Improved HPLC Determination of Hydroxycinnamic Acids in Orange Juice Using Solvents Containing THF†

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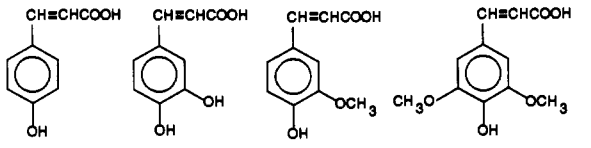
Solvent optimization techniques were used to maximize chromatographic resolution between sinapic, coumaric, ferulic, and caffeic acids and components in orange juice. Various reversed-phase solvent systems consisting of binary, ternary, and quaternary combinations of methanol, acetonitrile, and THF with water were evaluated. Greatest chromatographic resolution was realized from binary mobile phases consisting of THF and water and ternary solvents containing THF, methanol, and water. Resolution between the least resolved peak pair was improved 46% using the binary THF mobile phase compared to the best previously reported mobile phase. The THF, methanol, and water mobile phase produced the greatest resolution between the four acids of interest; however, unknown components in orange juice coeluted with both the sinapic and caffeic acid peaks. The mobile phase consisting of 21% THF/79% water (with 2% acetic acid) produced the best separation and allowed the most accurate quantitation of the four hydroxycinnamic acids from the components in orange juice. Chromatographic behavior may differ if acids other than acetic are used to suppress ionization.

INTRODUCTION

Hydroxycinnamic acids are commonly found in foods such as fruits, vegetables, and grains. Highest concentrations of these phenolics are typically found in the surface layers. Accordingly, it has been speculated that these compounds play some role in natural fungal resistance (Hahn et al., 1983) of these foods. These phenolic acids have also been associated with sour, bitter, and astringent flavors found in vegetable proteins (Huang and Zayas, 1991). A specific cinnamic acid, ferulic acid, has been identified as the odorless precursor to the highly malodorous *p*-vinylguaiacol (PVG) (Naim et al., 1988; Peleg et al., 1988). Hydroxycinnamic acids have also been associated with accelerated browning (Chen, 1988; Cilliers and Singleton, 1989). Because these acids can influence both the color and flavor of a variety of food products, many analytical procedures have been developed.

Early studies (Heimann et al., 1971; Schmidtlein and Herrmann, 1975) employed TLC to separate and quantify hydroxycinnamic acids. Spots were scraped off the plates and hydroxycinnamic acids quantified using spectrophotometric techniques. Later, GLC procedures were developed (Schulz and Herrmann, 1980) but required the acids to be derivitized into more volatile forms. HPLC is the current procedure of choice because it requires minimal sample preparation, is relatively rapid, and allows easy quantitation. It also has the ability to quantify esterified (conjugated) forms of hydroxycinnamic acids (Risch and Herrmann, 1988). The chromatographic determination of hydroxycinnamic acids is complicated by their ability to exist as both cis and trans isomers (Haug and Gierschner, 1979). HPLC procedures have been developed to determine hydroxycinnamic acids or hydroxycinnamic acid esters in orange and grapefruit juices and peels (Winter and Herrmann, 1984; Naim et al., 1988; Rouseff et al., 1990; Peleg et al., 1991), wine (Wulf and Nagel, 1976; Baranowski and Nagel, 1981), beer (Kenyhercz and Kissinger, 1977), tea (Bailey et al., 1990), apple juice (Singleton et al., 1977), eggplant (Lattanzio, 1982), and cereal grains (Pussayanawin and Wetzel, 1987; Hahn et al., 1983).

As shown by the structures, hydroxycinnamic acids are structurally very similar, and it is difficult to find conditions that will adequately resolve all four acids.



p-coumaric caffeic ferulic sinapic

Virtually all of the previous methods employ a C-18 column with a small amount of acid (usually acetic or phosphoric to suppress ionization). Earlier optimization studies (Wulf and Nagel, 1976) examined the effects of solvent strength (using only methanol) on the elution order of benzoic and hydroxycinnamic acids found in wine or optimized pH of water/methanol solvents using citrate buffers (Price et al., 1979). The latter two studies did not include sinapic acid.

Earlier HPLC procedures to determine hydroxycinnamic acids in fruits (Winter and Herrmann, 1984) required the use of polyamide columns to clean up the sample prior to HPLC analysis. Subsequent chromatographic procedures to quantify hydroxycinnamic acids in orange and grapefruit juices and fruit parts (Naim et al., 1988; Peleg et al., 1991) employed a combination of open column chromatography, TLC, and HPLC. Recent chromatographic studies (Rouseff et al., 1990) suggested acetonitrile was slightly superior to methanol, and an optimum acetonitrile concentration was established. However, the separation between coumaric, sinapic, and fer-

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Table I. Resolution Study Using Solvent Compositions of Similar Solvent Strength

solvent system	% MeOH	% CH_3CN	% THF	% H_2O[a]
1	30			70
2		20		80
3			21	79
4	15		10.5	74.5
5		10	10.5	79.5
6	15	10		75
7	10	6.7	7	76.3

[a] With 2% acetic acid.

ulic acids needed improvement because they eluted very close to each other. It was difficult to detect low levels of sinapic acid in the presence of high levels of ferulic acid (the typical situation in citrus).

Of all of the factors that influence chromatographic resolution, solvent selectivity is the single most influential feature. Procedures have been developed to optimize solvent selectivity (Glajch et al., 1980; Lehrer, 1981) on the basis of the separations observed from binary, ternary, and quaternary mixtures of solvents composed of widely differing solvents. This approach has been successfully used to improve the separation of limonin (a bitter component) in citrus juices (Shaw, 1986). Therefore, the purpose of this study was to apply solvent-optimizing techniques to select the best solvent or combination of solvents that would improve the separation of the four hydroxycinnamic acids found in orange juice.

MATERIALS AND METHODS

Reagents and Standards. Optima grade organic solvents from Fisher Chemical (Fair Lawn, NJ) were used. All water was doubly distilled. Ferulic, caffeic, and coumaric acids were obtained from Sigma Chemical Co. (St. Louis, MO), and sinapic acid was obtained from Aldrich Chemical Co. (Milwaukee, WI).

Instrumentation. A Waters Associates (Milford, MA) M-6000A pump was used with a Hewlett-Packard (Waldbronn, FRG) Series 1050 autosampler. Column effluent was monitored with a Laboratory Data Control Spectromonitor II variable-wavelength detector set at 300 nm. A Waters Model 990+ photodiode array detector was employed for spectral peak purity studies.

Chromatography. Separations were achieved using an Alltech (Deerfield, IL) Adsorbosphere HS 5-μm C-18 column, 25 cm × 4.6 mm i.d. Solvent flow rate was 1 mL/min. Mobile-phase solvent composition was mixed externally using graduated cylinders. Solvents were degassed with aspirator vacuum and sonication. Each new solvent was allowed to equilibrate at least 15 min prior to the first injection. Injection volume was usually 20 μL.

Solvent Optimization Studies. Using the solvent classification system of Snyder (1979) methanol was chosen from solvent group II, acetonitrile was chosen from solvent group VIb, and THF was chosen from solvent group III. Various water/methanol combinations were used (with 2% acetic acid to suppress ionization) to establish the solvent strength to elute the acids between $k' = 1$ and 10. Once this was established, the total reversed-phase solvent strength of the water/methanol solvent was calculated using the equation

$$S_r = \sum_0^i S_i \Psi_i = S_{\text{methanol}} \Psi_{\text{methanol}} + S_{\text{water}} \Psi_{\text{water}} \quad (1)$$

where S_r is the total reversed-phase solvent strength, S_i is solvent strength weighing factor for each component, and Ψ_i is the volume fraction for each component. Solvents of similar solvent strength were prepared using the above equation to calculate equivalent amounts of either acetonitrile or THF (also with 2% acetic acid). These three solvents plus three 50:50 binary mixtures of the first three solvents and one 33:33:33 mixture of the first three solvents were then used as mobile-phase solvents. The actual compositions of the seven solvents used are shown in Table I.

Standards were injected in duplicate, and average resolution between the four hydroxycinnamic acids was calculated.

Chromatographic Stability Studies. Ten successive 20-μL injections of 10 ppm standards containing all four hydroxycinnamic acids were injected after the column had equilibrated. The column was insulated with 0.5 in. of foam rubber, but there was no external control of temperature. Solvent system 3 was used at 1 mL/min.

Sample Preparation. Juice samples were prepared using a modification of the procedure of Naim and co-workers (Naim et al., 1988). Five milliliters of 2 N NaOH was added to 5 mL of juice and allowed to stand at room temperature for 4 h. Using a pH meter, the mixture was adjusted to pH 4.5 with concentrated phosphoric acid. The juice was centrifuged and the supernatant saved. The supernatant was extracted twice with 10 mL of ethyl acetate. Extracts were combined, evaporated to dryness, and redissolved in 1 mL of methanol. After filtering, the methanolic solution was stored in an amber vial for subsequent HPLC analysis.

RESULTS AND DISCUSSION

Snyder (1979) proposed a solvent classification system based on three attributes: hydrogen donors, hydrogen acceptors, and large dipoles. On the basis of these criteria, solvents were classified in eight solvent groups. Similar solvents were placed into similar groups. Since the greatest change in mobile-phase selectivity results when the various intermolecular interactions between solvent and sample are radically altered, solvents from three uniquely different solvent groups are usually selected. Since this was a reversed-phase study, miscibility with water was a further requirement. Typically methanol, acetonitrile, and THF are used to adjust k' and seven or more solvent mixtures are used to establish optimum resolution conditions (Glajch et al., 1980; Lehrer, 1981). Solvent selectivity can often be improved by utilizing ternary solvent mixtures.

Many HPLC gradient procedures have been developed to determine hydroxycinnamic acids (Lehtonen, 1983; Seo and Morr, 1984; Risch and Herrmann, 1988). Without exception they use combinations of water and methanol with acetic acid to effect the separation. A later study (Rouseff et al., 1990) employed a water/acetonitrile gradient to reduce the elution time and to sharpen the peak shape for the late eluting cinnamic acid and improve its quantitation at low concentrations. However, no cinnamic acid was observed in either orange or grapefruit juice. Therefore, an isocratic approach was chosen for this study since the four acids of interest elute at similar solvent strengths. Additionally, more analyses can be determined in the same time because it is not necessary to wait for the column to re-equilibrate, and less expensive HPLC equipment can be used.

Solvent Optimization. Methanol/water was used to determine the solvent strength necessary to elute the acids of interest in the optimum k' range 1–10. Since 30% methanol produced k' values in the optimum range, the binary compositions for acetonitrile and THF were calculated using eq 1 and reversed-phase solvent strength weighing factors of 0, 2.6, 3.2, and 4.5 for water, methanol, acetonitrile, and THF, respectively (Lehrer, 1981). The total solvent strength for methanol was calculated to be 0.78. The calculated equivalent concentrations of acetonitrile and THF were 24.4% and 17.3%, respectively. However, as also noted by Lehrer (1981), small amounts of acetic acid appreciably reduced k' values so that chromatograms from the predicted binary compositions had to be reformulated and rerun. The actual acetonitrile composition was 20% vs the predicted 24.4%. THF concentrations, on the other hand, had to be increased; the predicted value was 17.3%, whereas the experimental

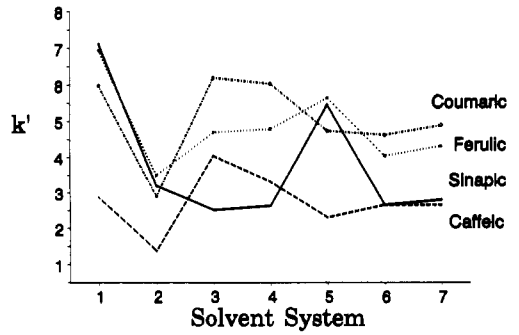

Figure 1. Solvent selectivity study using seven solvent systems. Solvent systems: 1, 30% MeOH/70% water; 2, 20% acetonitrile/80% water; 3, 21% THF/79% water; 4, 15% MeOH/10.5% THF/74.5% water; 5, 10% acetonitrile/10.5% THF/79.5% water; 6, 15% MeOH/10% acetonitrile/75% water; and 7, 10% MeOH/6.7% acetonitrile/7% THF/76.3% water. All water contained 2% acetic acid.

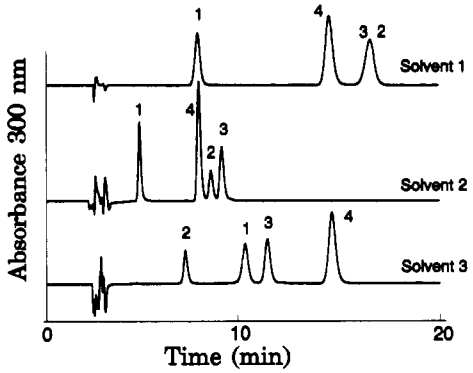

Figure 2. Chromatograms of 10 ppm cinnamic acid standards from solvents 1 (water/methanol), 2 (water/acetonitrile), and 3 (water/THF). Peak identification is as follows: 1, caffeic acid; 2, sinapic acid; 3, ferulic acid; 4, coumaric acid.

value was 21%. The final experimental binary compositions involving THF and acetonitrile with water were then used to formulate the final four solvent systems.

In Figure 1, the relative retentions ($k's$) for the four hydroxycinnamic acids with the seven solvent systems studied are shown. Perhaps the most remarkable feature is the number and extent of the peak elution order reversals with the different solvents. Elution order with methanol (solvent 1) was caffeic, coumaric, ferulic, and sinapic. It should be noted that sinapic and ferulic acids were not resolved with this solvent system ($k's$ for ferulic and sinapic acids were 6.9 and 7.1, respectively). These two compounds can be separated with this binary mixture by decreasing the solvent strength (decreasing the methanol concentration). However, the improved separation comes at the expense of greatly increased retention times and $k's$. A similar elution order was observed with the acetonitrile/water system, but there was a reversal between ferulic and sinapic acids. Furthermore, these two acids were completely separated even at this relatively high solvent strength. Elution order with THF was sinapic, caffeic, ferulic, and coumaric acids.

The three binary systems utilizing methanol, acetonitrile, and THF with water are shown in Figure 1 labeled as solvent systems 1–3, respectively. The actual chromatograms from these three binary solvent systems are shown in Figure 2. As noted from both Figures 1 and 2, the water/THF solvent resulted in the greatest resolution among the four acids of interest. Calculated resolution values are shown in Table II. Chromatographic resolutions ranged from essentially 0 to 9.8 with water/methanol. Similarly, resolutions ranged from 1.3 to 8.7 with water/acetonitrile. In comparison, resolutions ranged from 1.9 to 5.7 with the water/THF solutions. In chromatography, minimum resolution is more important than maximum resolution. Thus, there was a 46% increase (R_s 1.3 → 1.9) in minimum resolution between the best previous solvent system (acetonitrile/water) and the new water/THF system.

Table II. Resolution between Four Cinnamic Acids Using Various Solvents

peak[a]	solvent 1	solvent 2	solvent 3
1, 4	9.8		
4, 3	2.4		
3, 2	0		
1, 4		8.7	
4, 2		1.5	
2, 3		1.3	
2, 1			5.7
1, 3			1.9
3, 4			4.9

[a] Peak 1, caffeic acid; peak 2, sinapic acid; peak 3, ferulic acid; peak 4, coumaric acid.

Improved Separation with THF. The dramatic reshuffling of the elution order in the presence of THF is not easily explained. Ferulic acid is the major cinnamic acid in citrus. It was difficult to quantify small amounts of sinapic acid in the presence of large amounts of ferulic acid because sinapic acid elutes so close to it when using either water/methanol or water/acetonitrile. However, when using water/THF solvent, sinapic acid is the first acid to elute (and thus elutes as a sharp peak) and is well separated from the much larger ferulic acid peak.

Of the ternary and quaternary mixtures (solvents 4–7) (see Figure 1), solvent 4 (containing both THF and methanol) produced the greatest separation and resolution between the four acids of interest. As shown in Figure 1, the separation between ferulic and other hydroxycinnamic acids have been maximized. Ferulic acid is last to elute and is not well resolved from sinapic acid with solvent 5. Sinapic and caffeic acids are not resolved in solvents 6 and 7.

Separation of Hydroxycinnamic Acids in Orange Juice. As indicated in earlier experiments, optimum separation between the four acids of interest is achieved using solvents 3 or 4. It was initially thought that solvent 4 would be the solvent of choice because it produced the greatest separation between ferulic, the predominant cinnamic acid, and the other three acids. However, as shown in Figure 3, there are other orange juice components that must also be considered. Components a and b shown in Figure 3A (water/THF, solvent 3) are not observed in Figure 3B (water/THF/MeOH, solvent 4). There are fewer peaks observed with the ternary water/THF/MeOH system. As shown in Figure 3B only six distinct peaks are observed between 8 and 15 min, whereas nine peaks can be observed in approximately the same region in Figure 3A. This suggests serious overlapping of minor peaks in orange juice when solvent 4 (Figure 3B) is used. Peak areas for peaks 3 and 4 (ferulic and coumaric acids) were essentially identical (within 5%) when results from solvents 3 and 4 were compared. The peak area of sinapic acid was 48% greater in Figure 3B than in Figure 3A. Caffeic acid peak area was 130% greater in Figure 3B than in Figure 3A. This evidence strongly suggests that the unidentified peaks labeled a and b have merged with sinapic and caffeic peaks to produce the two larger peaks shown in Figure 3B.

Saunders and Olechno (1988) reported that cis–trans isomerism could be induced with UV light. In this study

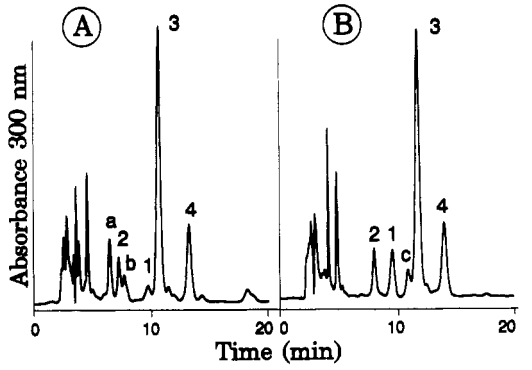

Figure 3. Comparison of orange juice extract using the two best solvent systems from the solvent optimization study. (A) Solvent 3 (water/THF); (B) solvent 4 (water/THF/MeOH). Note the minor peaks labeled a and b in chromatogram A and their apparent absence in chromatogram B.

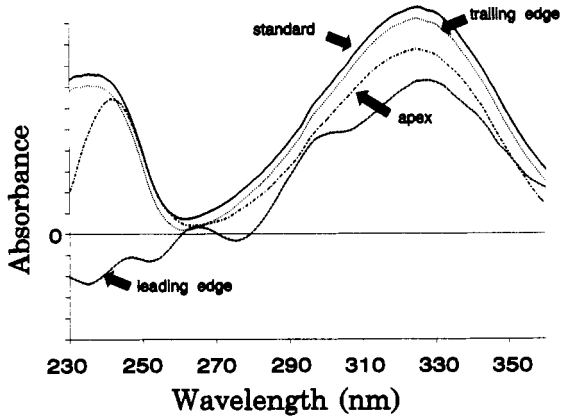

Figure 4. Normalized photodiode array spectra taken on peak 2 from chromatogram B in Figure 3. The spectra from halfway up the leading edge, the peak apex, and halfway down the trailing edge are compared with the spectrum of standard sinapic acid.

samples were stored in amber glass vials to prevent photochemically induced artifacts from forming. No cis isomers from the four acids of interest were detected.

Peak Purity Studies. To determine if the symmetrical peaks labeled 1 and 2 in Figure 3B were due only to sinapic and caffeic acids, the orange juice extracts were rechromatographed using a photodiode array detector and the peaks subjected to spectral analysis. One limitation of using solvents containing THF was the higher UV cutoff compared to that with either methanol or especially acetonitrile. Therefore, the spectra shown in Figure 4 were limited to the range 230–360 nm. Fortunately this region was entirely satisfactory to characterize the hydroxycinnamic acids of interest.

The normalized spectra from halfway up the leading edge, the peak apex, halfway down the trailing edge, and standard sinapic acid are shown in Figure 4. It can be seen that the maximum absorbance of the peak apex spectra is very similar to that of standard sinapic acid. However, there is not good agreement between the two spectra in the region below 260 nm, indicating an additional component with different spectral properties is also present in this peak. This indication was confirmed by comparing the spectra from the leading and trailing edges of the peak. These spectra are profoundly different, proving that this peak does not represent a single component. Furthermore, it can be determined that the impurity is concentrated at the front half of the peak as the spectrum of the trailing edge is almost identical to that from standard sinapic acid, whereas the spectra from the apex and leading edge are successively more dissimilar to the spectrum of standard sinapic acid. This impurity would explain the 48% greater peak area for the "sinapic acid" peak. Similar spectral studies on peak 1, the "caffeic acid" peak shown in Figure 3B, also indicated the presence of a significant impurity with an absorbance maximum in the region of 280 nm which is characteristic of flavanone glycosides typically found in orange juice.

Spectral peak purity studies were also carried out for peaks labeled 1 and 2 from Figure 3A (water/THF, solvent 3). Spectra from the leading edge and peak apex compared very favorably with the spectra from standard sinapic acid. However, the spectrum from the trailing edge did indicate a slight distortion from the peak labeled b. All three spectra from peak 1 (Figure 3A) were identical to the spectrum from standard caffeic acid. Therefore, the integrated peak areas from these peaks can be attributed to sinapic and caffeic acids, respectively, with a high degree of confidence.

Chromatographic Stability. To determine the stability of the chromatographic separation, retention times and areas from 10 successive injections of 10 ppm standards were compared. Retention times were stable with a very slight regular decrease in times with successive injections. Relative standard deviations ranged from 1.59% for caffeic acid to 1.91% for ferulic acid. Peak areas were also very reproducible with relative standard deviations ranging from 3.89% for sinapic acid to 1.58% for coumaric acid.

Conclusions. Previously reported chromatographic separations of hydroxycinnamic acids have utilized only binary aqueous methanol or acetonitrile solvent systems (with a small amount of acetic acid to suppress ionization). This is the first publication to show that mobile-phase solvents containing THF can substantially improve chromatographic resolution. Solvent systems employing THF produced improved separations between the four hydroxycinnamic acids of interest compared to solvents employing methanol or acetonitrile. Even though solvent 4 produced the greatest resolution between caffeic and ferulic acids, it did not adequately resolve unknown juice components from sinapic and caffeic acids. Therefore, the mobile phase that generates the maximum resolution between the components of interest may not be suitable when these components are separated from complex natural products where the number of components may not be known. Solvent 3, the binary system of THF and water, produced the most satisfactory separation in that it resolved all of the components of interest and all of the components in orange juice.

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Registry No. THF, 109-99-9; sinapic acid, 530-59-6; *p*-coumaric acid, 7400-08-0; ferulic acid, 1135-24-6; caffeic acid, 331-39-5.