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High Performance Liquid Chromatography of Phenolic Choline Ester Fragments Derived by Chemical and Enzymatic Fragmentation Processes: Analysis of Sinapine in Rape Seed

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High-performance liquid chromatography methods based on reversed-phase chromatography (RPC) and normal phase chromatography (NPC) were introduced for the separation of some representative phenolic acids, choline and betaine, which are the fragments of phenolic choline esters. Sinapine, which is the major phenolic choline ester found in rape seed, was quantitatively hydrolyzed to choline and sinapic acid upon treatment with a solution of sodium hydroxide at room temperature. Choline was further converted to betaine by incubating the base hydrolyzate with choline oxidase. Both sinapic acid and betaine formed the basis for the quantitative determination of sinapine in rape seed by RPC and NPC, respectively. The amounts of sinapine found in rape seed via either of the two fragments (i.e., sinapic acid or betaine) were in very close agreement.

KEYWORDS: HPLC; sinapine; phenolic acids; sinapic acid; choline; betaine; choline oxidase

1. INTRODUCTION

Phenolic acids, e.g., benzoic (C_6-C_1) and hydroxycinnamic acid (C_6-C_3) derivatives, are aromatic secondary plant metabolites belonging to the class of plant phenolics. Phenolic acids are found in almost every plant (*1*, *2*) and, therefore, are an integral part of the human diet. In fact, phenolic acids are important components in a wide variety of fruits (e.g., white grapes, tomatoes, apples, pears, cherries, plums, peaches, apricots, blueberries, etc.), vegetables (spinach, cabbage, asparagus, potatoes, etc.), coffee, olive oil, wheat, corn, wines, and rice to name a few crops (*3*, *4*).

Interest in phenolic acids is related to their diversity, biological significance and ecological role as secondary plant metabolites, use as chemotaxonomic markers, impact on fruit and vegetable quality, physiological effects, and various applications. Recent interest in food phenolic acids has increased greatly because of the antioxidant and free radical scavenging abilities associated with phenolic acids and their potential effects on human health (*5*). It is well-known that diets rich in fruits and vegetables are protective against cardiovascular diseases and certain forms of cancer (*6*−*8*) and perhaps against other diseases also.

Besides existing as free species, the majority of phenolic acids occur naturally in a wide group of combined forms. They occur in association with cyclohexane carboxylic acid (e.g., quinic acid), other organic acids (e.g., tartaric acid and malic acid), sugars (e.g., glucose), amines (e.g., choline as in sinapine and its derivatives), and also linked to other polyphenols (e.g., flavonoids) (*9*). Among all of these bound forms of phenolic acids, we are concerned here with phenolic choline esters, known as sinapines (*5*, *10*). Phenolic choline esters are especially abundant in the seeds of some glucosinolate-containing plants or crucifer seeds (*11*) such as rapeseed/canola (*10*). Typical phenolic choline esters are shown in **Figure 1**.

Phenolic choline esters are important natural products; therefore, methods for their accurate determination in plants are needed. Because standard phenolic choline esters are not available in commercial markets, the quantitative determination of phenolic choline esters in plants remains a problem. As shown in **Figure 1**, phenolic choline esters can be hydrolyzed in basic solution to choline and phenolic acid, the representatives of which are *p*-hydroxybenzoic acid (4-HBA), 3,4-dimethoxybenzoic acid (3,4-DBA), *p*-coumaric acid, ferulic acid, and sinapic acid (*12*). Also, choline can be enzymatically converted to betaine (*13*), see **Figure 1**. Because the standards of the phenolic acids and betaine are readily available, the amount of choline esters can be measured by determining the amount of phenolic acids or choline after hydrolysis of the rape seed extract. This article is concerned with developing high-performance liquid chromatography (HPLC) methods for the determination of phenolic choline esters based on their chemical and enzymatic fragments, which are reflective of the individual and total phenolic choline esters.

2. EXPERIMENTAL PROCEDURES

2.1. Instruments and Columns. The liquid chromatograph was assembled from (i) a model CM 4000 multiple solvent delivery system (Milton Roy, LDC division, Riviera Beach, FL), (ii) a model 7010 sample injector (20 μL) from Rheodyne (Cotati, CA), (iii) a diode array detector (DAD) model HP 1040A from Hewlett-Packard (Waldbronn, Germany) whose spectral range is from 190 to 400 nm, and (iv) a

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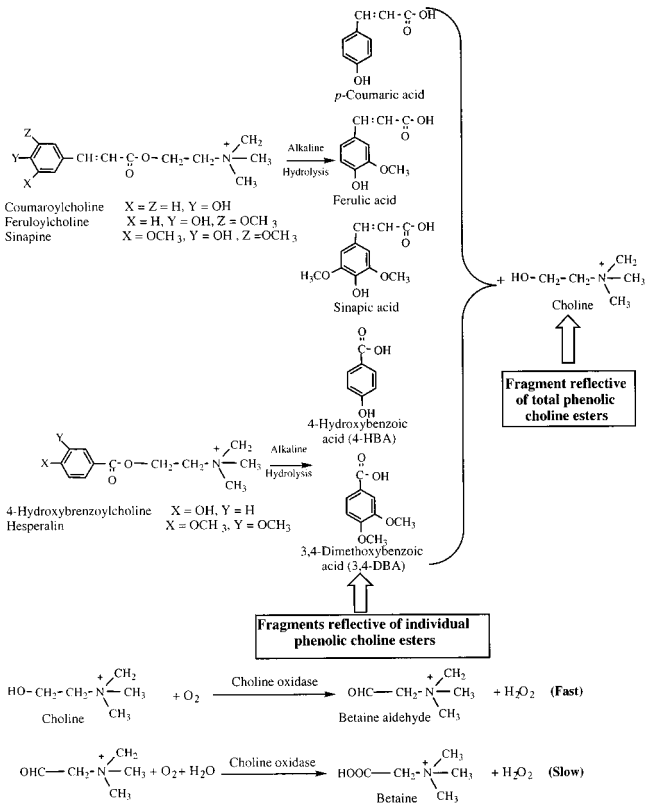

Figure 1. Structures of some sinapines found in crucifer seeds. Also shown in this figure are structures of the corresponding phenolic acids, choline and betaine fragments obtained by alkaline hydrolysis and enzymatic processes. While the phenolic acids are reflective of the individual sinapines and can be used for the quantitative determination of each phenolic choline ester, choline and its enzymatic derivative betaine can be used for the quantitative determination of total phenolic choline esters in plant extracts.

personal computer equipped with the software HP Chemstation for LC systems (Rev.A.04.01, Copyright Hewlett-Packard 1990−1996). The wavelength was set at 210 or 192 nm for sensing the column effluent with reversed-phase chromatography (RPC) or normal phase chromatography (NPC), respectively.

The columns for RPC were made of stainless steel tubing of dimensions 25 cm × 4.6 mm i.d. or 15 cm × 4.6 mm i.d. packed with narrow pore C18 of 5 μm mean particle diameter from J. T. Baker Inc. (Phillipsburg, NJ). The column for NPC was also made of stainless steel tubing of dimensions 15 cm × 4.6 mm i.d. and packed in-house with narrow pore bare Zorbax silica of 5 μm mean particle diameter. In all experiments involving RPC, we used an analytical guard column made from stainless steel tubing of dimensions 2 cm × 2 mm i.d. (Upchurch Scientific, Murrieta, CA) and dry-packed in-house with Zorbax C18 of 18 μm mean particle diameter. In NPC, there was no need for a guard column since the rape seed extract was subjected to a solid phase extraction (SPE) step.

2.2. Chromatographic Conditions. In all cases, the flow rate was set at 1.0 mL/min. Before running the experiments, the mobile phases were first filtered through S/P filter paper (grade 360 qualitative from Baxter, McGaw Park, IL) and then degassed in an ultrasonic bath. For gradient runs with the C18 column, mobile phase A_1 consisted of 10 mM $NH_4H_2PO_4$, pH 6.0, while mobile phase B_1 consisted of 80% MeOH and 20% mobile phase A_1. Linear gradients were run for 20 min from 100% A_1 to 100% B_1. For gradient runs with the silica column, mobile phase A_2 consisted of 98% acetonitrile (ACN) and 2% of 10 mM NH_4Cl, pH 3.6 (v/v), while mobile phase B_2 consisted of 50% ACN and 50% of 10 mM NH_4Cl, pH 3.6 (v/v). Again, linear gradients were run for 20 min from 100% A_2 to 100% B_2.

2.3. Reagents and Materials. Standards of choline, betaine, 4-hydroxybenzoic acid (4-HBA), and 3,4-dimethoxybenzoic acid (3,4-DBA) as well as choline oxidase (EC 1.1.3.17) were obtained from Sigma Chemical Co. (St. Louis, MO). Standards of *p*-coumaric acid, ferulic acid, and sinapic acid were from Aldrich Chemical Co. (Milwaukee, WI). For the structures of phenolic acids, choline and betaine, see **Figure 1**. Methanol of HPLC grade was purchased from EM Science (Gibbstown, NJ). ACN of HPLC grade was obtained from Fisher Scientific (Fair Lawn, NJ). Rape seed (Dwarf Essex) was from Dr. Melouk, Department of Plant Pathology, Oklahoma State University, Stillwater, OK. C18 Bakerbond SPE cartridges were from J. T. Baker.

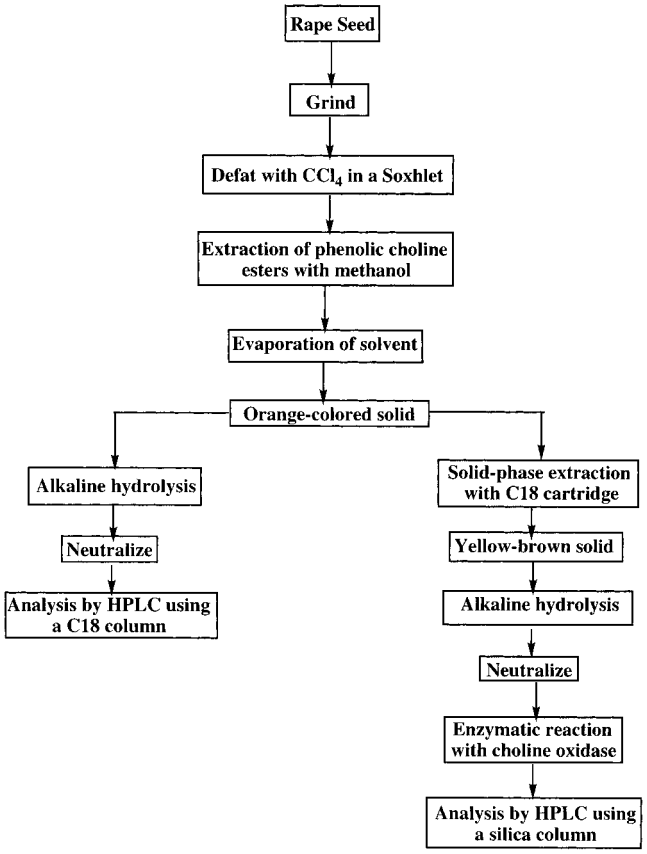

Figure 2. Steps involved in sample preparation and analysis.

2.4. Procedures. In the following sections, we describe the various steps involved in the sample preparation and the quantitative determination of the various components of interest by HPLC. **Figure 2** summarizes the various steps involved in sample preparation and analysis.

2.4.1. Rape Seed Extraction. An amount of 21.0 g of rape seed were ground thoroughly in a mortar until a paste formed. The slurry was then extracted with carbon tetrachloride in a Soxhlet apparatus for 4 h and then dried overnight at room temperature (*14*). Next, the defatted material thus obtained was extracted twice with 50 mL of HPLC methanol each. Finally, methanol was evaporated in a Savant speedvac at ~0 Torr (Savant, Holbrook, NY), and 0.50 g of an orange-colored solid was obtained.

2.4.2. SPE. The SPE was carried out as follows: the C18 SPE cartridge was activated with 80% methanol:20% H_2O (v/v), and then the cartridge was conditioned with deionized water and 0.1% (v/v) trifluoroacetic acid (TFA). Thereafter, the sample was applied and the cartridge was eluted with 50% methanol:50% deionized water (v/v) and 0.1% TFA (v/v). The eluent was collected from both the sample application step and the elution step in a vial. The collected fraction was then dried from methanol and TFA in a Savant vacuum system. The solid obtained was yellow-brown.

2.4.3. Alkaline Hydrolysis of Rape Seed Extract. A small amount of the solid extract from rape seed was dissolved in 1.0 mL of mobile phase A_1, which consisted of 10 mM ammonium phosphate, pH 6.0. To this solution, 100 μL of 2.0 M NaOH was added, and the alkaline

hydrolysis was carried out at room temperature for 2 h. The hydrolyzate was then neutralized by adding 100 μL of 2.0 M HCl..

2.4.4. Standard Calibration Curve. To quantitatively determine the amount of sinapic acid in the rape seed after hydrolysis, we used ferulic acid as an internal standard and made a standard calibration curve by plotting the ratio of peak height of sinapic acid and ferulic acid vs the concentration of standard sinapic acid. The standard solution of sinapic acid was made by dissolving 0.0052 g of sinapic acid into 1.0 mL of methanol that was added to a 10 mL volumetric flask, and the final volume was adjusted with mobile phase A_1 until it reached 10 mL. The final concentration of sinapic acid was 2.32×10^{-3} M. The standard solution of ferulic acid was made by dissolving 0.0098 g of ferulic acid into 1.0 mL of methanol that was added to a 50 mL volumetric flask, and the final volume was adjusted with mobile phase A_1 until it reached 50 mL. The final concentration of ferulic acid was 1.00×10^{-3} M.

The rape seed sample was prepared as follows: 0.0037 g of rape seed extract was added into a 25 mL volumetric flask followed by the addition of 2.0 mL of mobile phase A_1 and 100 μL of 2.0 M NaOH. After 2 h, 100 μL of 2.0 M HCl was added to neutralize the solution followed by the addition of 300 μL of standard ferulic acid and mobile phase A_1 to reach the 25 mL mark. The solutions for the calibration curve were prepared as follows: 300 μL of standard ferulic acid was pipeted into six 25 mL volumetric flasks, and then, 300, 400, 500, 600, 700, and 800 μL of standard sinapic acid were added, respectively. Thereafter, the volume was completed to 25 mL by adding mobile phase A_1. After the solution was sufficiently stirred (~15 min), the sample and standard solutions were injected into the HPLC system.

To quantitatively determine the amount of betaine converted from choline by choline oxidase, alanine was used as an internal standard. The standard calibration curve was made by plotting the ratio of peak height of betaine and alanine vs concentration of standard betaine. The standard solution of betaine was made by dissolving 0.0355 g of betaine into a 25 mL volumetric flask followed by adding 0.05 M Na_3PO_4 solution, pH 8.0, to reach the mark. The standard solution of alanine was made by dissolving a small amount of alanine (2.0 mg) into a 10 mL volumetric flask followed by adding 0.05 M Na_3PO_4 solution, pH 8.0, to reach the mark.

The rape seed sample was prepared as follows: 0.0402 g of rape seed extract was added into a small vial followed by the addition of 1.0 mL of water:methanol (3:2). After SPE was applied, the yellow-brown solid was dissolved in 0.8 mL of 0.05 M Na_3PO_4 solution, pH 8.0, in a small vial followed by the addition of 100 μL of 2.0 M NaOH. After 2 h, 100 μL of 2.0 M HCl was added to neutralize the unreacted NaOH in the solution so that its pH remained equal to 8.0. Thereafter, 200 μL of standard alanine solution and a known number of units of choline oxidase (10 or 50 units) were added into the vial, which was then put into a Thermolyne, Type 17600 DriBath (Dubuque, IA) that remained at a constant temperature of 37 °C for 18 h.

The solutions for the calibration curve for betaine were prepared as follows: 200 μL of standard alanine solution was pipeted into six small vials, and then, 200, 300, 400, 500, 600, and 700 μL of standard betaine solutions were added, respectively. Thereafter, 800, 700, 600, 500, 400, and 300 μL of 0.05 M Na_3PO_4, pH 8.0, were added, respectively. Under these conditions, the total volume of each of the six solutions was 1.200 mL.

3. RESULTS AND DISCUSSION

Because of the large differences in the polarity of solutes involved in this study, two different HPLC modes were utilized, i.e., RPC with C18-silica column and NPC with silica column. To be more specific, choline and betaine and also sinapine are more polar than the phenolic acids under investigation.

3.1. Chromatographic Behavior of the Standard Phenolic Acids and Betaine. In a series of experiments aimed at determining the optimum pH for the separation of the phenolic acid constituents of phenolic choline esters, 20 min linear gradients ran of 100% mobile phase A_1 to 100% mobile phase B_1. Mobile phase A_1 consisted of 10 mM $NH_4H_2PO_4$ at pH 2.5, 4.0, 4.5, 5.0, or 6.0 while mobile phase B_1 consisted of

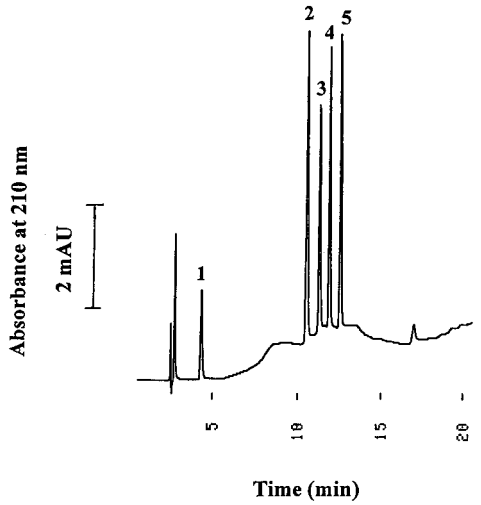

Figure 3. Chromatogram of the major phenolic acid constituents of phenolic choline esters obtained by RPC. Column, 250 mm × 4.6 mm i.d. packed with C18-silica; flow rate, 1.0 mL/ min; 20 min linear gradient elution from 100% A_1 to 100% B_1. Solutes: 1, 4-HBA; 2, *p*-coumaric acid; 3, 3,4-DBA; 4, ferulic acid; 5, sinapic acid.

80% MeOH and 20% solvent A_1. The pH of the solution was adjusted with phosphoric acid to pH values of 2.5 and 4.0 and with ammonium hydroxide to pH values of 4.0, 4.5, and 6.0. At pH 2.5, *p*-coumaric, ferulic, and sinapic acids coeluted while at pH 4.0, 4.5, and 5.0 ferulic and sinapic acids coeluted despite the structural difference among the three acids. At pH 6.0, all of the five acids were completely separated; see **Figure 3**. In addition to structural differences at pH 6.0, the acids are ionized to varying degrees, a fact that further differentiates their partitioning between the mobile and the stationary phases and brings about their separation. The optimum pH for the phenolic acids was thus set at pH 6.0. On the other hand, choline and its enzymatic derivative betaine were not retained in the pH range tested, i.e., pH 2.5−6.0.

On the basis of the above experiments, incorporation of a negatively charged ion-pairing agent (e.g., sodium octyl sulfate, SOS) was considered to bring about the retention of betaine and choline. Isocratic elution was conducted with a mobile phase of 10 mM ammonium phosphate, pH 6.0, at various SOS concentrations. In the absence of SOS, betaine and choline were not retained and only 4-HBA could be eluted while the other acids were very retarded. The dependence of solute retention factor k' on the concentration of SOS is shown in **Figure 4**. As can be seen in this figure, the retention factor of betaine increased substantially first from 0 to 0.55 upon adding 2.5 mM SOS and then leveled off as the SOS concentration was increased. Surprisingly, choline stayed unretained in the concentration range studied (0−10 mM SOS). The retention factor of the five phenolic acids decreased with increasing SOS concentration. The decrease in solute retention of phenolic acids is due to the fact that the ion-pairing agent carries the same charge as the solute, thus leading to solute repulsion from the stationary phase with adsorbed SOS. It is noteworthy that when surfactant concentration was at 0 mM, the retention of 4-HBA was longer than that of betaine. At 2.5 mM SOS, betaine and 4-HBA coeluted. After 5 mM, the retention of betaine became slightly longer than that of 4-HBA. This could be seen clearly at 10 mM; see **Figure 4**. Although the ion-pairing system allowed the simultaneous analysis of the phenolic acids and betaine constituents of phenolic choline esters, the retention of betaine was not sufficient and that of 4-HBA decreased

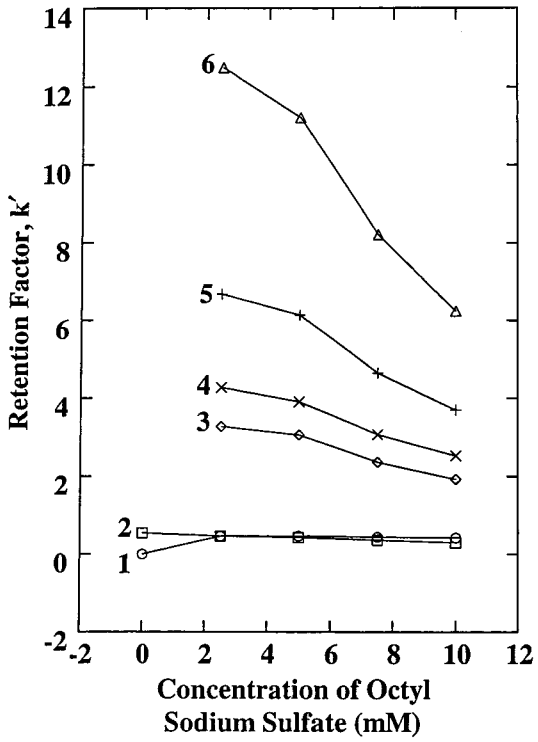

Figure 4. Plots of retention factors of betaine and some phenolic acids vs the concentration of SOS in the mobile phase. Column, 250 mm × 4.6 mm i.d. packed with C18-silica; flow rate, 1.0 mL/ min; isocratic elution with a mobile phase of 10 mM $NH_4H_2PO_4$, pH 6.0, at various concentrations of SOS. Curves: 1, betaine; 2, 4-HBA; 3, *p*-coumaric acid; 4, 3,4-DBA; 5, ferulic acid; 6, sinapic acid.

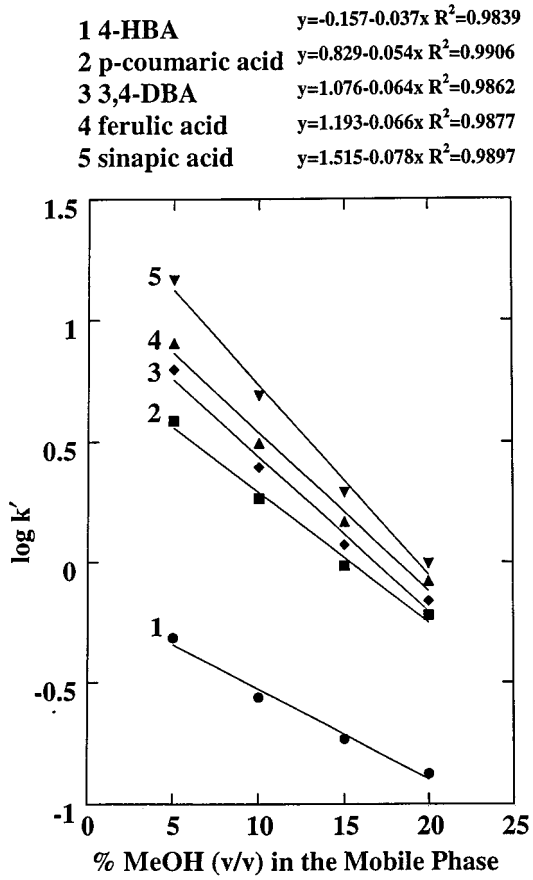

Figure 5. Plots of logarithmic retention factor (log *k*′) vs percent methanol in the mobile phase. Mobile phase, 10 mM $NH_4H_2PO_4$, pH 6.0, at various methanol contents. Curves: 1, 4-HBA; 2, *p*-coumaric acid; 3, 3,4-DBA; 4, ferulic acid; 5, sinapic acid.

significantly making both solutes elute near the dead time of the column where interferences of real samples may also elute.

Therefore, another chromatographic system more adequate for the analysis of choline and betaine was considered. On the other hand, the RPC chromatographic system based on gradient elution shown in **Figure 3** is very adequate for the analysis of the phenolic acid constituents. Also, the RPC system can be performed isocratically since the retention and selectivity of the system can be manipulated readily by the methanol content of the mobile phase; see **Figure 5**. As can be seen in this figure, for each phenolic acid, log *k*′ is linearly related to percent methanol and as expected, the absolute value of the slopes increased with the size of the molecule.

As stated above for the separation of betaine and choline, we had to use NPC with a bare silica column. **Figure 6** shows the chromatograms of standard choline and betaine obtained on a silica column of dimensions 15 cm × 4.6 mm i.d. run at a flow rate of 1.0 mL/min with a 20 min linear gradient from 100% solvent A_2 to 100% solvent B_2. Solvent A_2 consisted of 98% ACN and 2% of 10 mM NH_4Cl, pH 3.6 (v/v), while solvent B_2 consisted of 50% ACN and 50% of 10 mM NH_4Cl, pH 3.6 (v/v). As can be seen in **Figure 6**, choline and betaine elute at ca. 9.8 and 17.7 min, respectively, and they are far removed from the column dead time where many unretained species in real samples would elute.

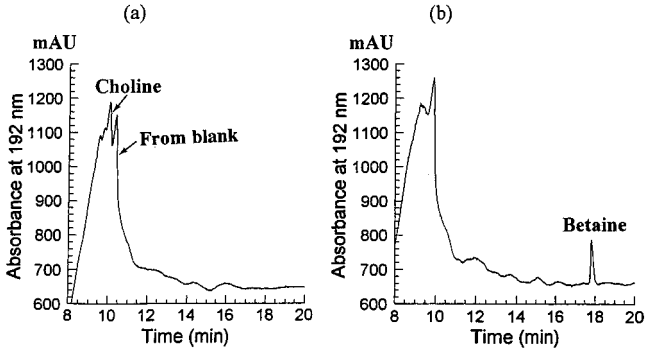

Figure 6. Chromatograms of (**a**) standard choline and (**b**) betaine obtained by NPC. Column, 150 mm × 4.6 mm i.d. packed with 5 μm silica particles; flow rate, 1.0 mL/min; 20 min linear gradient elution from 100% A_2 to 100% B_2. The words "from blank" mean from the gradient baseline.

3.2. Determination of Sinapine in the Extract of Rape Seed Via Its Degradation Products Sinapic Acid or Choline. *3.2.1. Analysis Via the Alkaline Hydrolysis of the Rape Seed Extract.* **Figure 7** shows the chromatograms corresponding to the analysis of rape seed extract before and after 2 h of alkaline hydrolysis, respectively. As can be seen in this figure, the peak eluting at 2.1 min disappeared while a large new peak eluting at 9.6 min appeared. When spiking the extract with standard sinapic acid, the sinapic acid peak coeluted with that eluting at 9.6 min, which increased proportionally in size indicating that the peak generated by alkaline hydrolysis is perhaps sinapic acid. Furthermore, the UV spectra from 190 to 400 nm generated by the DAD of the peak eluting at 9.6 min obtained upon alkaline hydrolysis and the peak of standard sinapic acid are exactly the same; see **Figure 8**. From this experiment, it can be assumed that the large peak is sinapic acid and the peak eluting at 2.1 min is sinapine whose spectrum obtained by DAD is shown in **Figure 8**. On the other hand, 4-HBA, 3,4-DBA, *p*-coumaric acid, and ferulic acid do not seem to exist in considerable amounts as compared with sinapic acid. Thus,

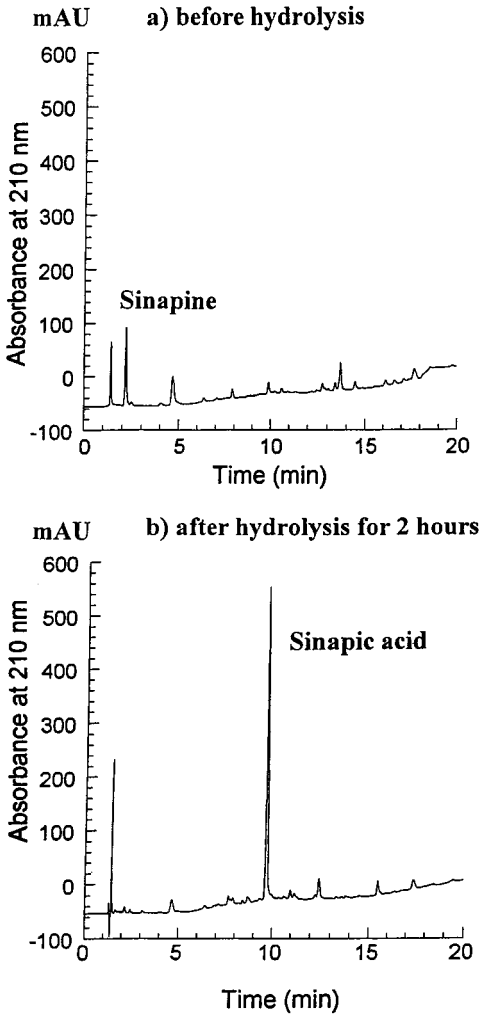

Figure 7. Chromatograms of (**a**) the intact and (**b**) the base hydrolyzed for 2 h rape seed extract obtained by RPC. Column, 150 mm × 4.6 mm i.d. packed with C18-silica. All other conditions as in **Figure 3**.

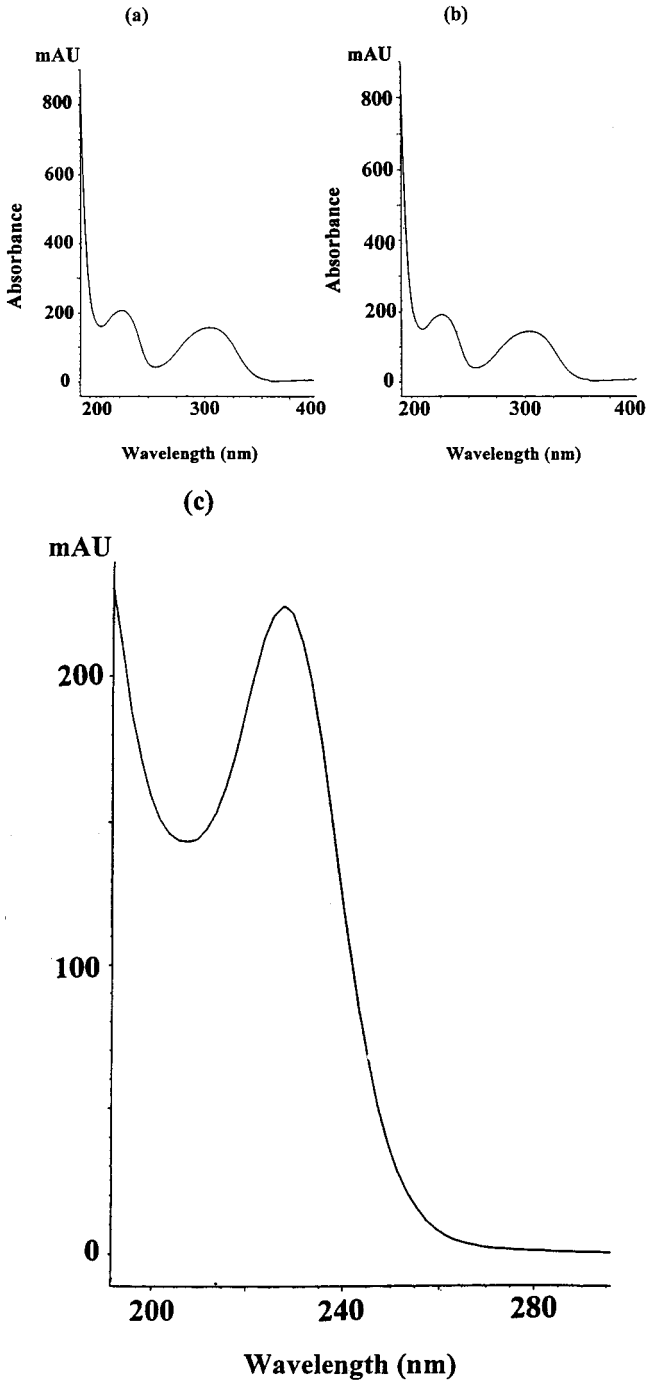

Figure 8. UV–vis spectra of (**a**) the standard sinapic acid peak, (**b**) the peak appearing after alkaline hydrolysis of rape seed extract as in **Figure 7b**, and (**c**) the peak labeled sinapine in **Figure 7a** obtained by the DAD used to monitor the column effluent.

sinapic acid is the dominant phenolic acid existing in rape seed extract after alkaline hydrolysis.

The alkaline hydrolysis of sinapine is completed in almost 30 min. The time course for the hydrolysis is shown in **Figure 9**. The peak area of sinapic acid increased dramatically during the first 30 min and after that it increased slowly. This means that hydrolysis of sinapine occurred mostly in the first 30 min. However, 2 h of hydrolysis time was chosen to make sure that all of the sinapine was converted to sinapic acid.

As explained in the Experimental Procedures, the determination of sinapic acid in the base hydrolyzate of the rape seed extract was based on the standard calibration curve using ferulic acid as the internal standard. The plot of peak height ratio of sinapic acid to ferrulic acid (i.s.) vs the concentration of sinapic acid was a straight line ($y = 0.081x - 0.052$, $R^2 = 0.9977$). From this standard calibration curve, the amount of sinapic acid in the extract was determined to be 7.3 μmol/g of rape seed.

Because sinapic acid is the alkaline hydrolysis product of sinapine, the molar concentration of sinapic acid equals that of sinapine. Thus, in each gram of rape seed, there is 7.3 μmol of sinapine assuming that hydrolysis goes to completion. This seems to be the case since the peak corresponding to sinapine disappeared totally after 2 h of subjecting the rape seed extract to alkaline hydrolysis.

3.2.2. Enzymatic Identification of Sinapine through Choline Oxidase. To further confirm the presence of sinapine in rape seed, the base-hydrolyzed extract was treated with choline oxidase, which converts choline to betaine (see Introduction). In fact, after choline oxidase was added into the base-hydrolyzed extract, a new peak appeared at 18 min. The chromatograms obtained in the presence or absence of the choline oxidase are shown in **Figure 10**. When spiked with betaine, the extract still yielded one peak at ca. 18 min. That is the standard betaine peak coeluted with the peak eluting at 18 min. This betaine peak must come from the oxidation of choline by choline oxidase. To further confirm the identity of the peak eluting at 18 min, its DAD spectrum was compared with that of standard betaine. Both spectra were identical.

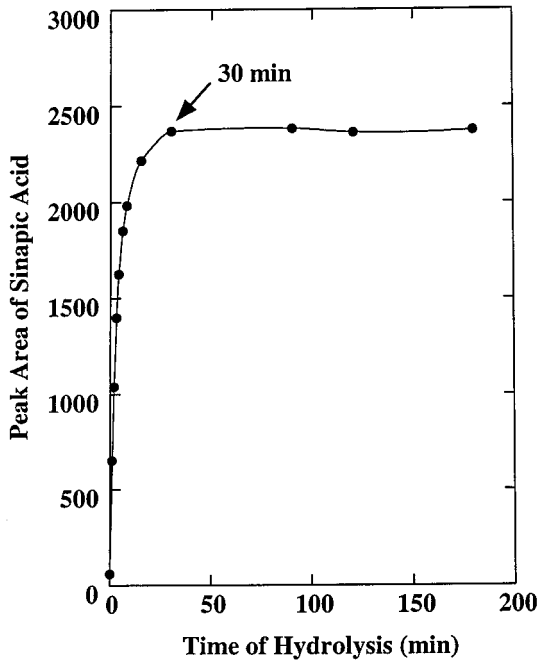

Figure 9. Time course for the appearance of the peak of sinapic acid in the rape seed extract upon alkaline hydrolysis.

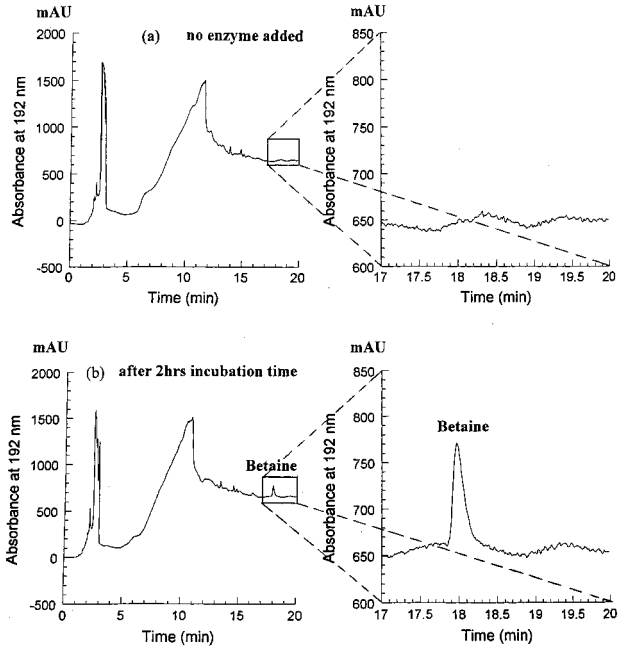

Figure 10. Chromatograms of the base-hydrolyzed rape seed extract in the (**a**) absence and (**b**) presence of choline oxidase enzyme. All conditions as in **Figure 7**.

The enzymatic conversion of choline to betaine was found to be slow, and as expected, its speed depended on the number of enzyme units added to the reaction. In fact, it took almost a day to completely convert choline to betaine upon adding only 10 units of choline oxidase to 1.0 mL of the base-hydrolyzed rape seed extract containing ca. 7.0 μmol choline. This time was reduced to almost 2.5 h when 50 units of choline oxidase was used.

Thus, sinapine could also be identified through betaine. Alanine was chosen as an internal standard. From the standard calibration curve ($y = 0.1216\ x + 0.0884$, $R^2 = 0.9997$), the amount of betaine generated by the enzymatic treatment of the base-hydrolyzed rape seed was determined to be 7.1 μmol/g of rape seed. This amount is that of sinapine since 1 mol sinapine produces 1 mol of choline/betaine.

4. CONCLUSION

RPC and NPC proved useful in the determination of phenolic choline esters via their chemical and enzymatic degradation products (i.e., via phenolic acids and betaine, respectively). Sinapine was found to be the major phenolic choline ester constituent of rape seed. The qualitative and quantitative determination of sinapine in rape seed can be readily done through the fragments derived from the alkaline hydrolysis and/or the enzymatic treatment of the rape seed extract. The amount of sinapine was determined to be 7.1−7.3 μmol/g of rape seed.

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