, and Z values do not have a direct proportion to real color intensities, they were converted to Hunter L a b values using the equations $L = 10 \cdot Y^{0.5}$, $a = 17.5 \cdot (1.02 \cdot X - Y)/Y^{0.5}$, and $b = 7.0 \cdot (Y - 0.847 \cdot Z)/Y^{0.5}$ (28). All the reported values were means of two determinations.

HPLC analysis. HPLC was used to monitor the structural changes in sinapic acid and sinapine bisulfate following the autoclave treatment. Chromatographic equipment consisted of two Waters (Milford, MA) pumps (models 501 and 510), an automated gradient controller model 680 (Waters), a Shimadzu (Kyoto, Japan) SPD-6A UV spectrophotometric detector, and a Hewlett-Packard (Avondale, PA) model HP3396II integrator connected with a peak 96 computer software (Hewlett-Packard Company, Avondale, PA) . A reverse-phase C_{18} column (Supelcosyl, 3-µm particle size, 33 × 4.6 mm i.d.; Supelco, Bellefonte, PA) was used. The elution solvent consisted of two buffers. Buffer A was a 0.05 M sodium 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 (29). Buffer A was filtered through a 0.45 µm filter. Buffer B was pure methanol. The column was maintained at 37°C and run at a constant flow rate of 1.4 mL/min.

For sinapic acid solutions before and after autoclaving, the initial elution solvent was 15% methanol and 85% buffer A. After a 10-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.

For the brown solid separated by filtration through a 0.45-µm filter, the initial elution solvent was 15% methanol and 85% buffer A. This composition was altered to 100% methanol with a 10-min linear gradient. The solvent composition was maintained at 100% methanol for 2 min, after which a 2-min linear gradient returned the solvent to the original composition. The brown solid was dissolved in methanol for the HPLC analysis. The same solvent system was used for sinapine bisulfate solutions. Sinapine bisulfate was eluted within the 10-min linear gradient. Mixtures of syringaldehyde and sinapic acid were separated by the same linear gradient solvent.

TLC. In order to qualitatively analyze the colored components, TLC was performed using silica gel TLC plates (Waterman, Clifton, NJ), with 100% ethyl acetate as elution solvent. Crude reproductions of the results were made using a computerized drawing program (Corel Flow, version 7, Corel Corporation Limited, Canada). R_f was measured manually.

Nuclear magnetic resonance and mass spectra. NMR and mass spectral analyses were performed on several materials obtained during the isolation procedure. Samples were dissolved in acetone D_6, filtered through a pipet filled with Kimwipes paper (Kimberly-Clark Corp., Roswell, GA) and collected in NMR tubes for analysis.

The ^1H and ^{13}C NMR spectra were recorded using a Brucker AM-300 spectrometer (Karlsruhe, Germany) with tetramethylsilane as internal standard. Acetone D_6 was used as solvent for all of the NMR analyses. Mass spectra were obtained on a vacuum generator, model VG 7070E-HF instrument (Manchester, England).

RESULTS AND DISCUSSION

Sinapic acid. Spectral and color changes during autoclaving (development of yellow appearance). UV and visual spectral changes for sinapic acid solutions before and after autoclaving are shown in Figure 1 A,B. The UV spectrum of the sinapic acid solution changed from two major peaks (maxima around 230 and 320 nm) to three major peaks (maxima around 210, 225, and 310 nm). There was an increased absorbance in the region above 380 nm after autoclaving (Fig. 1A). It appears that about 40% of the sinapic acid was con-

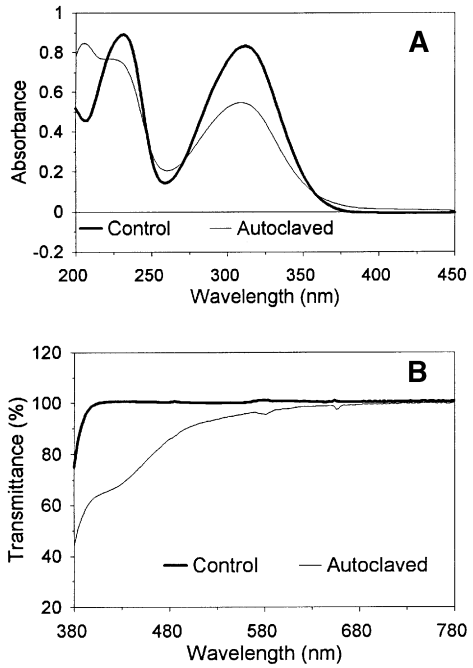


FIG. 1. (A) Effect of autoclaving (121°C, 0.1 MPa, 15 min) on the ultraviolet spectrum (diluted 10 times), and (B) visual transmittance spectrum of a 100 µg/mL sinapic acid solution.

verted to new substances during autoclaving, and that the UV spectrum still mainly represented sinapic acid.

The effects of autoclaving on the visual transmittance spectrum of a 100 µg/mL sinapic acid solution were more dramatic (Fig. 1B). After autoclaving, the solution exhibited a decreased percentage transmittance (increased absorbance), especially in the range from 380 to 480 nm. This would be consistent with the yellow appearance of the solution, since the maximum absorbance occurred in the blue wavelength region. A more precise determination of color was done by calculating the CIE 1931 tristimulus values, X, Y, and Z, and converting to the Hunter L a b values.

The effect of autoclaving time on the Hunter L a b values of a 100 µg/mL sinapic acid solution is shown in Figure 2. Autoclaving treatment decreased whiteness (L value), while significantly increasing yellow (b value) and slightly increasing green color. As a result, the appearance of the solution turned from colorless to yellow after autoclaving. Increasing the heat treatment time from 15 to 30 min resulted in an increase in the intensity of yellow. However, whiteness and green seemed not to be affected by autoclaving time. In addition, further increases in time after 30 min seemed to have no significant effects on the yellow intensity (b value).

Before the autoclaved solution was analyzed by HPLC, it was filtered through a 0.45-µm filter. A brown solid was retained on the filter. This suggested that some of the colored substances, formed during autoclaving, were water insoluble. Therefore, this filtration was used as a technique to separate the brown solid from the yellow liquid, as indicated in Scheme 1.

Analysis of the brown solid phase. HPLC chromatograms of standard sinapic acid and the colored substances in the brown solid phase are shown in Figure 3 A,B. These substances were dissolved in methanol for the HPLC analysis. Figure 3A shows the HPLC chromatogram of the standard sinapic acid and Figure 3B that of the brown solid. A number

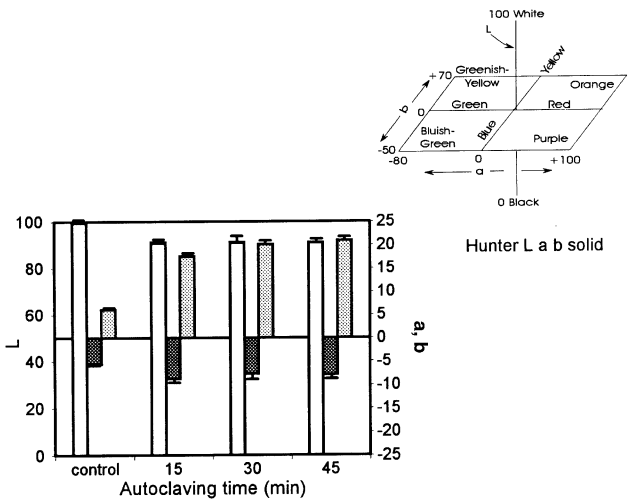


FIG. 2. Effect of autoclaving on the Hunter L a b values of a 100 µg/mL sinapic acid solution. L, white bars; a, gray bars; b, dark bars.

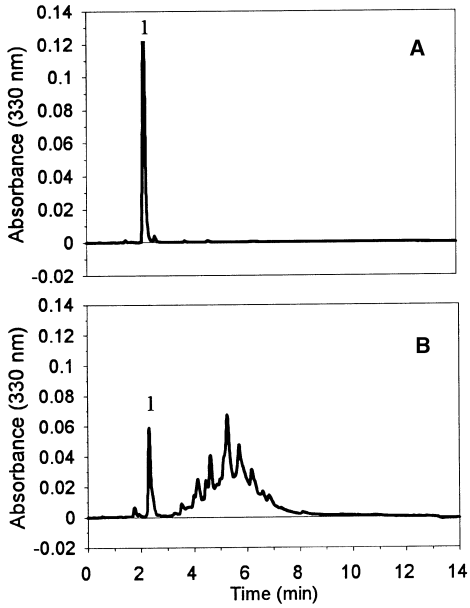


FIG. 3. High-performance liquid chromatography (HPLC) chromatograms of the solid phase. Samples were applied to HPLC column as methanol solutions. (A) control sinapic acid, (B) brown solid separated by filtering through a 0.45 µm filter. 1. Sinapic acid, other peaks were unidentified.

of peaks were present in the HPLC chromatogram (Fig. 3B), suggesting the presence of several new compounds. Determination of the structures of these compounds required further experimentation.

Based on the HPLC results indicating that there were several compounds present in the solid phase after filtration (Fig. 3A,B), TLC was used to separate three strong bands, which were yellow, orange and purple (Fig. 4). These three bands had retention factors (R_f) of 0.95 ± 0.01, 0.91 ± 0.01, and 0.72 ± 0.02, respectively, with the yellow band being the major fraction. In addition, it was noted that the yellow and orange compounds were UV light-sensitive and turned purple when exposed to UV light for a few minutes. There were also some other faintly colored bands found using TLC analysis. The compounds found in the TLC analysis do not necessarily correspond to the compounds detected by HPLC.

The results of NMR analysis of the yellow, orange, and purple bands gave poorly resolved spectra with many peaks.

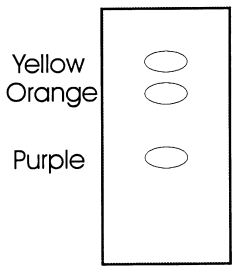


FIG. 4. Thin-layer chromatography of the solid phase with 100% ethyl acetate as eluant.

The only conclusion that could be drawn was that the yellow substance seemed to be a dimer of sinapic acid. There appeared to be two sets of signals for the protons on the aromatic ring and two sets of signals for the methoxyl groups for the yellow substance, in comparison with only one set of each of these signals for the sinapic acid. It was also possible that the yellow substance may be composed of two major products as evidenced by signals in both the aromatic and methoxyl region.

Analysis of the yellow liquid phase. The results of the HPLC analysis for the control sinapic acid and the liquid phase of the autoclaved samples after filtration are shown in Figure 5A,C. Immediately following autoclaving, a small peak corresponding to thomasidioic acid and another peak (unknown) were found in the liquid phase (Fig. 5B). Sinapic acid, however, remained as the predominant component after autoclaving (Fig. 5B). HPLC results indicated that about 40% of the sinapic acid was lost during autoclaving. Therefore, only part of the sinapic acid was converted to new substances during autoclaving, and these new substances may be present only in small amounts since almost no peak other than sinapic acid stood out. After 20 d at 22°C, with daily loosening of the cap of the small bottle for air, the peak of thomasidioic acid was more pronounced whereas the unknown peak seemed to disappear. The peak height of the sinapic acid also decreased (Fig. 5C). The pH of the solution increased from 4.3 to 5.3 during this period of storage. The increase in thomasidioic acid content may be due to the conversion of sinapic acid to thomasidioic acid at higher pH values, although the final pH value was still in the acid region. Rubino *et al.* (27) reported the formation of thomasidioic acid when sinapic acid was exposed to neutral or alkaline conditions. In detailed studies on the conversion of sinapic acid to thomasidioic acid, it was shown that oxygen was necessary for the conversion (27,30). During the storage test, air was introduced occasionally. The formation of thomasidioic acid was confirmed by NMR spectroscopy. Some thomasidioic acid was formed during the autoclave treatment, although the mechanism of this conversion is unknown.

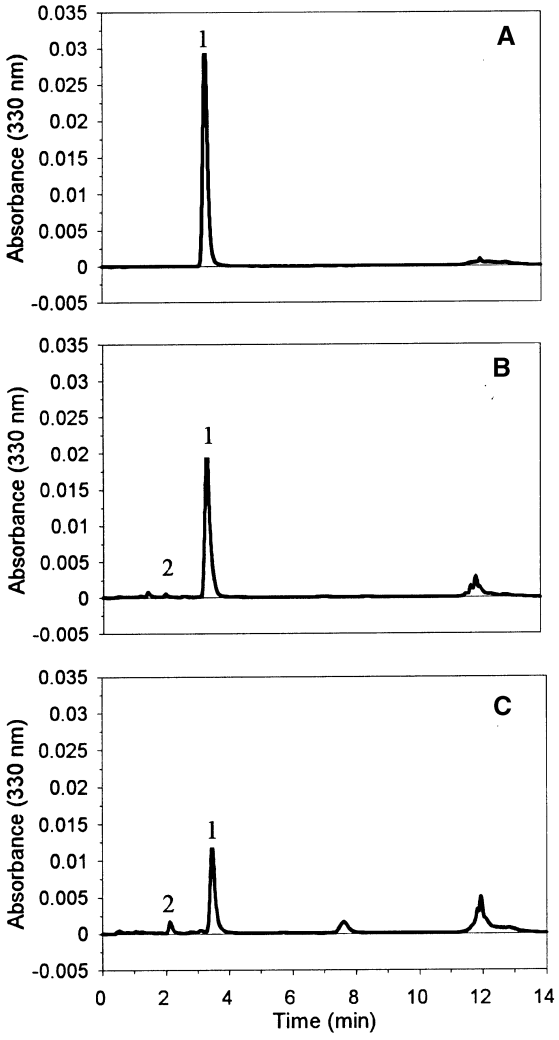


FIG. 5. HPLC chromatograms of the yellow liquid phase. Samples were applied to the HPLC column as an aqueous solution. (A) Control sinapic acid (sample pH 4.3), (B) liquid phase separated by filtering through a 0.45 µm filter (sample pH 4.3), (C) sample of filtered liquid phase after standing at 22°C for 20 d (sample pH 5.3). 1. Sinapic acid, 2. thomasidioic acid, other peaks were unidentified. For abbreviation see Figure 3.

When the sinapic derivatives in the liquid phase were recovered and chromatographed on silica gel, a new substance, syringaldehyde, was identified by ^{1}H and ^{13}C NMR, by mass spectral analysis, and by HPLC analysis of one of the major fractions.

The identification of syringaldehyde in the liquid phase was made by NMR and mass spectroscopic investigation of the major fraction recovered from silica gel chromatography. NMR spectral data for standard sinapic acid, NMR and mass spectral data for standard syringaldehyde, and the spectral data for the unknown compound present in the liquid are shown in Table 1. The NMR spectrum and mass spectral data of the unknown were identical to those of the standard syringaldehyde. This indicated that syringaldehyde had formed during autoclaving.

The identification of syringaldehyde was not readily apparent, since the unknown sample was a mixture of syringaldehyde and several other substances. In fact, the results of ^{1}H NMR spectrum of the whole residue showed the sample to be the mixture of three major substances, sinapic acid, syringaldehyde, and thomasidioic acid. After the residue was recrystallized from CH_2Cl_2 and chromatographed through the silica gel, the major fraction still contained two substances, sinapic acid and syringaldehyde (NMR). This meant that the separation of syringaldehyde from sinapic acid was not complete, although the separation involved several steps. This may be, in part, due to the similarity of the polar nature for both substances. Because of the incomplete separation, the presence of syringaldehyde was not easily confirmed by ^{1}H NMR spectroscopy alone. However, ^{13}C NMR spectroscopy provided the typical nine-carbon spectrum. In addition, the ^{1}H NMR spectrum exhibited a typical low-field aldehyde proton signal. With this information at hand, NMR spectra

TABLE 1
Nuclear Magnetic Resonance and Mass Spectra of Standards and Compounds Produced During Autoclaving

Description	Spectrum technique	Spectra
Sinapic acid	^{1}H NMR (acetone D_6)	7.58 (*d*, 1H, *J* = 15.9 Hz), 7.01 (*s*, 2H), 6.39 (*d*, 1H, *J* = 15.8 Hz), 3.91 (*s*, 6H, OCH_3)
	^{13}C NMR (acetone D_6)	167.98 (CO), 148.39 (2C),146.00 (C), 139.20 (C), 126.01 (CH),116.08 (CH), 106.63 (2CH), 56.52 ($2CH_3$)
Syringaldehyde	^{1}H NMR (acetone D_6)	9.79 (*s*, 1H), 7.21 (*s*, 2H), 3.90 (*s*, 6H, OCH_3)
	^{13}C NMR (acetone D_6)	191.12 (CO), 148.94 (2C),142.93 (C), 128.96 (C),107.73 (2CH), 56.63 ($2CH_3$)
	Mass spectrum *m/z* (relative intensity)	182 (100), 167 (20), 153 (8), 139 (13), 111 (16), 96 (11), 79 (12), 65 (15)
Compound identified	^{1}H NMR (acetone D_6)	9.81 (*s*, 1H), 7.23 (*s*, 2H), 3.92 (*s*, 6H, OCH_3)
	^{13}C NMR (acetone D_6)	190.93 (CO), 148.84 (2C), 142.82 (C), 128.87 (C), 107.63 (2CH), 56.73 ($2CH_3$)
	Mass spectrum *m/z* (relative intensity)	182 (100), 167 (20), 153 (7), 139 (13), 111 (20), 96 (9), 79 (15), 65 (18)

were examined more closely. Two groups of peaks, representing sinapic acid and syringaldehyde with a molar ratio of around 1:0.27, were found in both ^{1}H and ^{13}C NMR spectra for the major fraction from silica gel chromatography. Three groups of peaks, representing sinapic acid, syringaldehyde molar and thomasidioic acid with a ratio of around 1:0.1:0.03, were found in ^{1}H NMR spectrum of the ethyl acetate extract from the liquid phase. The major fraction from silica gel chromatography was also analyzed using mass spectroscopy. The major mass peaks corresponded to syringaldehyde alone since syringaldehyde has a lower boiling point than does sinapic acid. Only syringaldehyde was evaporated and recorded in the spectrum under the test condition. The spectrum of the unknown was almost perfectly matched with that of the standard. The unknown was therefore identified to be syringaldehyde.

Based on NMR and mass spectral results, HPLC analysis was conducted to confirm the presence of syringaldehyde in the liquid phase. To ensure that the formation of syringaldehyde was not due to other treatments following the autoclaving, a blank control sinapic acid was also analyzed. Figure 6A–D shows the results of HPLC analysis for a recrystallized control sinapic acid (Fig. 6A), a standard sample of syringaldehyde, (Fig. 6B), the mixture of the standard sinapic acid, and the standard syringaldehyde (Fig. 6C), and the unknown sample from the column chromatographic step (Fig. 6D). Two peaks were found in the unknown sample, which were identified as sinapic acid and syringaldehyde. The identification was done by comparing the chromatogram of the unknown sample (Fig. 6D) with that of the mixture of the standard sinapic acid and the standard syringaldehyde (Fig. 6C). A sinapic acid standard (not shown, result similar to Fig. 6A) and a syringaldehyde standard (Fig. 6B) were also examined separately to ensure that no interaction of the two substances was interfering with the elution times. To prove that syringaldehyde was produced during autoclaving but not during the sample preparation following the autoclaving, the standard sinapic acid was used as a blank sample and run through the same experimental procedure but without autoclaving. To do this, the sinapic acid was dissolved in water, then recovered by ethyl acetate extraction followed by recrystallization from CH_2Cl_2. After removing the solvent under vacuum, the substance was analyzed by HPLC. The HPLC chromatogram of this blank sinapic acid is given in Figure 6A. The single peak was identified as sinapic acid. There was no evidence of syringaldehyde in the control sample. This proved that syringaldehyde was formed during autoclaving. Attempts were made to improve resolution of the HPLC analysis by using two other gradients for the mixture of sinapic acid and syringaldehyde. However, similar results with no improvement in resolution were obtained. Further experimentation for this purpose was terminated since the presence of syringaldehyde was firmly confirmed based on NMR and mass spectral analysis. HPLC analysis was mainly conducted to confirm the results of the NMR and mass spectral analysis. Thomasidioic acid found in the previous HPLC chromatograms (Fig. 5A–C) was not detected in the major fraction from silica gel column chromatography. This may indicate a good separation between thomasidioic acid and syringaldehyde during the silica gel chromatography.

Retention time for syringaldehyde was very close to that for sinapic acid. In addition, the concentration of syringalde-

hyde in the liquid phase was low. It was estimated from HPLC data that 40% of the sinapic acid was converted to other substances during a 30-min autoclaving. The starting material was a 100 µg/mL sinapic acid aqueous solution. The autoclaved solution contained about 60 µg/mL sinapic acid, about 6 µg/mL syringaldehyde, about 2 µg/mL thomasidioic acid (HPLC, NMR), and the rest were undetermined substances. The earlier HPLC analysis of the yellow liquid phase (Fig. 5B,C) did not show the presence of syringaldehyde. This is due to the excess of sinapic acid present, which masks the signal from syringaldehyde owing to their similar retention time.

In order to evaluate the color significance of syringaldehyde, the UV and transmittance spectra of syringaldehyde were compared with those of the sinapic acid (Fig.7 A,B). The UV spectra for both substances are shown in Figure 7A. Syringaldehyde had a spectrum with two maxima of around 220 and 310 nm. Both maxima are at lower wavelengths than those of the sinapic acid. While sinapic acid shows almost no absorbance in the wavelength region from 275 to 400 nm, syringaldehyde shows a slight absorbance in this region. This is consistent with the dull yellow appearance of syringaldehyde. The transmittance spectra for syringaldehyde and sinapic acid are shown in Figure 7B. Syringaldehyde shows a relatively lower percentage transmittance than does sinapic acid in the whole visual light region, especially in the wavelength region from 380 to 400 nm. Therefore, the formation of syringaldehyde would contribute a yellowness and a darkness to the sinapic acid solution. However, comparison of the transmittance spectrum of syringaldehyde solution (Fig. 7B) with that of the autoclaved sinapic acid solution (Fig. 1B), leads to the

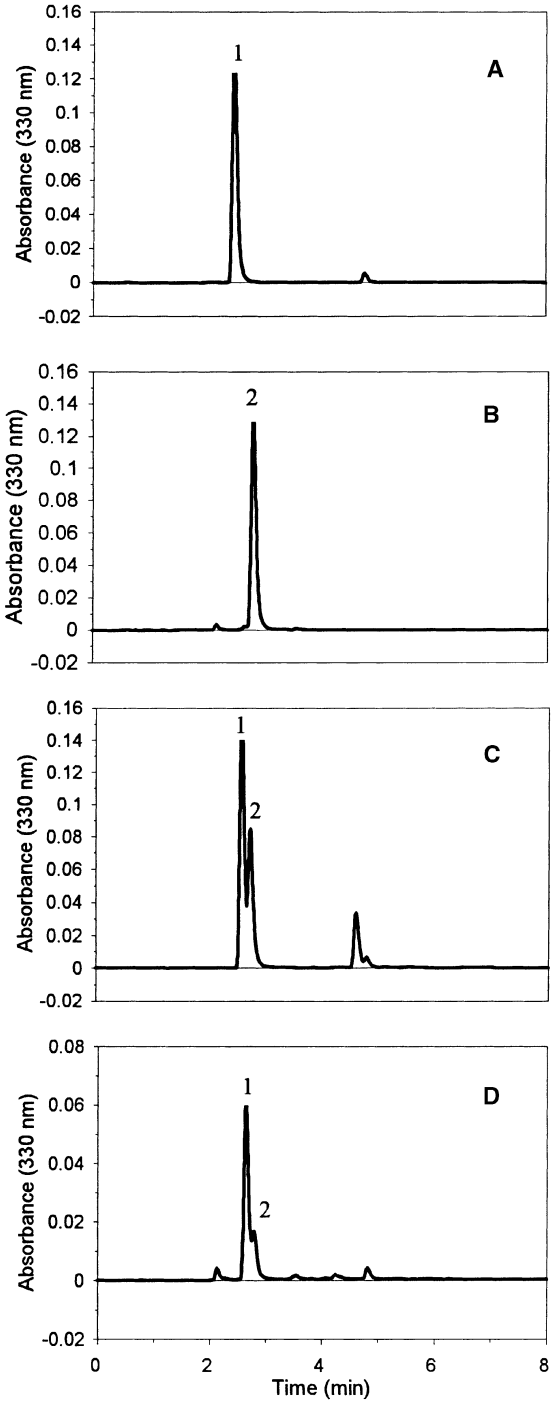


FIG. 6. HPLC chromatograms of the major fraction from silica gel chromatography (A) sinapic acid blank, (B) standard syringaldehyde, (C) mixture of standard sinapic acid and syringaldehyde, and (D) unknown sample identified to be a mixture of sinapic acid and syringaldehyde. 1. Sinapic acid. 2. Syringaldehyde. For abbreviation see Figure 3.

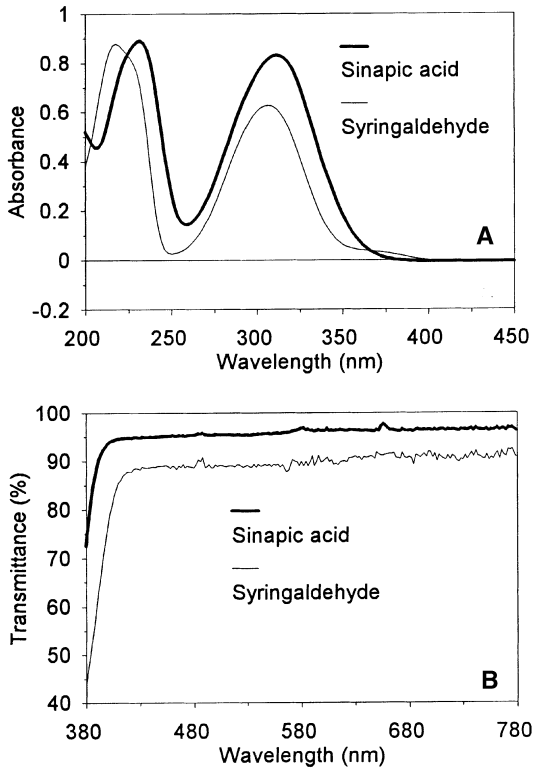


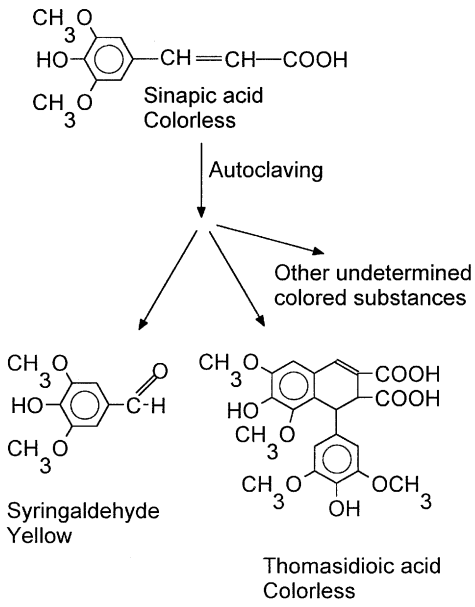
FIG. 7. (A) Comparison of ultraviolet spectra (dilute 10 times and (B) visual transmittance spectra of 100 µg/mL syringaldehyde and 100 µg/mL sinapic acid aqueous solutions.

conclusion that syringaldehyde contributes only a very small amount of yellowness and darkness to the solution. Other undetermined components, especially those three colored substances shown by TLC, could be the major components responsible for color change of sinapic acid during autoclaving. The concentration of these highly colored compounds may be much lower than that of syringaldehyde.

Sinapine bisulfate. The results of UV and visual spectral analysis for sinapine bisulfate solution before and after autoclaving are shown in Figure 8 A,B. Spectra of sinapine bisulfate for the control and autoclaved sample were perfectly matched. This would suggest that no changes occurred during autoclaving.

Both control and autoclaved sinapine bisulfate solutions (100 μg/mL) were analyzed using HPLC for a storage period of 20 d at 22°C with a daily loosening of the cap of the small bottle for air. No differences were found between the two groups of samples (HPLC chromatograms not shown). This also suggested that no structural changes occurred during autoclaving.

In conclusion, it has been demonstrated that a yellow coloration developed when a 100 μg/mL sinapic acid aqueous solution was autoclaved for 15 min at 121°C and 0.1 MPa. A newly formed yellow substance was identified to be syringaldehyde by NMR, mass spectral, and HPLC analysis. Thomasidioic acid was also formed during autoclaving. There were at least three other colored substances estimated to be present by TLC analysis. However, their identities have not been determined. The structural and color changes of a 100 μg/mL sinapic acid aqueous solution during autoclaving are summarized in Scheme 2. Sinapine bisulfate, under the same conditions, showed no evidences of any structural or color changes.



SCHEME 2

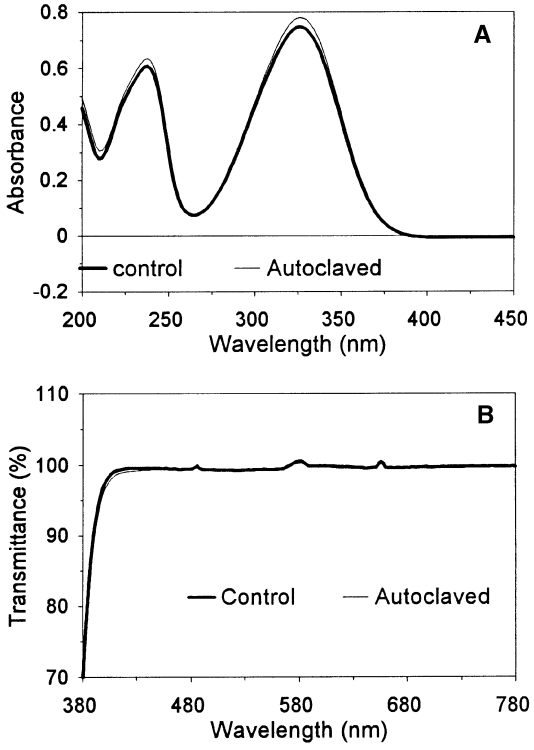


FIG. 8. (A) Effect of autoclaving on the UV spectrum (diluted 10 times, and (B) visual transmittance spectrum of a 100 μg/mL sinapine bisulfate bisulfate aqueous solution.

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