FOOD COMPOSITION AND ADDITIVES

Analytical Monitoring of Citrus Juices by Using Capillary Electrophoresis

PAUL F. CANCALON
Florida Department of Citrus, 700 Experiment Station Rd, Lake Alfred, FL 33850

A capillary electrophoretic method was developed to analyze simultaneously most citrus juice components in a single procedure. After filtration, sample components are separated with an uncoated capillary tubing and a 35 mM sodium borate buffer (pH 9.3) containing 5% (v/v) acetonitrile. Analyses were run at 21 kV and 23°C. Compounds monitored regularly were the biogenic amine synephrine, some flavonoids (didymin, hesperidin, narirutin, neohesperidin, and naringin), the polyphenol phlorin, 3 UV-absorbing amino acids (tryptophan, phenylalanine, and tyrosine), ascorbic acid, an unidentified peak generated by heat and storage, and the preservatives sorbate and benzoate that can be added to citrus products. Separation can be achieved in 20 min, and each compound can be subsequently quantitated. Didymin, narirutin, and phlorin peaks were used with an artificial neural network to assess the volume of added pulp wash, a by-product of juice preparation. This method allows rapid monitoring of citrus juices, giving information on quality, freshness, and possible adulteration of the product. Similar procedures could be used to monitor other fruit juices and quantitate diverse juice blends.

The past few years have seen considerable developments in capillary electrophoresis (CE; 1, 2). Food analysis is one area in which CE has seen a major expansion (2–7), with most compounds in food products having been examined by CE: sugars (8, 9), saccharides (10, 11), pectins (12), proteins (13–15), vitamins (16–18), retinoids (19), organic acids and ions (20–23), flavonoids (24, 25), limonoid glucosides (26), polyphenols (27, 28), coumarins (29), anthocyanins (30, 31), preservatives (32), and lipids (33, 34).

Many of these compounds are present in citrus juices and are regularly monitored to assess product quality. Until recently, compounds in citrus juices were analyzed individually, usually by liquid chromatography (LC; 35). Furthermore, quantitation of pulp wash (PW) added to citrus juice was done with a method (36) requiring UV/visible spectrophotometric and fluorescence analyses. PW is "water-extracted soluble fruit solids recovered in the presence of water from unfermented excess fruit pulp removed during the production of citrus juice products" (37). It is an orange extract very similar to the juice itself but of a somewhat lower quality. It has less color and is much more bitter than the juice. PW is usually used to produce orange drinks, but because it is cheaper than the juice, it is used as an adulterant often in conjunction with sugar addition.

In the United States, PW produced during extraction (in-line PW) can be added back to the juice before concentration, but it is not allowed in not-from-concentrate (NFC) juices. In-line PW represents between 3 and 7% of the final juice volume. Its presence in a juice at a much higher percentage could be due only to illegal addition of out-line PW. It is therefore important to determine accurately the level of PW in an orange juice.

In the present study, quantitation of PW was performed with an artificial neural network (ANN). These pattern recognition programs have been used to detect complex, nonlinear relationships in multivariate data (38). In food analysis, an ANN has been used to monitor adulteration of butter fat (39). More recently, an ANN has been used to determine the geographical origin of wine vinegars (40). Measuring PW in orange juice amounts to quantitating a mixture of 2 very similar juices, and the method could be adapted for other blends of juices. The procedure described here has been used routinely for the past 2 years to assess citrus juice quality.

Experimental

Analyses were performed with a Spectra-Physics 1000 electrophoresis apparatus (Thermo Quest, Fremont, CA) equipped with high-speed scanning detection in the UV and visible range. Separation was achieved with uncoated fused-silica capillary tubings 70 cm × 50 μm (Polymicro Technologies, Phoenix, AZ), with a 35 mM sodium borate buffer (pH 9.3) containing 5% (v/v) acetonitrile. Electrophoretic analyses were run at 21 kV and 23°C. Before and after daily runs, the capillary was washed at 23°C with water for 3 min, with 0.1N NaOH for 3 min, and with water for 5 min. The capillary also was washed for 3 min with running buffer before each run. New capillary columns were conditioned by washing them for 10 min at 60°C with 1N NaOH, 10 min at 60°C with water, and 10 min at 23°C with the running buffer.

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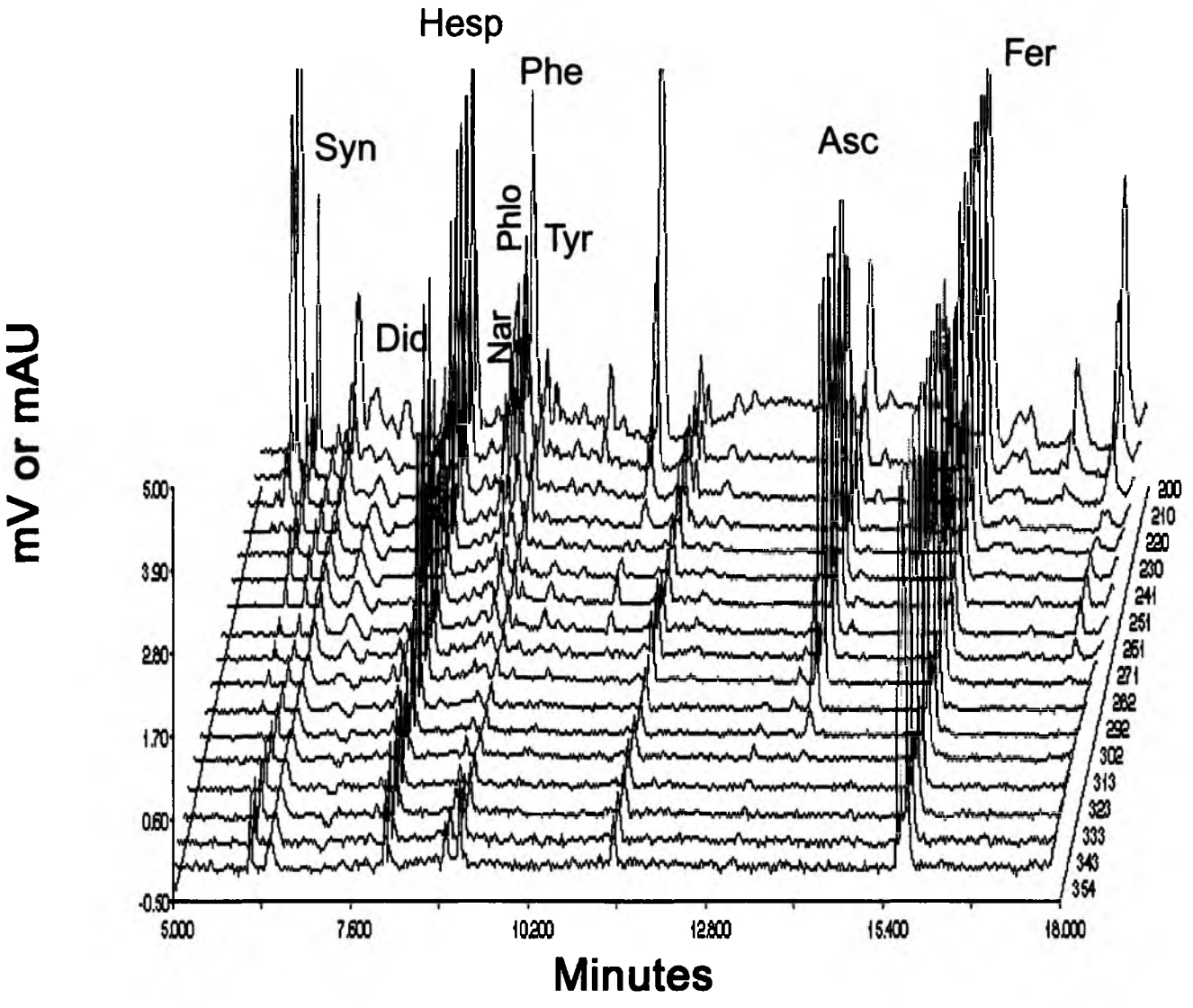

Figure 1. Three-dimensional electropherogram of orange juice. Each wavelength can be extracted and examined separately. Syn = synephrine, Did = didymin, Hesp = hesperidin, Nar = narirutin, Phlo = phlorin, Phe = phenylalanine, Tyr = tyrosine, Asc = ascorbic acid, Fer = ferulic acid (IS).

Flavonoid standards were obtained from Extrasynthese (Lyon, France). Identification of phlorin was performed with a sample originally provided by R.L. Johnson (CSIRO, Division of Food Technology, North Ryde, Australia). However, phlorin is not commercially available and recently several laboratories have started to quantitate phlorin as phloroglucinol.

Unless otherwise expressed, juice samples were diluted 4-fold with LC-grade water (4 mL juice diluted to a final volume of 16 mL) and filtered through 25 mm GD/X Whatman cellulose acetate filter (Whatman, Clifton, NJ). No centrifugation was necessary. One hundred microliters of a solution of ferulic acid (Sigma, St. Louis, MO; final concentration = 31.25 mg/L) was added to the juice as an internal standard (IS). Although, trace amounts of ferulic acid were found in some juices, amounts were too small to affect quantitation. Ferulic acid absorbs relatively constantly over the entire UV spectrum, providing a peak of relatively constant size for use as an IS for all compounds examined.

Samples were injected hydrodynamically for 10 s. Quantitations by the IS procedure were performed with the Thermo Quest PC 1000 software. Standard samples containing 31.25 mg ferulic acid/L and, depending on the quantitation to be performed, 10–200 mg synephrine/L, 2–200 mg flavonoids/L, 1–80 mg phloroglucinol/L, 10–200 mg amino acids/L, or 5–600 mg ascorbic acid/L, were run to establish standard curves. To examine the effect of fruit maturity, we examined synephrine levels in 3 juice samples from early and late Hamlin oranges picked in October 1995 and January 1996 and in 3 samples from early and late Valencia oranges picked in January and June 1996. A specific calculation program was developed for PW quantitation to calculate peak area ratios of didymin, narirutin, and phlorin to that of ferulic acid.

For subfractionation, fruits were collected from the Citrus Research and Education Center "teaching grove" (Valencia on Swingle root stock and Hamlin on Carriso root stock). The flavedo was removed from the fruit with an electrical peeler,

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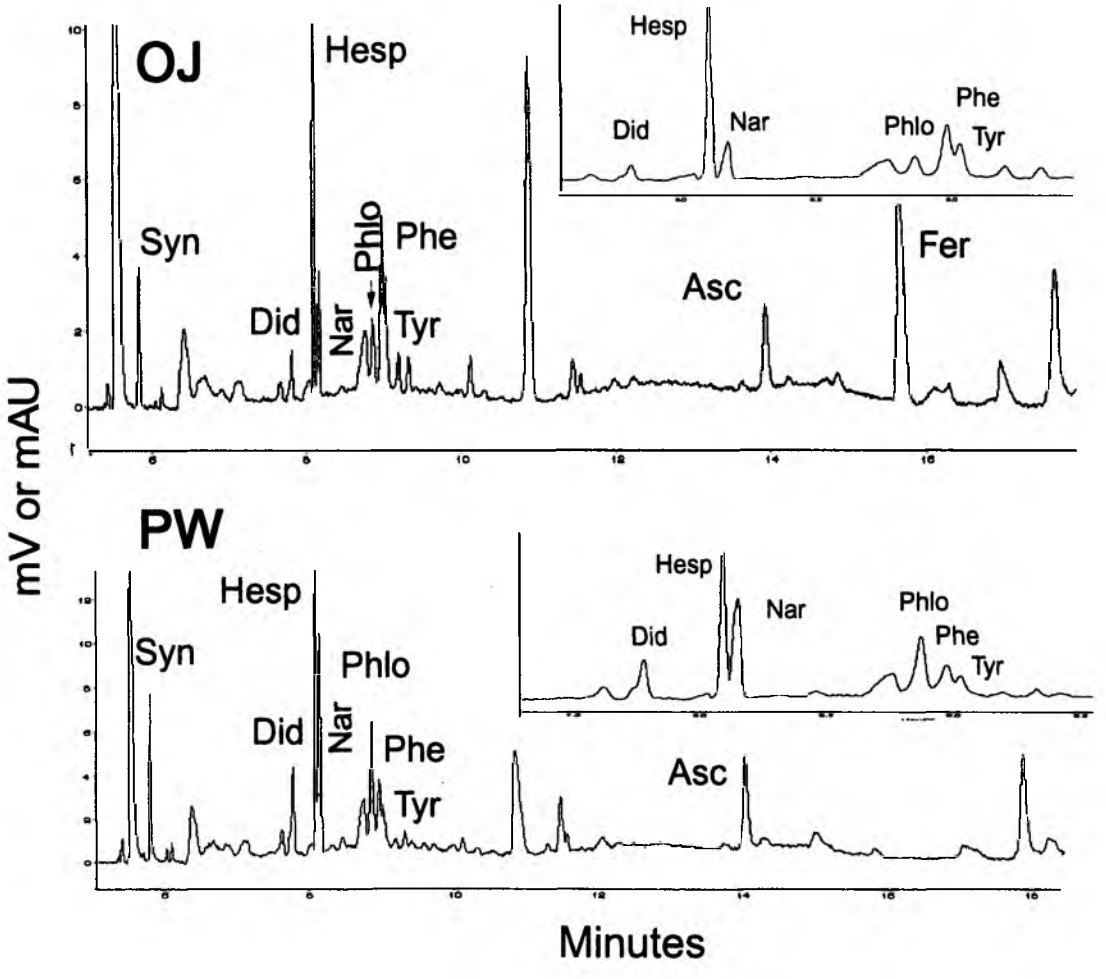

Figure 2. Electropherogram (200 nm) of good-quality orange juice (freshly squeezed OJ) and pulpwash (PW). The differences in the amounts of didymin (Did), narirutin (Nar), and phlorin (Phlo), and amino acids can be seen.

and the juice was squeezed manually. Segment wall membranes were cut off the albedo, rinsed, and blotted to remove the remaining juice. The albedo was then cleaned of any remaining membrane or flavedo fragments. Solid fractions were homogenized for 5 min with a rotary homogenizer at maximum speed and centrifuged at 475 × *g* for 5 min to remove remaining solids, and then the supernatant was analyzed. To improve homogenization, water was added to the flavedo (1:1 by weight) and to the albedo (1:2 by weight). Membranes were not diluted.

PW levels were determined by analyzing the CE data with an ANN trained by back-propagation (NeuroShell, Ward Systems, Frederick, MD). The net architecture consisted of 3 input neurons, 4 hidden neurons, and 2 output neurons. The learning rate was set at 0.6, and the momentum at 0.5. Iteration was maintained until the learning threshold was reached. Learning was performed by presenting to the ANN the ratios of the surface areas of didymin, narirutin, and phlorin to that of the IS ferulic acid. The training set was established from frozen concentrated juices and from PW from Florida, Brazil, and California (8, 5, and 4 samples, respectively). To guarantee authenticity, most citrus juices were produced at the Florida Department of Citrus pilot plant. PW samples and various types of commercial juices and juices with in-line PW were obtained from industrial sources. Mixtures containing 0, 10 , 20, 30, 40, and 100% PW were examined. Blends were made by mixing pure juice and pure PW diluted to 11.8°Brix.

Results and Discussion

Our goal was to separate as many compounds in citrus juice as possible in a single experiment. As a consequence, it was not possible to achieve the best separations for all compounds examined. The method provides information on the quality of the juices. The best juice is one made from mature fruits squeezed and processed under mild conditions. NFC juices meet this definition. Nonrefrigerated products such as canned juices represent the low-quality end of the spectrum, where yield is the main concern. In addition to fruit quality, many treatments affect juice quality, including squeezing and finishing pressures (41), addition of PW (42), and heat treatment and storage (43). These procedures modify the chemical composition of the juice and alter its flavor.

Citrus Juice Components

A preliminary report of the CE method was published previously (44). Since then, details for monitoring of citrus juices

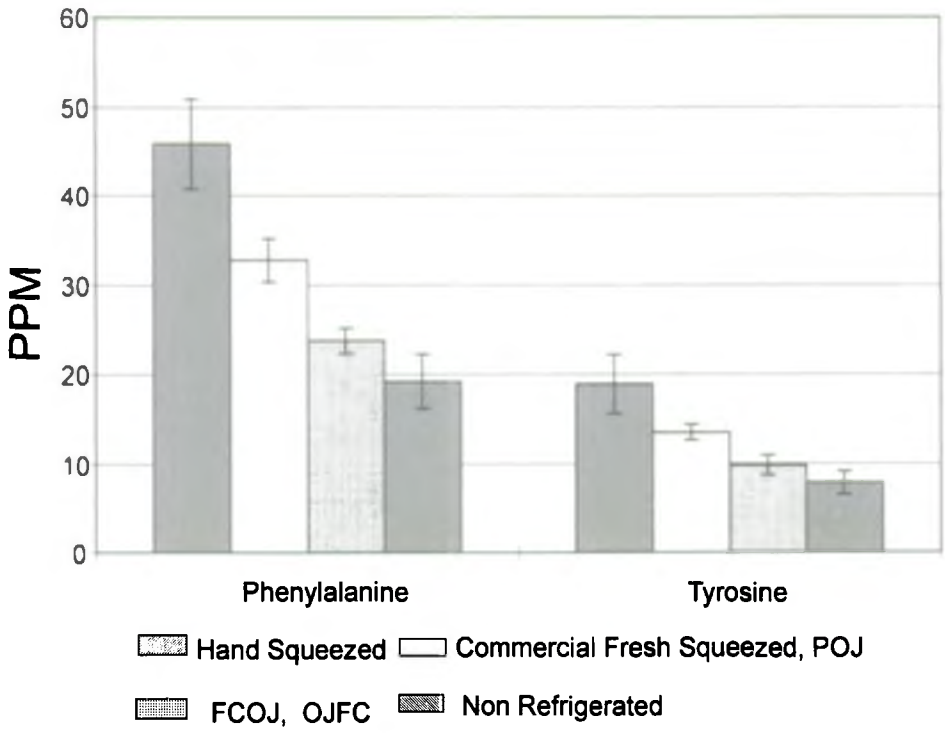

Figure 3. Differences in amounts of phenylalanine and tyrosine among different types of orange juice (mean and standard deviation of 8 samples). Commercial freshly squeezed orange juice and pasteurized orange juice (POJ) were combined. Likewise, frozen concentrated orange juice (FCOJ) and orange juice from concentrate (OJFC) were combined.

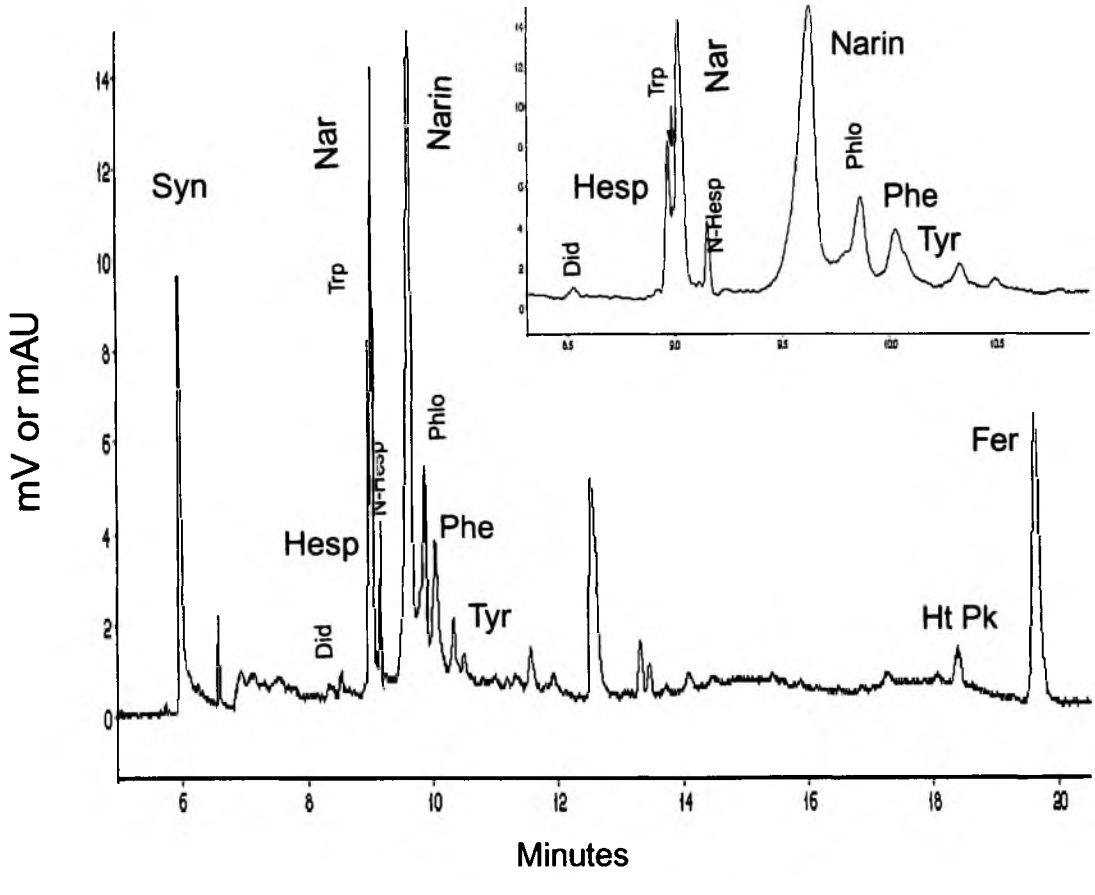

Figure 4. Electropherogram of grapefruit juice: Trp = tryptophan, N-hesp = neohesperidin, Narin = naringin.

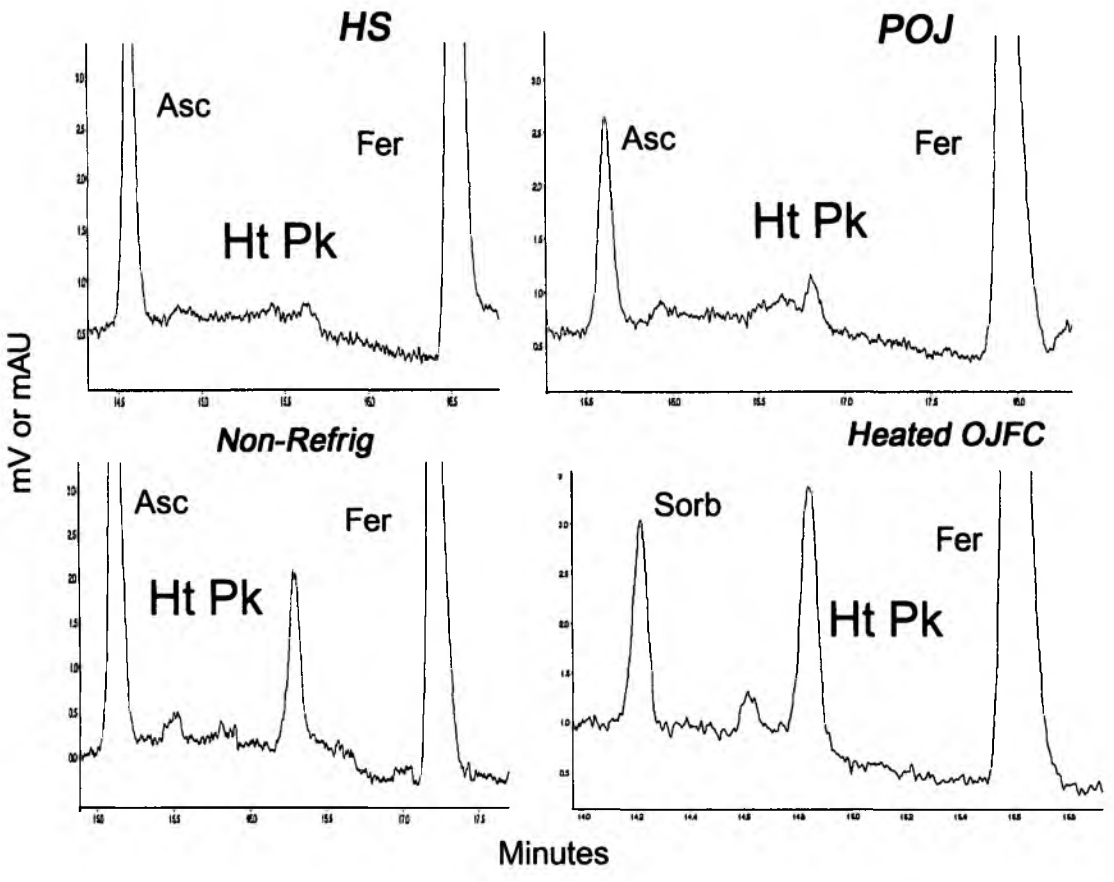

Figure 5. Changes in the size of heat peak (Ht Pk) in hand-squeezed (HS), pasteurized (POJ), nonrefrigerated orange juice from concentrate (Non-Refrig), and heated juice (Heated OJFC; 15 min at 50°C).

have been developed and some modifications to the procedure have been introduced. Addition of acetonitrile to the buffer improved separation but modified the migration times of some components, particularly the amino acids, which migrate more slowly under the present conditions (4). The presence of acetonitrile made it imperative to use capped buffer vials (Scientific Resources, Eatontown, NJ). A new buffer vial has to be used after a maximum of 3 runs to ensure good separation, particularly in the area of phlorin. Without capped vials, compounds such as phlorin, phenylalanine, and tyrosine merge into a single peak and phlorin can be overestimated. Washing of the capillary is also very important, because the accumulation of residues induces changes in migration time.

Figure 1 shows a 3-dimensional electropherogram of orange juice. Using high-speed scanning, the instrument produces a UV scan every 10 nm, between 192 and 360 nm. Stacking of these traces introduces a third dimension that reveals the spectrum associated with each peak. An individual scan at any of the recorded wavelengths can be extracted from the electropherogram to quantitate a specific chemical (Figure 2). Not all compounds identified in the juice are mentioned; only those used in the monitoring procedure will be discussed. Among those, the biogenic amine synephrine is more than 3 times more abundant in juice from immature Hamlin and Valencia (92.2 ± 6.5 ppm) than in juice from mature fruits (28.6 ± 3.7 ppm) and could be used as an index of fruit maturity.

Other information is provided by the 3 amino acids absorbing in the UV: trytophan (Trp), phenylalanine (Phe), and tyrosine (Tyr). The method gives only partial separation of hesperidin (Hesp) and Trp. This is not a major problem for orange juice because the juice is rich in Hesp and relatively poor in Tyr. In grapefruit juice, however, the 2 peaks are of similar size and a minimal separation is desirable. This can usually be done by injecting very small volumes. High levels of Phe and Tyr are characteristic of fresh juice. Levels of these amino acids decrease steadily as the extent of processing increases. Similar changes occur for Trp in grapefruit juice; early microbial activity could be responsible for the decline in the levels of this amino acid (Figure 3). Free amino acid concentration of fresh and canned orange juices have been compared by Kampfl et al. (45) who showed that fresh juices have a higher concentration of Trp and a much lower concentration of proline than canned juice. These results show that much work remains to be done to fully understand the changes in the free amino acid concentrations of stored citrus juices.

Phlorin (phloroglucinol glucoside) is one of the main components of the albedo and the membranes. It is almost totally absent from hand-squeezed juices and is the component most indicative of the presence of PW. When the method was first being developed, we referred to phlorin as unknown I (4). Its identity was established only recently (46).

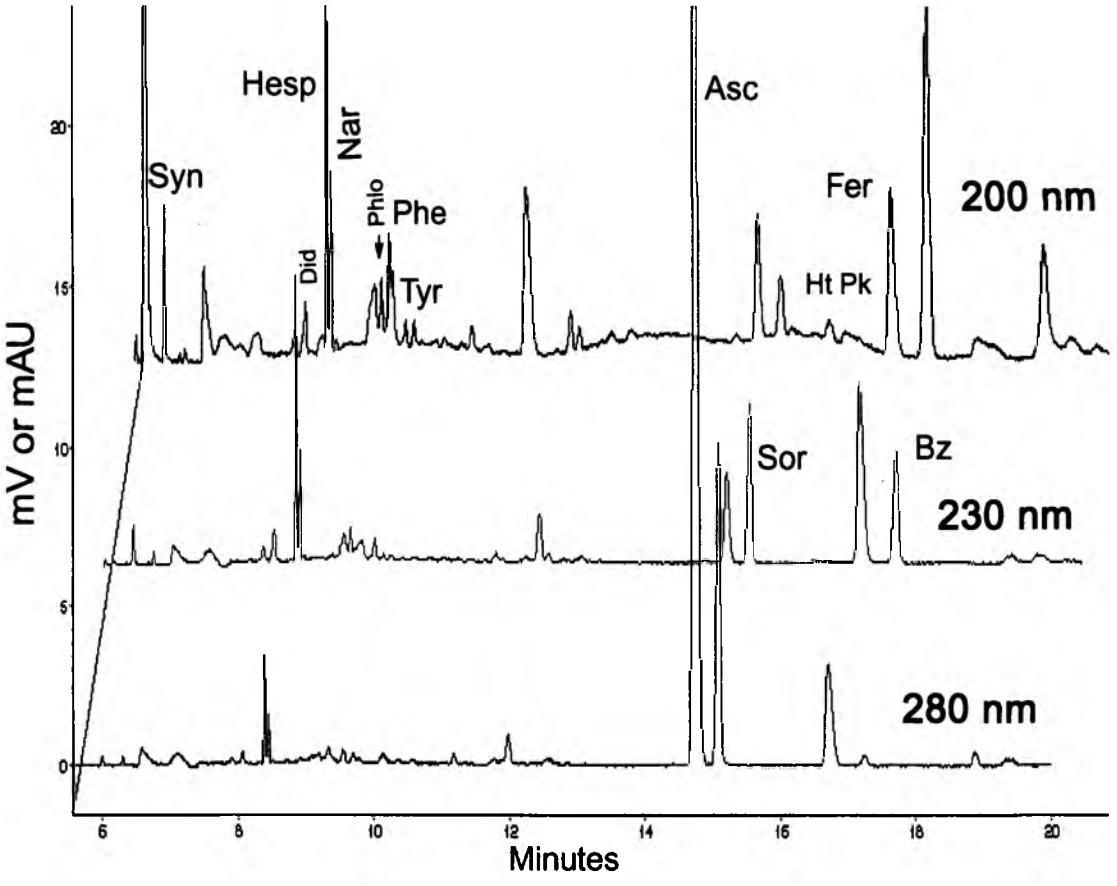

Figure 6. Example of orange juice screening. Examination at 3 wavelengths (200, 230, and 280 nm) allows rapid monitoring of samples. Bz = benzoate, Sor = sorbate.

The flavonoids didymin and narirutin are important in determining juice quality. High amounts reflect the harshness of the squeezing process and the presence of PW. The Hesp peak reflects only the soluble part of the total Hesp content. It is relatively constant in juice from concentrate. In fresh juices, the very high level of Hesp found initially decreases rapidly because of precipitation. Grapefruits, K-early tangelos, and sour oranges contain naringin, neohesperidin, and other related compounds in various amounts (35). Compared with orange juice, grapefruit juice contains large amounts of narirutin, phlorin, and naringin and small amounts of neohesperidin (Figure 4). If present in a juice, naringin and neohesperidin reveal the presence of citrus juice other than that of citrus sinensis. These compounds can be examined in a single analysis. We also examined ascorbic acid routinely.

A peak, not yet characterized, is induced by thermal processing. This peak is absent from hand-squeezed or fresh-squeezed juices but appears in pasteurized juice, and the size of the peak increases in orange juice from concentrate (OJFC). It can be generated by heating a fresh juice, and it develops over time in juices kept at room temperature (Figure 5). This compound absorbs only in the low UV, and its spectrum could not be correlated with any compounds associated with juice heat abuse such as hydroxymethylfurfural.

The differences between a good-quality NFC and PW can be seen in Figure 2. The NFC has low didymin, narirutin, and phlorin peaks and high amino acid peaks. The opposite is true of PW. The low amino acids may be attributed to some microbial activity during storage because PW is not always handled with the same speed as the juice.

Some compounds that do not originate from the fruit are also examined. Sorbate and benzoate are monitored because they are used as preservatives. Trace amounts of benzoate are

Table 1. ANN estimation of PW[a]

PW added, %	PW found, %	
	Same juice and PW	Different juices and PWs
10	9.8 ± 0.6	9.6 ± 1.9
15	14.7 ± 0.7	15.9 ± 2.9
20	21.1 ± 1.6	23.6 ± 3.3
30	30.9 ± 1.9	32.7 ± 3.9

[a] For each concentration, 8 values were measured from blends of the same juice and PW or from 8 mixtures of different juices and PWs. Values are means ± standard deviations. The juices were prepared at the pilot plant. PWs were from industrial origin.

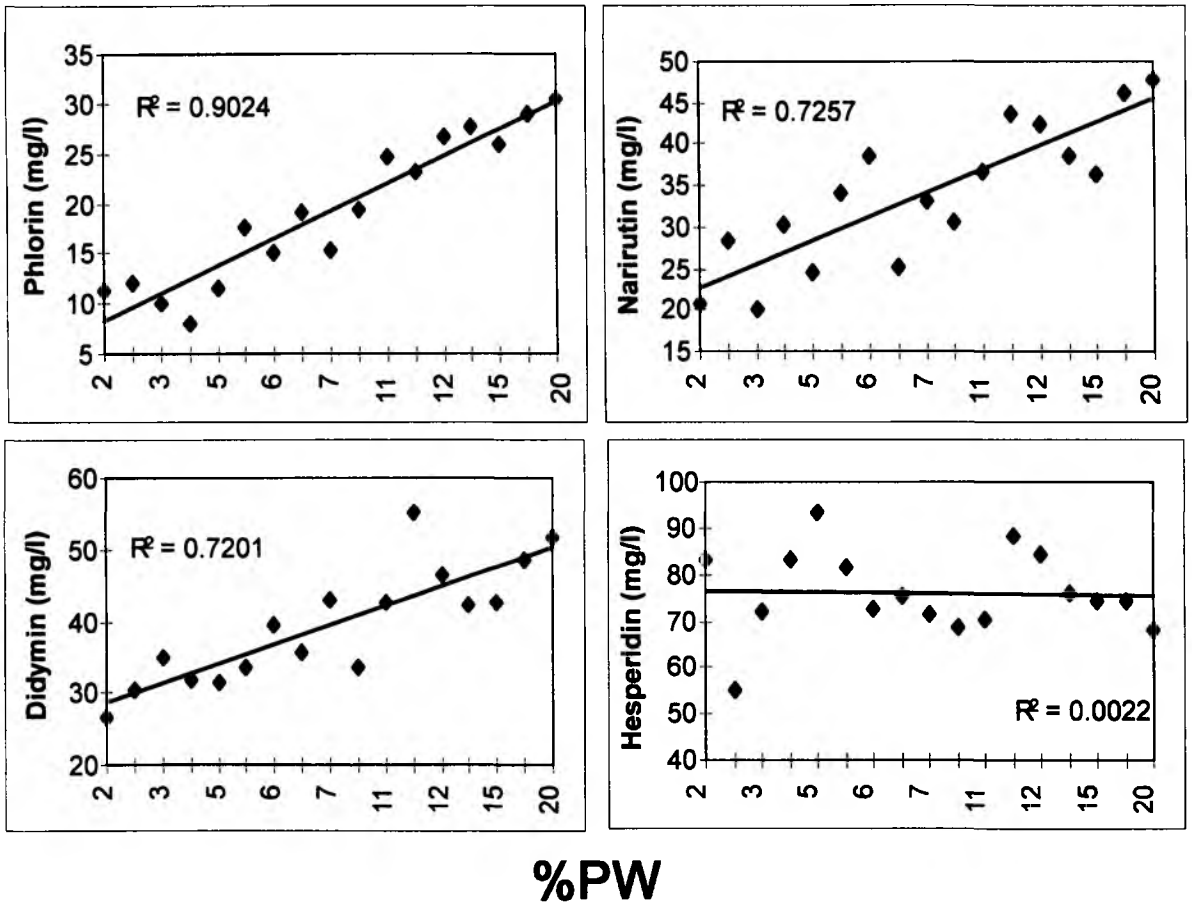

Figure 7. Regression analyses indicate positive relationships between PW and phlorin, narirutin, and didymin but not hesperidin. Dilutions were made with 5 different juices and PWs.

found naturally in the juice, and benzoate also is added to outline PW at 5–10 ppm as a marker in drinks.

Analysis of Juice and PW

In the routine juice analysis that was developed, data are collected as high-speed-scan electropherograms. Most of the citrus juice compounds scrutinized (amines, flavonoids, polyphenols, and amino acids) can be seen at 200 nm. However, the benzoate peak is noted at 230 nm, and the sorbate and ascorbic acid peaks at 280 nm. For juice monitoring, it is convenient to extract these 3 wavelengths simultaneously (Figure 6). The 3-wavelength graphs allow screening of samples for further analyses or calculations. The use of an IS (ferulic acid) eliminates some variations introduced by these analyses. Flavonoids, preservatives, ascorbic acid, and PW are the compounds most frequently quantitated. However, comparison of results obtained by CE with those obtained by other methods will be examined separately.

In the past, PW was monitored by the Petrus method, which involved examining the UV, visible, and fluorometric scans of the whole juice (35). The method provided mostly qualitative information, and the influence of the color could produce false-positive results. In the present method, data from high-speed scans generated during juice monitoring by CE were used to quantitate PW.

PW concentration was assessed by measuring the peak size of compounds more particularly concentrated in PW (PW compounds) and processing the ratios of PW compound peaks to that of ferulic acid with an ANN. Results are shown in Table 1. PW percentages were estimated for 8 groups of 8 samples containing 10, 15, 20, or 30% PW made from the same or different juices and PWs. Calculated values were close to actual percentages, and standard deviations (SDs) were small when mixtures of the same juice and PW were examined. Estimated percentages for mixtures of different juices and PWs were still similar to actual values, but SDs were significantly larger. The ANN correctly estimated PW concentrations even for blends of dif-

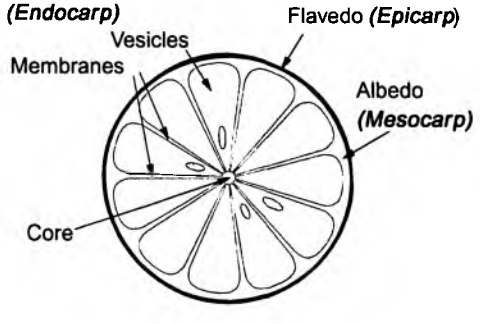

Figure 8. Cross-section of orange fruit.

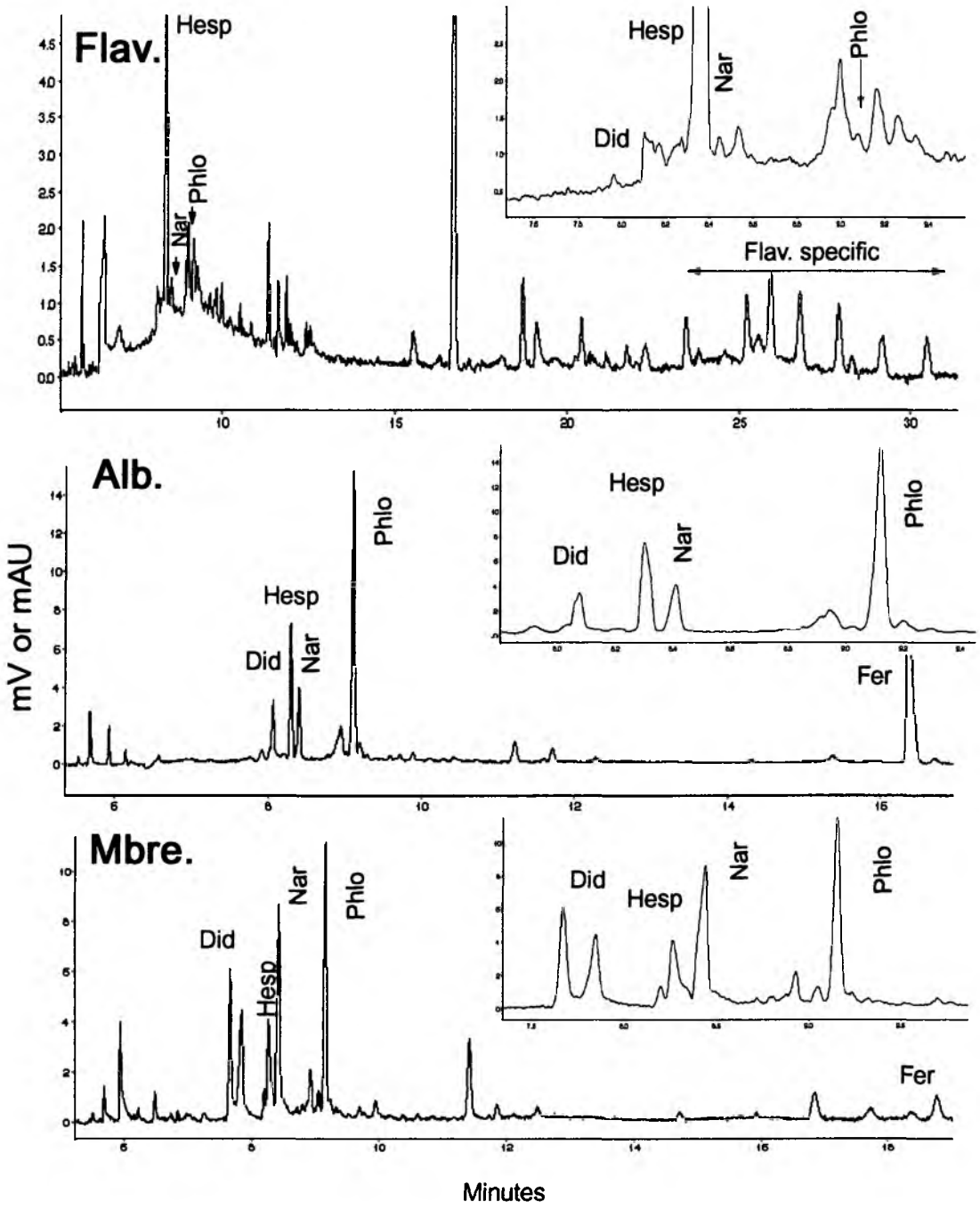

Figure 9. Electropherograms (200 nm) of orange fruit parts: Flav. = orange flavedo diluted 32-fold (flavedo-specific compounds are clearly visible although the maximum absorption of their spectra is at 320 nm); Alb. = orange albedo diluted 48-fold; Mbre. = orange membranes diluted 40-fold.

ferent products. In these mixtures, variations in the quality of the PW was responsible for the larger SD.

At the beginning of this study, several compounds were found to be present in larger amounts in PW than in juice, namely synephrine, didymin, Trp, narirutin, Hesp, feruloyl and sinapyl glucose, and 2 unknowns (I and II; 44). The number of PW characteristic compounds was rapidly reduced to 5. Synephrine levels depend too much on fruit maturity. Trp varies mainly with freshness. Feruloyl and sinapyl glucose do not show sufficient variations. More recently, the best evaluations of PW have been obtained with only didymin, narirutin, and unknown I, now identified as phlorin (Figure 7).

Analysis of Subfractions

PW compounds were determined empirically. They were present only in very small amounts in the juice itself and had to originate from other parts of the fruit. To determine the origin of these extraneous components, several fruit subfractions (Figure 8) were analyzed: the flavedo, the albedo, the segment membranes (Figure 9), and the juice itself (Figure 2). Electropherograms of orange subfractions have been described previously (4). The albedo is the main source of phlorin, but it also contains significant amounts of narirutin, didymin, and Hesp. The membranes contain very large amounts of didymin, nariru-

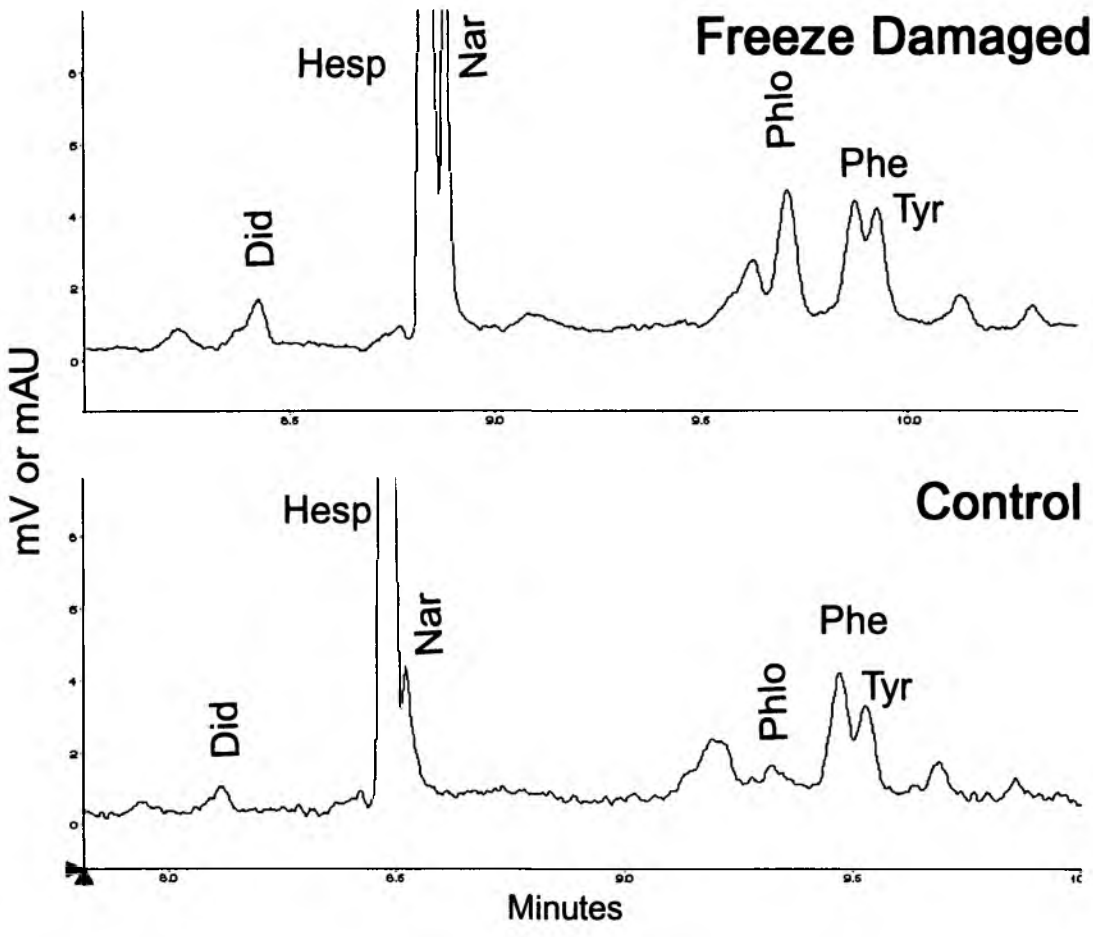

Figure 10. Influence of freezing on concentration of PW components in juice. Fruits were frozen for 16 h and allowed to thaw for 48 h before the juice was extracted. Juice from freeze-damaged fruits no longer looks like that from a freshly squeezed juice but appears more like a juice containing PW.

tin, and phlorin. By contrast, the flavedo is low in narirutin and contains almost no phlorin. The small amount of phlorin found in the flavedo may come from contamination from the albedo. Phlorin is present in large amounts in peel extract and is being used as a marker for this preparation (46). This is only possible because the peel extract originates from the flavedo and the albedo. The main characteristic of the flavedo is a series of unidentified peaks, with similar spectra, not found anywhere else in the fruit. There are preliminary indications that these compounds could be related to carotenoids. These compounds make identification of peel extract particularly easy at 320 nm. This study shows that compounds characteristic of PW originate from contamination by the albedo and the segment wall membranes.

Factors Affecting Measured PW Concentration

The percentage of PW measured by ANN is determined by the concentration of albedo and membrane molecules (PW chemicals) present in the juice. It is therefore important to determine how and to what extent PW compounds can be released into the juice. Three processes, and to some extent the fruit cultivar, were found to influence the amounts of PW compounds in the juice. Material can leak into the juice when freeze-damaged fruits are extracted. They can also be released mechanically by pressure when fruits are squeezed. Finally water extraction of the pulp frees some of these compounds.

Most fruit varieties did not seem to influence greatly the amount of PW compounds released into the juice. A high level of narirutin has been reported in some juices from navel oranges, but Widmer (personal communication) reported a narirutin concentration of 34 ± 11 ppm in California navel juice, similar to values measured for Hamlin or Valencia oranges. Furthermore, navel juice is usually debittered before being added to commercial products. The level of PW components (narirutin, didymin, and phlorin) is higher in pure Ambersweet juice (47) than in other oranges, and pure Ambersweet juice can give a false-positive indication for PW.

When a fruit freezes, water crystals break the fruit cellular walls and allow PW molecules to leak into juice sacs. Laboratory experiments show that the level of these molecules depends on the extent of the freeze (time and temperature) and on the time between the freeze and the processing after thawing. Fruits frozen for 4 h and processed after 8 h at room temperature showed in-line PW values (as if 3–7% PW had been added to the juice) or below. Fruits frozen solid and then allowed to thaw for 24 h show about 20% PW (Figure 10). When thawing time reaches 48 h, the components indicate 40% PW. When a freeze occurs in the grove, fruits are rapidly processed, produc-

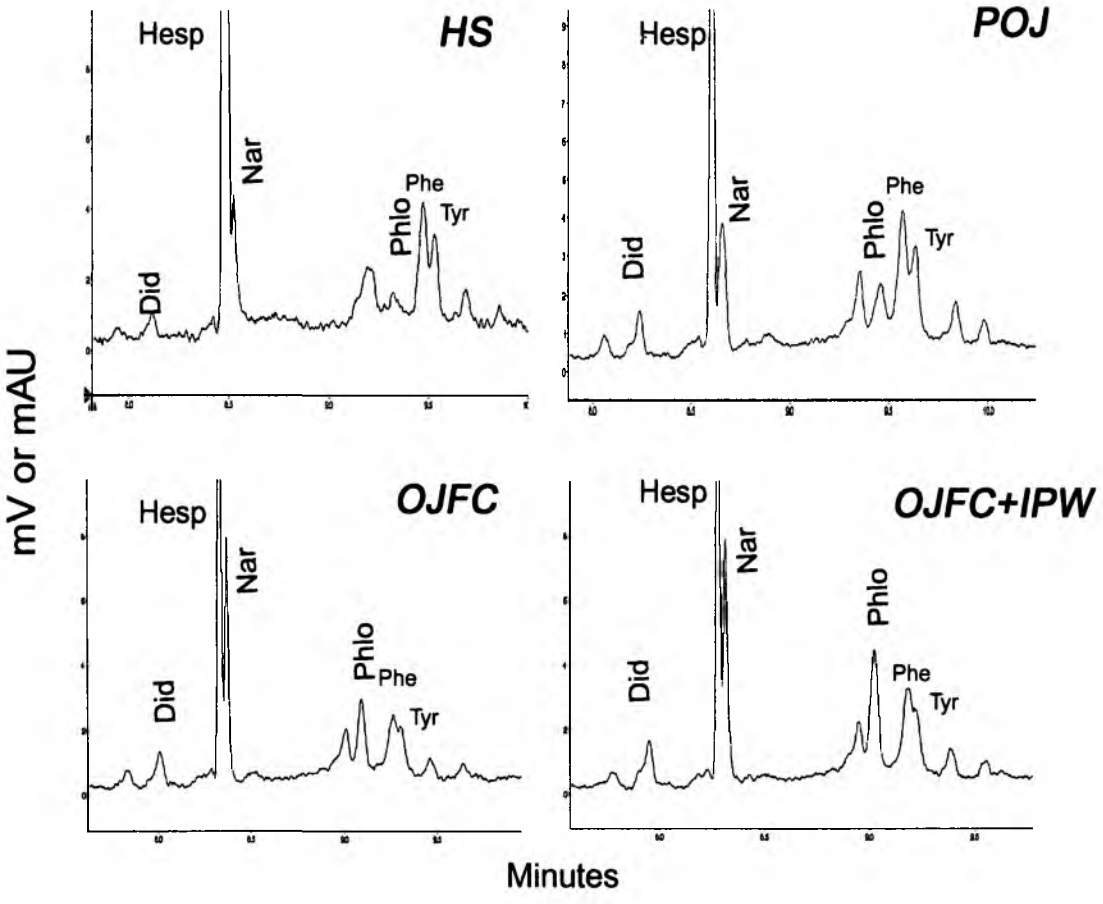

Figure 11. Electropherograms of different types of juices: HS = hand squeezed, POJ = pasteurized orange juice, OJFC = orange juice from concentrate (reconstituted juice), OJFC + IPW = OJFC containing in-line PW.

ing a juice that contains nonendocarp compounds at a level similar to or below that produced by in-line pulp washing.

Mechanical treatment of fruits (squeezing and finishing) strongly influences juice composition. High-quality juices, particularly NFC, are extracted under gentler conditions than OJFC. Thus, the concentration of PW components released varies with the type of commercial orange juice, from freshly squeezed juice to canned juice. In each case, different levels of didymin, narirutin, and phlorin can be measured, and from these data PW concentration can be estimated.

These differences are illustrated in Figure 11. Orange juices prepared in our pilot plant under very mild conditions of squeezing and finishing contained small amounts of "PW compounds" and the level of PW estimated by the neural network was close to zero ($0.6 \pm 0.4\%$). Hand-squeezed juices gave very similar results: phlorin is present only as a trace and the ANN program extrapolates a PW value between 0 and 1%. Commercial pasteurized juices are also produced under mild conditions, and the small amount of PW compounds pasteurization generates results in a calculated PW of about 2–3%. For OJFC, obtaining a high yield is the major concern, and these juices contain a little more PW chemicals than other juice types, about 5–7%. Any PW blended with an OJFC will add PW compounds to those generated by juice processing. OJFC with in-line PW contain 10–14% PW. Therefore, this PW has 2 origins: 5–7% due to the fact that the juice is an OJFC and an extra 5–7% generated by the presence of PW (Figure 12).

If the ANN database had been built from commercial OJFC data, the ANN would predict 0% PW for OJFC and the calculated amount of PW would be identical to the true PW level. The question of PW compounds being released into the juice by juice sacs in the case of juice containing added pulp also has been raised. Commercial frozen concentrated orange juices with or without pulp showed the same level of apparent PW ($3.7 \pm 1.1\%$ for juices with pulp as compared with $3.3 \pm 0.9\%$ for juices without), and they contain similar amounts of nonendocarp chemicals. When 40 g pulp/L was added to an NFC juice, the apparent PW level was not affected by 12 h of shaking at 28°C or by 3 days of shaking at 4°C.

From values in its database, the ANN calculates what volume of average PW at 11.8°Brix has to be added to the average juice at 11.8°Brix to produce the same concentrations of PW compounds as those present in the sample being examined. Table 1 shows that the ANN can accurately predict the correct percentage of added PW when the same preparation is analyzed. But depending on their quality, juices and PWs contain variable amounts of PW compounds that can affect the estimated volume of PW. It would be more meaningful to monitor juice quality by establishing limits on the concentration of these chemicals rather than by using the traditional volume estimation. Under such conditions, it also would no longer be neces-

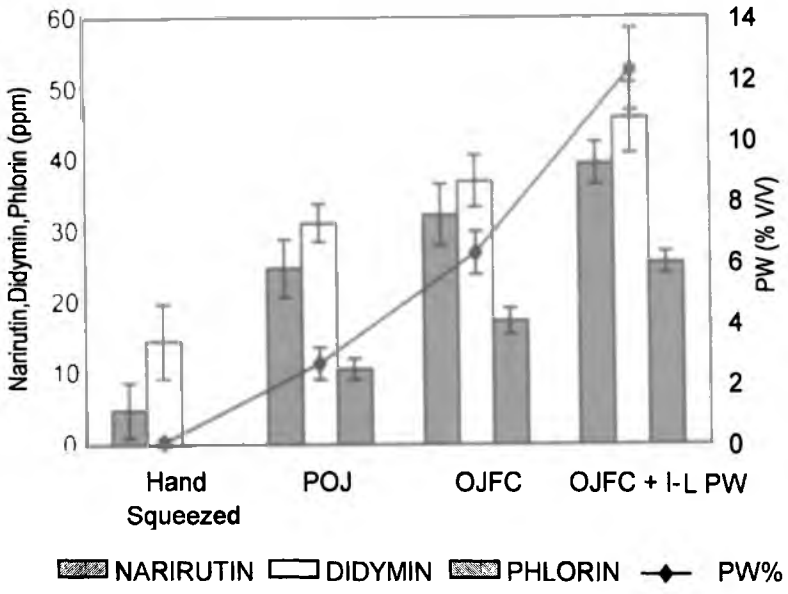

Figure 12. Changes in concentrations of narirutin, didymin, and phlorin (as phloroglucinol) and apparent PW percentage in several types of juices (mean and standard deviation of 14 samples). Hand-squeezed juices were prepared in the laboratory; others were of industrial origin.

sary to take into account the origin of the chemicals (diffusion or mechanical extraction).

Conclusion

We have taken advantage of the qualities of CE to develop a monitoring technique for citrus juices. Using UV detection, the analysis provides information on flavonoid and polyphenol contents of the juice, the concentration of vitamin C, and the presence of the preservatives sorbate and benzoate. Other compounds such as amines and amino acids also are seen but are not monitored. The presence of PW, addition of which to some types of juices is allowed under certain conditions, is determined by analyzing CE results with an ANN. Because PW estimation corresponds to quantitation of a mixture of 2 very similar juices, the same method could be used to determine the proportions of a blend of 2 juices. The total analytical time is one-half that of the previous procedure involving PW determination and LC measurement of flavonoids and preservatives.

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