Conversion of Phenolics to Lignans: Sinapic Acid to Thomasidioic Acid

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ABSTRACT: Changes in sinapic acid when exposed to aqueous alkaline conditions were elucidated. Sinapic acid was exposed to a volatile buffer (pH 8.5) for 24 h, lyophilized, acidified, extracted, and characterized using nuclear magnetic resonance and mass spectroscopy. The product obtained was identified as the lignan thomasidioic acid. This identification was confirmed by comparison with a synthesized authentic sample of thomasidioic acid. Conversion of sinapic acid to thomasidioic acid under alkaline conditions previously has not been reported. Thomasidioic acid was present after exposure of sinapic acid to pH 8.5 for as few as 6 h. Thomasidioic acid also was formed at pH 7.

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Canola is one of the main sources of vegetable oil in Canada. The seeds of this crop contain approximately 40% edible oil, and the meal produced after oil extraction contains 40% protein (1). Presently, the meal is used for animal feed, although there have been several studies targeted at isolating the protein for human consumption (2–5). Despite the fact that the amino acid profile for canola protein is similar to that for soybean (though higher in methionine and lower in lysine than soybean), extraction and utilization of canola protein have been retarded by higher fiber content and presence of antinutritional factors, such as glucosinolates, phytic acid, and phenolic compounds (6). The present research focuses on the phenolic compounds.

Phenolic compounds in canola meal are responsible for bitter taste, brown color, and digestibility problems. Some studies have shown that the distasteful flavor in meat and milk, as a consequence of including canola meal in animal feed, is mainly due to the presence of sinapine (7,8), a phenolic ester of sinapic acid and choline. Also, a fishy taint in brown-shelled eggs has been attributed to the presence of sinapine in the meal (9,10). Phenolic compounds also may interact with proteins, such as enzymes and functional storage proteins. This may inactivate some digestive enzymes, inhibiting protein utilization, thereby lowering the nutritional value of canola products (11,12).

The phenolic acids in canola are present in free, esterified, and insoluble-unbound forms. The esterified form constitutes 80% of the total phenolics present, and the free phenolic makes up 16% (13,14). Sinapic acid is the predominant free phenolic acid and the principal phenolic acid released from esters and glucosides (13–15). In a survey of the composition of free hydrolyzable phenolic acids in ten defatted oilseeds, canola contained the highest level of sinapic acid, followed by mustard (15). In all other materials, sinapic acid represented only a minor portion of the phenolic compounds.

In ongoing work looking at the interactions between canola protein and sinapic acid, we noted that there were significant changes in sinapic acid when exposed to alkali. These changes included a color change as well as a shift in the elution time during high-pressure liquid chromatography (HPLC) when samples were adjusted to pH 7 or 8.5. Furthermore, the presence of lignan-type products during processing of canola has been previously reported (16). The objective of this work, therefore, was to elucidate the changes in sinapic acid that occur at alkaline pH. This will provide basic information required for examining more complex systems, such as processed canola meal or protein isolates.

MATERIALS AND METHODS

Materials. Sinapic acid (3,5-dimethoxy-4-hydroxycinnamic acid) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Aldrich silica gel (230–400 mesh, 60 Å) was used for column chromatography. Thin-layer chromatography (TLC) was performed using premade silica gel TLC plates (Whatman, Clifton, NJ). All chemicals used for HPLC were HPLC-grade.

Instrumental methods. The ^{1}H and ^{13}C NMR (nuclear magnetic resonance) spectra were recorded using a Brucker AM-300 spectrometer (Karlsruhe, Germany) with tetramethylsilane as internal standard (residual protonated dimethyl sulfoxide [DMSO], δ 2.5, was used as internal standard in

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DMSO-D_6). Mass/mass spectra were obtained on a Vacuum Generator, model VG 7070E-HF instrument (Manchester, England).

HPLC was used to rapidly identify thomasidioic acid and sinapic acid. Chromatographic equipment consisted of two Waters (Milford, MA) pumps (model 501 and 510) and automated gradient controller model 680, a Shimadzu (Kyoto, Japan) SPD-6A ultraviolet (UV) spectrophotometric detector, and a Hewlett-Packard (Avondale, PA) model HP3396II integrator. The UV detector was set at 330 nm. A reverse-phase column (Supelcosyl, 3-μm particle size, 33 × 4.6 mm i.d.; Supelco, Bellefonte, PA) was used. The buffer (buffer A) used for elution was a 1:100 water dilution of a stock pH 4.7 acetate buffer, prepared by adjusting 5M acetic acid to pH 4.7 with NaOH and filtering through a 0.22 μm filter (17). The initial elution solvent was methanol/buffer A (15:85, vol/vol). After 8 min of isocratic flow at 1.7 mL/min, a 3-min linear gradient was used to change the solvent composition to 100% methanol to clean the column. The column was maintained at 37°C and run at a constant flow rate of 1.7 mL/min.

For standards, 2 mg of thomasidioic acid (synthesized as described below) and 2 mg of sinapic acid were dissolved in 5 mL of methanol/buffer A (15:85, vol/vol). Volumes ranging 1–5 μL of this mixture were injected by using a 20-μL sample loop.

Sample preparation for HPLC analysis. Two samples of sinapic acid, 5 mg in 40 mL of water, were adjusted to pH 7 and 8.5 using 0.2M NaOH. After the solutions reached the desired pH, they were left for at least 6 h stirring at room temperature in air. The solutions were then evaluated by HPLC as described above. Samples at both pH values were examined at least in triplicate.

Identification of thomasidioic acid. The procedure adopted for identifying the products formed from sinapic acid when exposed to alkaline condition is outlined in Figure 1. Fifty milligrams of sinapic acid, whose ^{13}C NMR spectrum was recorded in DMSO-D_6 (Table 1), was dissolved in 200 mL of 0.3 M aqueous volatile buffer containing NH_4HCO_3 and NH_4OH (pH 8.5) and stirred for 24 h in air. The solution was lyophilized, and ^1H and ^{13}C NMR spectra were recorded using DMSO-D_6 as solvent (Table 1). At least three separate batches of sinapic acid were treated to give this product. To further identify the compound formed, part of a sample also was acidified to pH 2 with 1N HCl, extracted three times with ethyl acetate after saturating the water phase with NaCl, dried using Mg_2SO_4, and evaporated under vacuum. An ^1H NMR spectrum in DMSO-D_6 was recorded (Table 1) using part of this sample. The remainder (20 mg) was exposed overnight to diazomethane (18) in a mixture of ethyl acetate and methylene chloride and then evaporated under vacuum to dryness. The residue was dissolved in methylene chloride (5 mL), and chromatographed through a silica gel open column (1 × 30 cm) under pressure, eluting with ethyl acetate and hexane (40%/60%, vol/vol) and collecting in 5 mL fractions. The fractions were analyzed by TLC in the same solvent mixture. Two compounds were separated (9.6 and 9.4 mg), and ^1H NMR spectra were recorded for both using deuterated chloroform ($CDCl_3$) as solvent. The ^1H NMR and mass spectra for the first eluted sample are reported in Table 1. Sinapic acid (25 mg) also was exposed to aqueous alkaline buffer (150 mL) for 6 h, lyophilized, and the ^{13}C NMR spectrum in DMSO-D_6 recorded (Table 1).

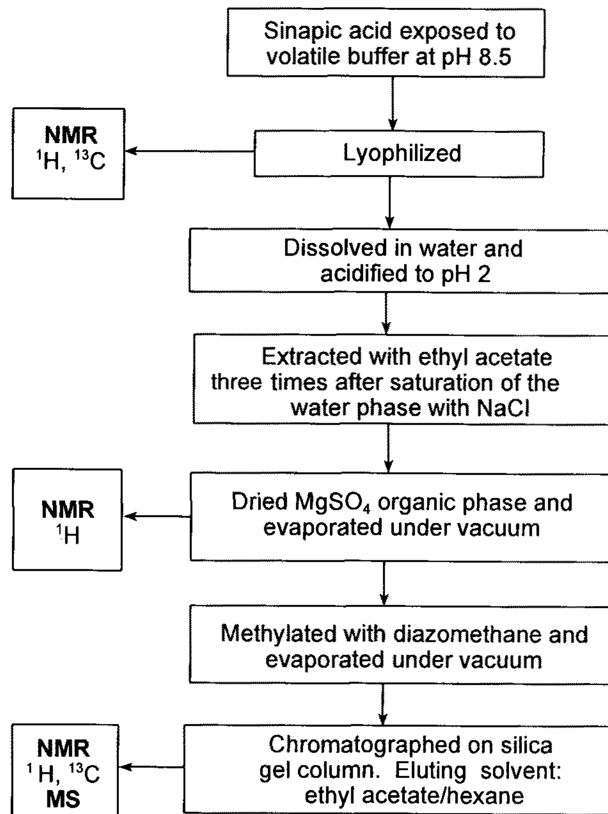


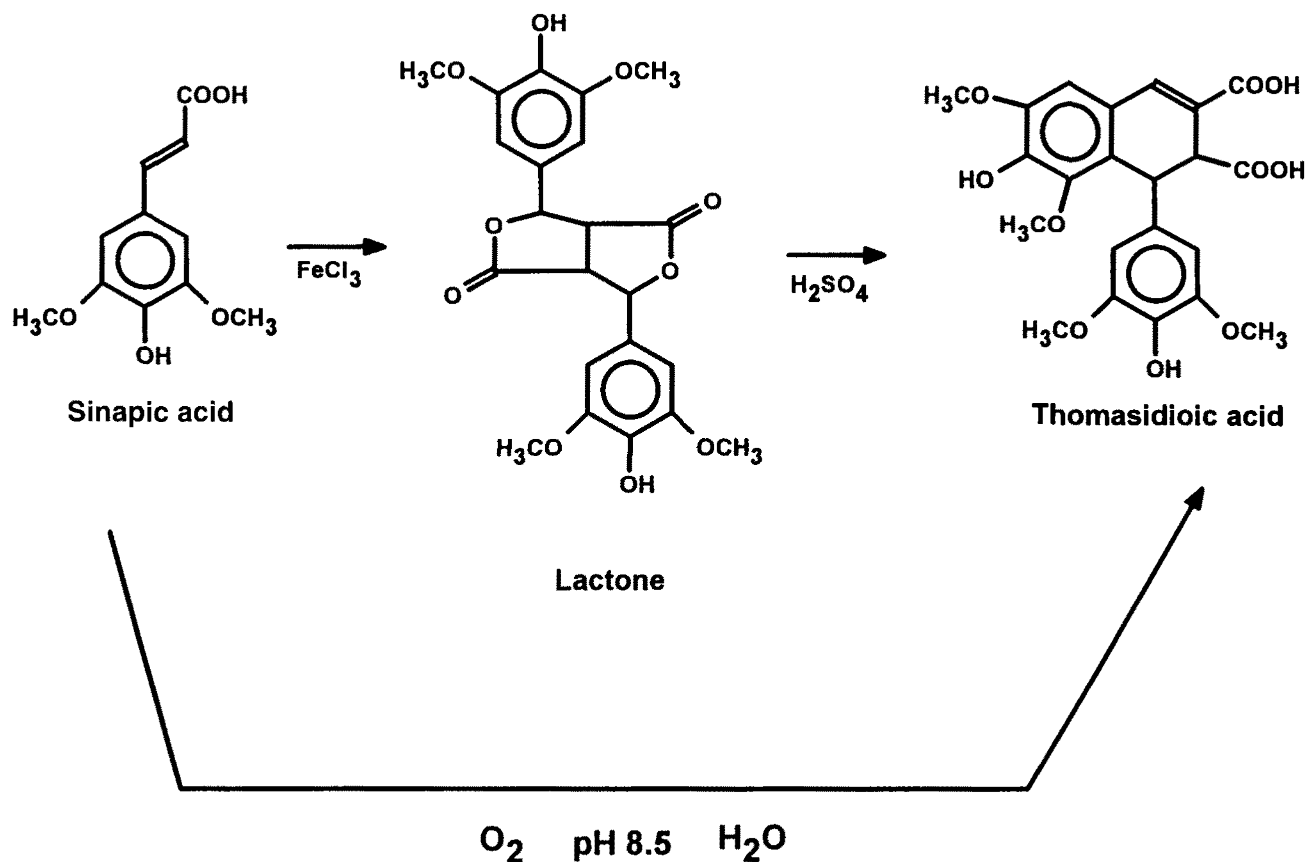
FIG. 1. Flow chart of protocol used in identifying thomasidioic acid; NMR, nuclear magnetic resonance; MS, mass spectrometry.

Synthesis of thomasidioic acid. Sinapic acid (40 mg) was converted to dehydrosinapic acid dilactone (Scheme 1) by treating with $FeCl_3$ in the presence of O_2, using the method previously described (19,20). The lactone was converted to thomasidioic acid by a brief treatment (30 s) with concentrated H_2SO_4, followed by dilution with cold water. While the original method converted the lactone to thomasidioic acid using HCl in dioxane, it was found that the treatment with H_2SO_4 was more efficient. The resulting mixture was extracted with ethyl acetate three times, dried with $MgSO_4$, and evaporated under vacuum. Ethyl acetate was used in place of methanol/chloroform as suggested in the original paper. The residue was dissolved in methanol, charcoal added, the mixture filtered, and the methanol evaporated under nitrogen. The residue was recrystallized from ethyl acetate. The ^1H NMR spectrum was determined using DMSO-D_6 as a solvent (Table 2). The ^{13}C NMR spectrum was determined by dissolving the sample in NH_4HCO_3 buffer (pH 8.5), evaporating and recording the spectrum in DMSO (Table 2).

TABLE 1
Nuclear Magnetic Resonance (NMR) and Mass Spectra of Compounds Produced in the Identification of Thomasidioic Acid[a]

Treatment of sinapic acid	Spectrum technique	Spectra
Sinapic acid	^{13}C NMR (DMSO)	δ 168.15 (CO), 148.19 (2C), 144.97 (CH), 138.22 (C), 124.81 (CH), 116.23 (CH), 106.19 (2CH), 56.21 (2CH_3)
Sinapic acid exposed 24 h to aerated buffer at pH 8.5	^{1}H NMR (DMSO)	δ 6.94 (*s*, 1H), 6.76 (*s*, 1H), 6.24 (*s*, 2H), 4.90 (*s*, 1H), 3.78 (*s*, 3H, OCH_3), 3.76 (*s*, 1H), 3.60 (*s*, 6H, OCH_3), 3.58 (*s*, 3H, OCH_3), 3.34 (*s*, 1H)
	^{13}C NMR (DMSO)	δ 172.88 (CO), 172.02 (CO), 147.50 (2C), 147.11 (C), 145.52 (C), 140.35 (C), 135.03 (C), 134.00 (C), 130.29 (CH), 129.40 (C), 123.94 (C), 123.04 (C), 107.37 (CH), 105.26 (2CH), 59.45 (CH_3), 56.02 (3CH_3), 50.27 (CH), 37.88 (CH)
Sinapic acid exposed 24 h to aerated buffer at pH 8.5, acidified and extracted	^{1}H NMR (DMSO)	δ 9.15 (*s*, 1H), 8.20 (*s*, 1H), 7.54 (*s*, 1H), 6.98 (*s*, 1H), 6.21 (*s*, 2H), 4.81 (*s*, 1H), 3.82 (*s*, 3H, OCH_3), 3.74 (*s*, 1H), 3.60 (*s*, 6H, OCH_3), 3.49 (*s*, 3H, OCH_3)
Sinapic acid exposed 24 h to aerated buffer at pH 8.5, acidified, methylated, and chromatographed. First eluted compound	^{1}H ($CDCl_3$)	δ 7.64 (*s*, 1H), 6.72 (*s*, 1H), 6.25 (*s*, 2H), 4.99 (*s*, 1H), 4.06 (*d*, 1H, *J* = 1.2), 3.89 (*s*, 3H, OCH_3), 3.88 (*s*, 3H, OCH_3), 3.78 (*s*, 3H, OCH_3), 3.77 (*s*, 3H, OCH_3), 3.72 (*s*, 6H, OCH_3), 3.67 (*s*, 3H, OCH_3), 3.64 (*s*, 3H, OCH_3)
	Mass spectrum *m/e* (relative intensity)	503 (23), 502 (M^+, 82), 411 (100), 396 (24), 384 (74), 380 (48), 369 (16), 35 (16, 303 (30)
Sinapic acid exposed to buffer at pH 8.5 for 6 h	^{13}C NMR (DMSO)	δ 172.85 (CO), 171.97 (CO), 147.49 (2C), 147.11 (C), 145.52 (C), 140.33 (C), 135.04 (C), 134.00 (C), 130.25 (CH), 129.43 (C), 123.94 (C), 123.05 (C), 107.37 (CH), 105.26 (2CH), 59.45 (CH_3), 56.01 (3CH_3), 50.27 (CH_3), 37.87 (CH)

[a]DMSO, dimethyl sulfoxide.



SCHEME 1

TABLE 2
NMR Characterization of Thomasidioic Acid[a]

Description	Spectrum technique	Spectra
7-Hydroxy-6,8-dimethoxy-1-(4′-hydroxy-3′,5′-dimethoxyphenyl)-*trans*-1,2-dihydronaphtalene-2,3-dicarboxilic acid, thomasidioic acid	^{1}H NMR (DMSO)	δ 12.45 (*br s*, 2H), 9.13 (*s*, 1H), 8.17 (*s*, 1H), 7.54 (*s*, 1H), 6.98 (*s*, 1H), 6.21 (*s*, 2H), 4.82 (*s*, 1H), 3.83 (*s*, 3H, OCH_3), 3.75 (*s*, 1H), 3.61 (*s*, 6H, OCH_3), 3.49 (*s*, 3H, OCH_3)
	^{13}C NMR (DMSO)	δ 172.82 (CO), 171.81 (CO), 147.49 (C), 147.11 (C), 145.50 (C), 140.33 (C), 135.01 (C), 1433.99 (C), 130.31 (CH), 129.34 (C), 123.91 (C), 123.06 (C), 107.37 (CH), 105.24 (2CH), 59.46 (CH_3), 56.01 ($3CH_3$), 50.21 (CH), 37.88 (CH)

[a]See Table 1 for abbreviations.

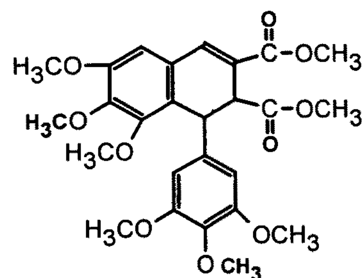
RESULTS AND DISCUSSION

Identification of thomasidioic acid. Changes in the solution color and elution time for sinapic during HPLC analysis were noted when aqueous solutions of sinapic acid were exposed to pH 7 and 8.5. To identify the compound responsible, a volatile buffer was used so that the pH could be maintained during conversion, yet be easily removed during lyophilization for analysis of the product. The ^{1}H and ^{13}C NMR spectra of the product resulting from exposure of sinapic acid to pH 8.5 in this manner were significantly different from those for sinapic acid, so it was clear changes were taking place. The identity of the new compound, however, was not readily apparent.

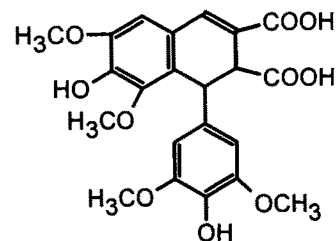
Acidification of the sample was used as a means to ensure that the new compound was in the acid form and, therefore, easier to identify with NMR; but identifying the new compound from the ^{1}H NMR spectrum (Table 1) was again unsuccessful. Furthermore, inspection of the NMR spectrum suggested that more than one compound may have been present. The polar nature of the material, however, made it impossible to purify by chromatography on silica gel. Treating the sample with diazomethane was to methylate all of the acidic hydroxyl groups to increase solubility and facilitate chromatography. Two compounds were separated from the methylated sample upon chromatography on silica gel using ethyl acetate/hexane as eluent. The ^{1}H NMR spectra of these compounds were recorded in $CDCl_3$. From these spectra, it was observed that the first compound had eight methoxyl groups, and we concluded that the precursor to this compound must have been a dimer of sinapic acid. The mass of the compound was determined by mass spectrometry (MS) and tentatively identified as the dimethyl ether/dimethyl ester of thomasidioic acid (Scheme 2). The second compound, which was not fully characterized, corresponded to partial methylation of the thomasidioic acid, since it only had seven methoxyl groups. The unmethylated compound was identified as thomasidioic acid.

The identification was confirmed by comparison with an authentic sample of thomasidioic acid synthesized from sinapic acid. The NMR spectrum of the synthetic thomasidioic acid coincided with the spectrum found in the literature (19–21) and was identical to the thomasidioic acid produced from sinapic acid in basic buffer (Tables 1 and 2).

From the experiments outlined above, the conversion of sinapic acid to thomasidioic acid can be accomplished following two routes as shown in Scheme 1. The reaction under acidic conditions with dehydrosinapic acid lactone as an intermediate previously has been reported, but the unexpected conversion of sinapic acid to thomasidioic acid under basic conditions has never previously been observed. As shown in Tables 1 and 2, the ^{13}C spectra of thomasidioic acid synthesized by the modified literature procedure, and of the thomasidioic acid which resulted from the exposure of sinapic acid



Dimethyl ether dimethyl ester of thomasidioic acid



Thomasidioic acid

SCHEME 2

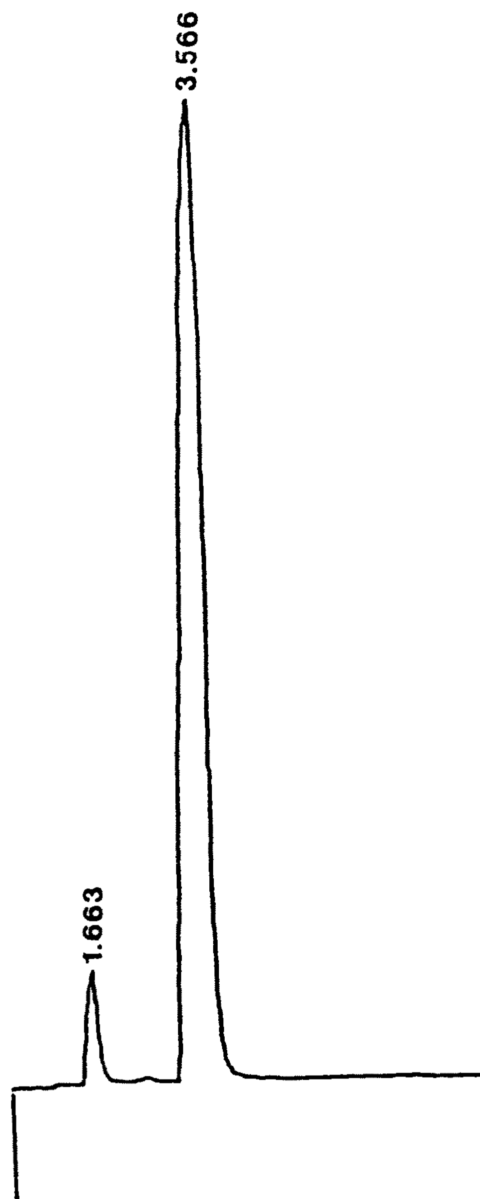


FIG. 2. High-pressure liquid chromatography chromatograms of thomasidioic acid standard (1.663 min) and sinapic acid standard (3.409 min).

to the aqueous alkali buffer, were identical. Furthermore, we also observed that under the same conditions thomasidioic acid also was partially formed after only 6 h of exposure of sinapic acid to the alkali buffer (Table 2).

Detection of thomasidioic acid. We felt that it would be valuable to develop a procedure which could identify thomasidioic acid in the presence of other phenolics, specifically sinapic acid, since both could be present in the sample. An HPLC method was developed to identify thomasidioic acid. The peaks of sinapic acid and thomasidioic acid standards were well separated using this chromatographic method (Fig. 2). The chromatograms of sinapic acid exposed to pH 8.5 and 7 are shown in Figure 3. Each solution required several pH adjustments until they were pH stable. In both chromatograms, a peak corresponding to thomasidioic acid was observed. There was, however, evidence that sinapic acid was present in the sample at pH 7.

It has been demonstrated that sinapic acid can readily transform into thomasidioic acid when exposed to alkaline conditions and that this reaction can take place even at pH 7. This information is significant since there are many procedures that have been proposed where canola meal is exposed to solvent systems which include alkali, such as ammonia, to extract antinutrients or to produce protein isolates (22–24).

The incidence of thomasidioic acid in the canola meal needs to be assessed. Future research will focus on the degree of toxicity of thomasidioic acid, the levels of thomasidioic acid present in the meal when exposed to various alkaline conditions, and developing an understanding of the mechanism by which the sinapic acid is converted into thomasidioic acid under alkaline conditions.

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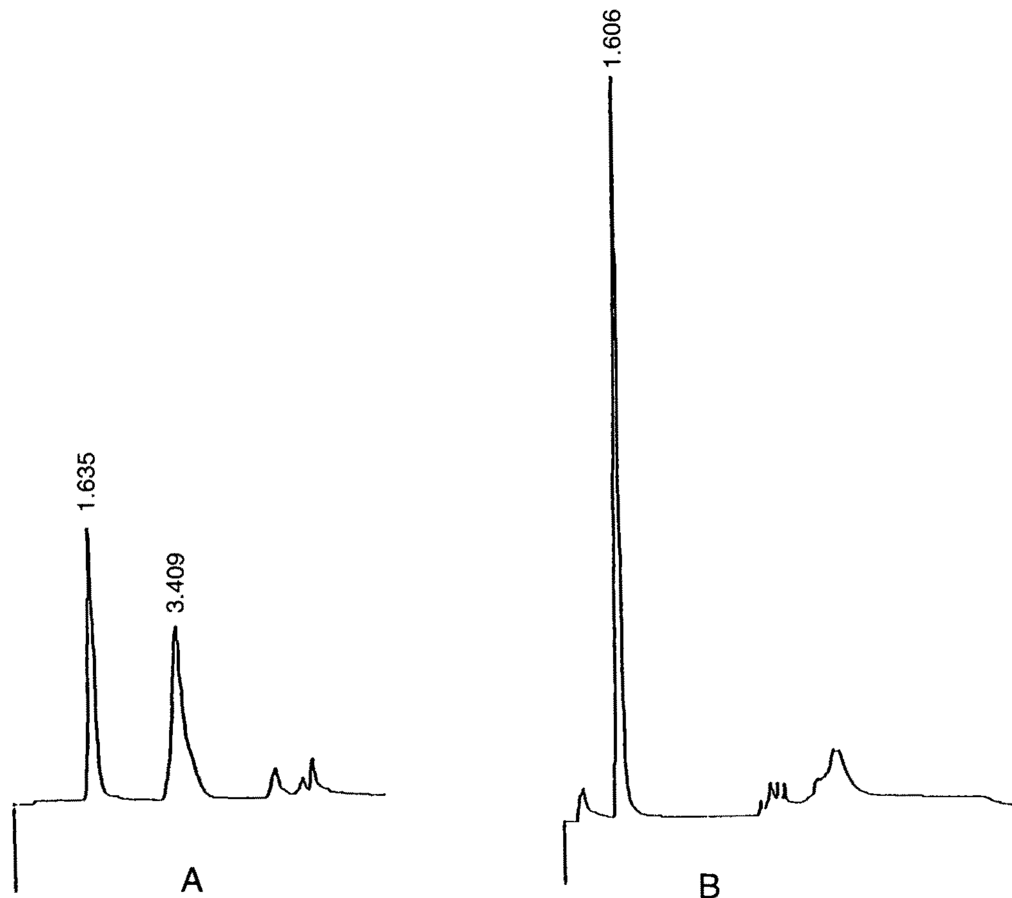

FIG. 3. High-pressure liquid chromatography chromatograms of A, sinapic acid exposed to pH 7 [some sinapic acid (3.409 min) was converted to thomasidioic acid (1.635 min)]; B, sinapic acid exposed to pH 8.5 [all sinapic acid was converted to thomasidioic acid (1.606)].

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