J.; Kearney, P. C., Eds.; Pergamon Press: Oxford, New York, Toronto, Sydney, Paris, and Frankfurt, 1983a; Vol. 3, pp 97-102.
Lambert, R.; Sandmann, G.; Böger, P. *Pestic. Biochem. Physiol.* **1983b**, *19*, 309-320.
Matsunaka, S. *Residue Rev.* **1969a**, *25*, 45-58.
Matsunaka, S. *J. Agric. Food Chem.* **1969b**, *17*, 171-175.
Orr, G. L.; Hess, F. D. *Pestic. Biochem. Physiol.* **1981**, *16*, 171-178.
Orr, G. L.; Hess, F. D. *Plant Physiol.* **1982**, *69*, 502-507.
Sandmann, G.; Böger, P. In "Biochemical Responses Induced by Herbicides"; Moreland, D. E.; St. John, J. B.; Hess, F. D., Eds.; American Chemical Society: Washington, DC, 1982a, ACS Symp. Ser. No. 181, pp 111-130.
Sandmann, G.; Böger, *Lipids* **1982b**, *17*, 35-41.
Sandmann, G.; Böger, P. *Weed Sci.* **1983**, *31*, 338-341.
Sandmann G.; Kunert, K.-J.; Böger, P. *Z. Naturforsch. C: Biosci.* **1979**, *34C*, 1044-1046.
Sandmann, G.; Lambert, R.; Böger, P. *Z. Naturforsch., C: Biosci.* **1981**, *36C*, 633-637.
Urbach, D.; Suchanka, M.; Urbach, W. *A. Naturforsch., C: Biosci.* **1976**, *31C*, 652-655.

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Direct Gas Chromatographic Analysis of Aqueous Citrus and Other Fruit Essences

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An analytical method has been developed in which the characteristic flavor and aroma constituents of natural fruit essences can, for the first time, be quantitatively and qualitatively determined from a direct injection of aqueous essence into a gas chromatograph. This objective method can be used to evaluate essence strength and quality, determine effects of fruit varieties, temperature, storage, and unusual weather, control seasonal blending and production efficiency, and detect adulteration. Commercial aqueous orange essence samples were analyzed to determine strength, quality, processing differences, and effects of mild freeze damage to the fruit. Although this method was developed for evaluating citrus essences, we have also demonstrated its applicability to essences from apples, grapes, pineapples, strawberries, and bananas.

Citrus essence (aroma) is an aqueous distillate collected from the first stage of an evaporator during the production of juice concentrate from the corresponding fresh juice. The aqueous essence is separated from an oily layer (essence oil) prior to storage. Citrus juice volatiles concentrated in the aqueous fraction reflect both quantitatively and qualitatively the flavor and aroma of the parent juice. The aqueous fraction is thus a desirable flavoring material. Although aqueous essences are produced from all major citrus fruit, orange essence is the most commercially important and widely used. It is added to orange concentrate to restore "fresh" flavor and aroma and to synthetic drinks and other products to impart a natural flavor and aroma.

Ten million pounds of aqueous orange essence are currently being used annually in the United States. The potential annual production, based on the quantity of orange concentrate produced, is approximately 20 million lb. Realization of the full commercial potential of orange and other citrus essences has not materialized primarily because of the difficulty in evaluating their strength and quality and thus in producing a consistent, standard product (Shaw, 1977). Strength and quality characteristics of aqueous essences vary from lot to lot because of variations in processing methods, cultivar, season of harvest, and maturity. Many of the analytical methods previously reported for evaluating aqueous essences have included concentration of essence constituents before analyses. These include solvent extractions (Wolford et al., 1962; Shultz et al., 1964; Moshonas and Shaw, 1973). Other means of concentration included liquid–liquid extraction (Attaway et al., 1962) and adsorption on organic polymer powders (Moshonas and Lund, 1971; Shultz et al., 1971; Dravnieks and O'Donnell, 1971). Colorimetric techniques were reported by Attaway et al. (1967), Braddock and Petrus (1971), Peleg and Mannheim (1970), and Ismail and Wolford (1970). Attempts to obtain accurate quantitative and qualitative analysis by injecting orange essence on packed columns was reported by Lund and Bryan (1977). However, these and other methods have had inherent limitations and have not been satisfactory to the citrus industry for calculating essence strength and quality. Consequently, subjective organoleptic evaluations are still necessary to adequately evaluate these essences for use in flavoring citrus products.

The present study reports a simple, objective method for evaluating strength and quality of aqueous fruit essences. This gas chromatographic (GC) method makes it possible, for the first time, to obatin detailed quantitative and qualitative analyses of these essences from a direct injection of a small quantity of the whole essence. The production of fused silica capillary columns coated with a cross-linked, nonpolar liquid phase helped in the development of this method. These columns resist bleeding and degradation associated with aqueous sample analyses attempted on earlier GC columns.

EXPERIMENTAL SECTION

Gas Chromatography. GC data were obtained with a Hewlett-Packard Model 5880A instrument equipped with a flame ionization detector, a 50-m, wide-bore (0.031–0.032-mm i.d.) capillary fused silica cross-linked SE-54 column (Hewlett-Packard, Avondale, PA), and a capillary inlet system fitted with a splitless liner that allows helium to flow down through the liner to the head of the column. There the flow divides, with 1.5 mL/min going through the column while the rest is vented. The normal three-stage operation of the splitless mode was not used.

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A capillary split inlet system was used for the GC when interfaced to a mass spectrometer (see below). Injection port and detector temperatures were 250 °C. The column temperature was held at 40 °C for 3 min, then programmed to 175 °C at 6 °C/min, and held there for 3 min. Upon completion of the run the temperature was automatically advanced and kept at 210 °C for 20 min. The threshold was set at 1, peak width at 0.02, and chart speed at 1 cm/min.

Procedure. With the attenuation set at 2↑9, a 1-μL sample of whole aqueous essence is injected into the GC and the oven program started. After the large, first peak (mostly ethanol) has eluted, the attenuation is reduced to 2↑0 for the remainder of the run.

Mass Spectra. A Finnigan Model 4021 gas chromatograph–mass spectrometer (GC–MS) was used to separate and identify the constituents of aqueous orange essence. The GC was equipped with a split liner in the injection port and a 0.02 mm i.d. by 50 m fused silica column, coated with SP-2100 (Hewlett-Packard). The flow rate was 1 mL/min and the injection port split ratio was 100:1. Initial oven temperature was held at 50 °C for 10 min and then programmed at 6 °C/min to 220 °C. The MS was equipped with a jet separator and mass units were monitored from 40 to 300 at 70 eV. Mass spectral identifications were made by comparison of mass spectra and retention times with those of authentic compounds.

Flavor Evaluations. For taste tests, orange essences were added to a bland solution consisting of 1500 mL of distilled water, 180 g of sugar, 6.3 g of citric acid, 2.1 g of sodium citrate, and 0.63 g of pectin. The triangle and paired comparison tests used were reported by Boggs and Hanson (1949). For triangle tests, there were three samples per presentation, two of which were identical. Judges were asked to indicate which sample had a different flavor. In paired comparison tests, judges were asked to indicate which sample they prefered.

RESULTS AND DISCUSSION

With the analytical method now being reported, whole aqueous fruit essences can, for the first time, be directly analyzed to yield detailed quantitative and qualitative data on flavor and aroma constituents. These data can be used to objectively determine the strength and quality of these essences. Commercial citrus essences have been generally collected so that alcohol content ranges from 11 to 15% by volume. Alcohol content has been traditionally used as a measure of essence strength (Wolford et al., 1968). However, because of variations in processing methods, season of harvest, cultivar differences, and maturity differences, the percent alcohol ofter does not accurately reflect the flavor strength or quality of aqueous essences being produced.

When significant differences in essence strength are present, GC profiles readily show these differences. Each of three gas chromatograms shown in Figure 1 were obtained from a 1-μL sample of whole aqueous orange essence. Chromatograms A and B show typical separations of commercial essence constituents (11–15% alcohol), while GC curve C resulted from analysis of an experimental orange essence (40% alcohol). The stronger essence (based on alcohol content) also contains much higher levels of other volatile compounds including many too small to detect in the weaker, more typical essence profiles. Detailed quantitative and qualitative data obtained from such GC chromatograms can be used to evaluate flavor strength and quality of aqueous fruit essences.

A commercial essence with 14.5% alcohol content was used to determine reproducibility (Figure 2). The coefficient of variation for each of the 26 major peaks was determined from 6 consecutive gas chromatographic runs. For 22 peaks the coefficient of variation was less than 10%. The two smallest peaks (9 and 24) had the highest coefficients. The remaining peaks (3, 9, 23, and 24) showed coefficients of variation 18, 22, 22, and 39%, respectively. As expected, the smaller peaks had the greater coefficients of variation.

Strength differences, observed in evaluating the GC curves of essences, can be determined objectively from the

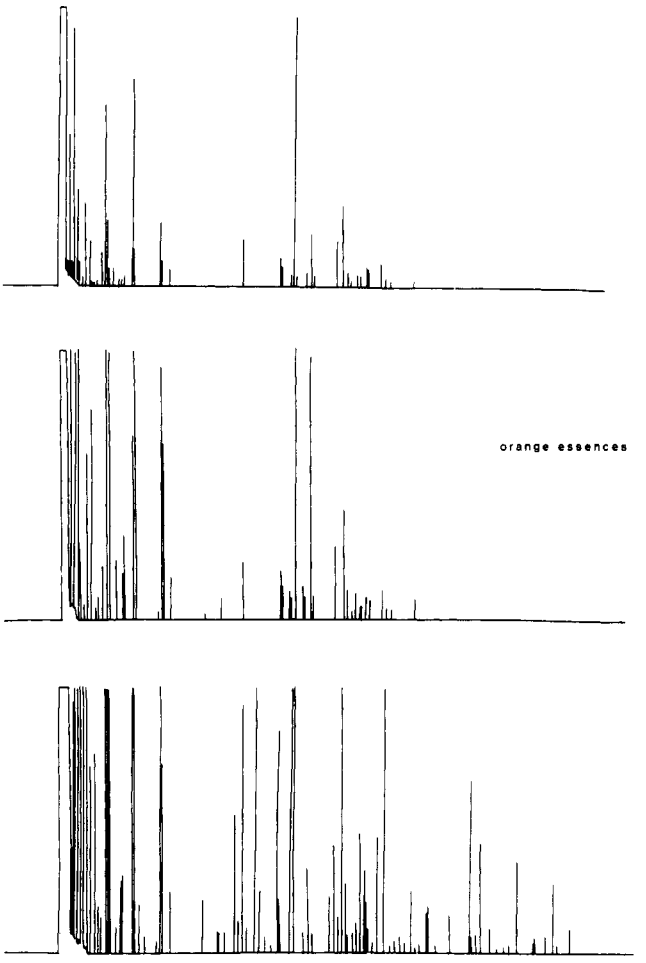

Figure 1. Gas chromatograms obtained from whole aqueous orange essences (A, upper curve; B, middle curve; C, lower curve).

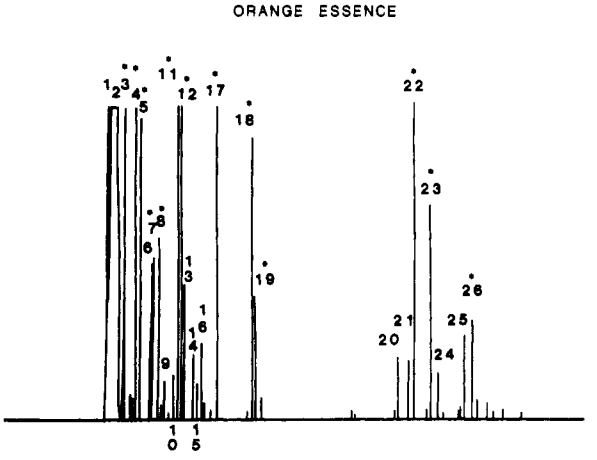

Figure 2. GC profile of aqueous orange essence showing constituents used to determine reproducibility. Components used to determine efficiency of essence recovery units (indicated by asterisks) and their GC area percent values are as follows: 3, 0.059; 4, 0.095; 5, 0.019; 7, 0.011; 8,0.022; 11, 0.032; 12, 0.030; 17, 0.035; 18, 0.022; 19, 0.014; 22, 0.032; 23, 0.028; 26, 0.009.

Table I. Constituents of Aqueous Orange Essence

1, acetaldehyde methanol	10, 1-octanal	20, α-terpineol
2, ethanol	11, *p*-cymene	21, 1-decanal
3, acetal	12, limonene	22, *trans*-carveol[a]
4, *trans*-2-pentenal[a]	13, *trans*-linalool oxide[a]	23, neral
5, 1-hexanal	14, 1-octanol	24, geraniol
6, ethyl butyrate	15, *cis*-linalool oxide[a]	25, geranial
7, *trans*-2-hexenal	16, 1-nonanal	26, perillaldehyde
8, 3-hexen-1-ol[a]	17, linalool	27, 1,1-diethoxyoctane[b]
9, 1-hexanol	18, ethyl 3-hydroxyhexanoate	28, thymol[b]
	19, terpinene-4-ol	

[a] Tentative identification. [b] Identified for the first time as orange constitutents.

total peak area percent of 13 major constituents indicated by asterisks in the GC curve for a typical commercial orange essence shown in Figure 2. Peak area percentages of the individual numbered compounds of this sample are also reported. Four major volatile constituents were omitted from the flavor strength calculations. These were ethanol and methanol (peaks 1 and 2), which are not important contributors to citrus flavors, acetaldehyde, which does not separate from the methanol peak, and limonene (absent in this sample), which varies widely in concentration depending on how efficiently the essence oil (95% limonene) layer is separated from the aqueous essence.

To demonstrate a practical application of this GC method, flavor strength analyses were made to determine the efficiency of two different essence recovery units. Four aqueous orange essence samples each were collected from essence recovery units A and B, from the same lot of oranges, on the same day, and at the same plant. Comparison of the total peak area percentages of the 13 chosen compounds from unit A (average 0.458%, SD 0.06) with unit B (average 0.320%, SD 0.02) showed a significant difference in the two values (>99% confidence level) and a quantitative difference of 31% less flavor and/or aroma constituents in essence from unit B compared to that from unit A. The analysis also showed that samples from both units were qualitatively identical and that constituent relationships were also the same. This information led to processing modifications of the unit processing the weaker essence, thus increasing the flavor strength of the weaker essence to the level of the stronger essence.

Determination of flavor strength by quantitative analysis of essence constituents does not provide enough information for evaluating the overall quality. Data provided by this GC method provides additional information in two areas needed to accurately make a quality evaluation. The first area is identification of essence constituents so they can be routinely monitored by their retention times. Any significant qualitative change in which new constituents appear or established constituents are lost would indicate an essence of quentionable quality. Aqueous orange essence constituents identified in this study are numbered in the chromatogram obtained from a concentrated orange essence (25% ethanol) shown in Figure 3 and listed in Table I. Thymol and 1,1-diethoxyoctane are being reported as orange product constituents for the first time. Thymol is an important tangerine constituent and was probably found in orange essence because processors are permitted to use up to 10% tangerine juice in the production of orange concentrate. 1,1-Diethoxyoctane can be formed from two other known juice and essence constituents, octanal and ethanol, in the acidic juice medium during distillation.

A second area that can be examined by this analytical method for effects on essence quality is the quantitative relationship of individual components. The quantitative relationship of constituents in any flavor fraction can have a dramatic effect on flavor and aroma, and any significant

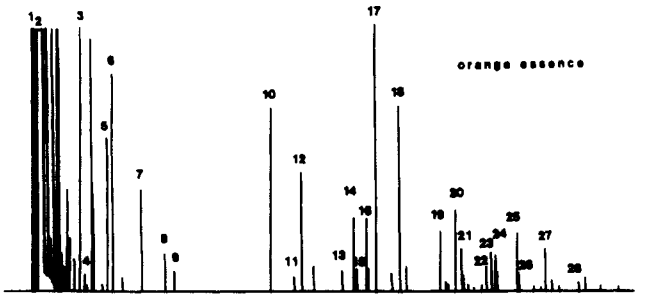

Figure 3. Gas chromatogram of commercial aqueous orange essence.

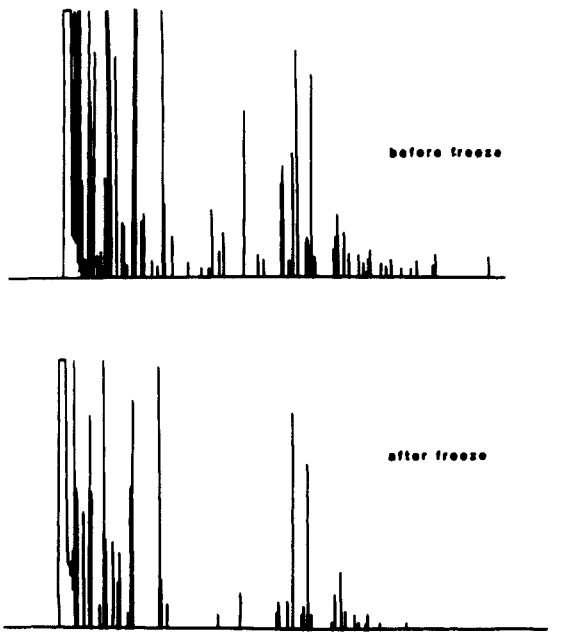

Figure 4. Gas chromatogram of aqueous orange essences obtained from oranges before and after the 1982 freeze.

shift in this relationship would indicate an essence of questionable quality.

Analysis of samples of aqueous orange essences provided by a commerical processor illustrates the type of quality evaluation that can be quickly made using this GC method. Two essences obtained from oranges harvested from the same grove and processed in the same plant with the same equipment were analyzed and compared (Figure 4). The only difference was that one essence was obtained from oranges harvested before the Jan. 1982 freeze, while the second essence came from oranges harvested after the freeze. The GC profile of the before-freeze essence sample compared well with the profile of a typical, good-quality commercial orange essence. The profile of the after-freeze essence sample showed quantitative and qualitative differences from normal orange essence, indicating an adverse affect on quality. To confirm this conclusion, taste tests were conducted using an experienced taste panel. The panel found a significant difference (99% confidence level)

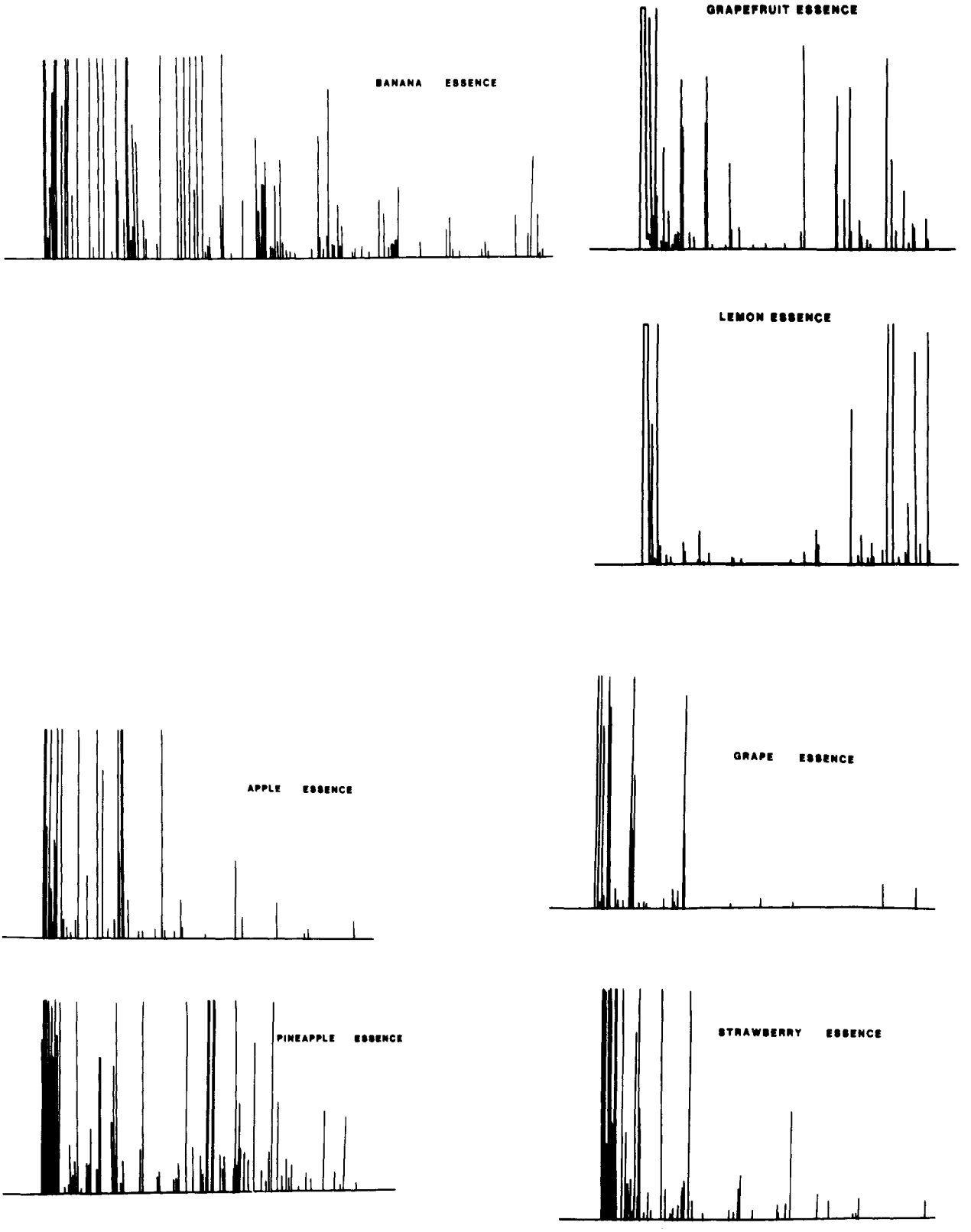

Figure 5. Gas chromatograms of commercial aqueous fruit essences.

in the flavor of the two samples and, through a second taste test, determined a significant preference (95% confidence level) for the essence obtained from oranges before the freeze.

Some citrus processors are currently using this analytical method to help evaluate strength and quality of aqueous citrus essences (Johnson and Vora, 1983; Hickey, 1983). It has the potential to (a) help determine the proper mix for blending essences to produce a more uniform product, (b) provide a flavor profile of a given essence so that any storage changes can be monitored, (c) help determine any adulteration of essences, and (d) determine through the size of the limonene peak whether enough essence oil has been removed to prevent flavor deterioration due to degradation of limonene during storage (Guadagni et al., 1970).

Although our primary interest and emphasis were in finding a simple objective method for evaluating citrus essence, the method also makes it possible, for the first time, to get detailed quantitative and qualitative analyses on flavor and aroma constituents directly from other aqueous fruit essences. Figure 5 shows the favor profiles of a number of commercially available natural fruit es-

sences obtained by this procedure. These essences can also be evaluated in the same manner described for orange essences.

Registry No. 1, 75-07-0; 2, 64-17-5; 3, 105-57-7; 4, 1576-87-0; 5, 66-25-1; 6, 105-54-4; 7, 6728-26-3; 8, 544-12-7; 9, 111-27-3; 10, 124-13-0; 11, 99-87-6; 12, 138-86-3; 13, 11063-78-8; 14, 111-65-9; 15, 11063-77-7; 16, 124-19-6; 17, 78-70-6; 18, 2305-25-1; 19, 562-74-3; 20, 98-55-5; 21, 112-31-2; 22, 1197-07-5; 23, 106-26-3; 24, 106-24-1; 25, 141-27-5; 26, 2111-75-3; 27, 54889-48-4; 28, 89-83-8; methanol, 67-56-1.

LITERATURE CITED

Attaway, J. A.; Wolford, R. W.; Dougherty, M. H.; Edwards, G. J. *J. Agric. Food Chem.* **1967**, *15*, 688.
Attaway, J. A.; Wolford, R. W.; Edwards, G. J. *J. Agric. Food Chem.* **1962**, *10*, 102.
Boggs, M. M.; Hanson, H. L. *Adv. Food Res.* **1949**, 222.
Braddock, R. J.; Petrus, D. R. *Proc. Fla. State Hortic. Soc.* **1971**, *84*, 223.
Dravnieks, A.; O'Donnell, A. *J. Agric. Food Chem.* **1971**, *19*, 1049.
Guadagni, D. G.; Bomben, J. L.; Mannheim, H. C. *J. Food Sci.* **1970**, *38*, 279.
Hickey, M., Intercit, Inc., Safety Harbor, FL, personal communication, 1983.
Ismail, M. A.; Wolford, R. W. *J. Food Sci.* **1970**, *35*, 300.
Johnson, J. D.; Vora, J. D. *Food Technol. (Chicago)* **1983**, *37*, 92.
Lund, E. D.; Bryan, W. L. *J. Food Sci.* **1977**, *42*, 385.
Moshonas, M. G.; Lund, E. D. *J. Food Sci.* **1971**, *36*, 105.
Moshonas, M. G.; Shaw, P. E. *J. Food Sci.* **1973**, *38*, 360.
Peleg, Y.; Mannheim, C. H. *J. Agric. Food Chem.* **1970**, *18*, 176.
Schultz, T. H.; Flath, R. A.; Mon, T. R. *J. Agric. Food Chem.* **1971**, *19*, 1060.
Schultz, T. H.; Teranishi, R.; McFadden, W. H.; Kilpatrick, P. W.; Corse, J. *J. Food Sci.* **1964**, *29*, 790.
Shaw, P. E. "Citrus Science and Technology"; Nagy, S.; Shaw, P. E.; Veldhuis, M. K., Eds.; Avi Publishing Co.: Westport, CT, 1977; Vol. 1, Chapter 12.
Wolford, R. W.; Alberding, G. E.; Attaway, J. A. *J. Agric. Food Chem.* **1962**, *10*, 297.
Wolford, R. W.; Dougherty, M. H.; Petrus, D. R. *Ber.—Int. Fruchtsaft-Union, Wiss.-Tech. Komm.* **1968**, *9*, 151.

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Improved High-Performance Liquid Chromatographic Analysis of Phenolic Acids and Isoflavonoids from Soybean Protein Products

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An improved analytical high-performance liquid chromatographic (HPLC) method was developed for recovering, fractionating, and quantitating individual phenolic acids and isoflavonoids from soybean products. This improved method includes ethanol extraction of the phenolics, mild hydrolysis of phenolic acid esters, separation of phenolic acids from isoflavonoids by C_{18} Sep-PAK, and C_{18} reverse-phase HPLC analysis of the two phenolics fractions using a water–acetic acid and methanol–acetic acid gradient. This developed method provides substantially greater recovery of phenolic compounds, requires much less time, separates phenolic acids from isoflavonoids, and improves the resolution of individual phenolic compounds. Defatted soy flakes contained a total of 4 mg of total phenolics/g of sample, which was distributed as about 28% phenolic acids and 72% isoflavonoids. Genistin was the major isoflavonoid, accounting for about 75% of the total isoflavonoids. Major phenolic acids in defatted soy flakes were syringic, ferulic, and sinapic acids. Control and commercial soy protein isolates contained substantially lower concentrations of phenolics. Ion-exchange and activated carbon treatments both removed ≥90% of the total phenolics from defatted soy flakes and were equally effective for removing both phenolic acids and isoflavonoids.

Soybeans contain a number of important phenolic compounds including free phenolic acids, phenolic acid esters, isoflavones, and their glucosides. Maga (1978) and Sosulski (1979) reviewed the literature dealing with the composition and chemistry of these compounds in foods and oilseed protein products. They also discussed the key relationship that these compounds exhibit to flavor and color defects in oilseed proteins and related food products.

The isoflavonoid compounds have been isolated and quantitated by gas–liquid chromatography (GLC) and high-performance liquid chromatography (HPLC). For example, Naim et al. (1974) reported on the use of GLC and Murphy (1981) and Eldridge (1982) reported on the use of HPLC for quantitating phenolic compounds in soybeans. Maga and Lorenz (1974) also used GLC to study the phenolic acids in soybean flakes and reported a total of 256 μg of phenolic acids/g of defatted soy flakes.

How and Morr (1982) used an HPLC method that was slightly altered from that developed by Wulf and Nagel (1976) to study the effectiveness of several processing treatments to remove phenolic compounds from soy protein isolates. HPLC patterns of How and Morr (1982) revealed about 40 peaks, 25 of which were distinct and well resolved, for unfractionated phenolic compounds, e.g., phenolic acids and their esters and isoflavonoids.

The major objectives of the present study were (a) to fractionate the phenolic acids from neutral phenolic compounds prior to HPLC analysis and (b) to improve the resolution and quantitate the important phenolic compounds in defatted soy flakes and soy protein isolates by HPLC. These efforts included (a) mild alkaline hydrolysis of extracted phenolic compounds to free phenolic acids from their esters, (b) development of an improved recovery method based upon alcohol extraction and subsequent

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