Journal of Chromatography, 635 (1993) 143-150
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 901

Short Communication

Analysis of phenolic and flavonoid compounds in juice beverages using high-performance liquid chromatography with coulometric array detection

P. Gamache, E. Ryan and I.N. Acworth*

ESA Inc., 45 Wiggins Avenue, Bedford, MA 01730 (USA)

(First received December 7th, 1992; revised manuscript received January 25th, 1993)

ABSTRACT

Analysis of phenolics and flavonoids in juice beverages using reversed-phase HPLC with coulometric array detection is described. Sixteen serial coulometric detectors were used for on-line resolution of co-eluting compounds and generation of voltammetric data. Within each class of compounds, oxidation potential corresponded to specific substitution patterns where: catechol < methoxycatechol < monohydroxyl C methoxyl. Twenty-seven standard compounds were resolved in a 45-min run. The limits of detection were in the low ng/ml range with a linear response range of at least three orders of magnitude. Intra-run retention time variation was < 1% (R.S.D.) and adjacent sensor response ratios varied by <5% (R.S.D.). The utility of this technique in generating multivariate data for differentiation of juices and juice mixtures is shown.

INTRODUCTION

Phenolics and flavonoids are common substituents of plant-derived beverages. There is great diversity in the natural occurrence and distribution of these compounds reflecting differences in metabolism and stability. Interest in these metabolites is primarily based on their contribution to such properties as: color (e.g. anthocyanins), flavor (e.g. flavanone glycosides), fragrance (e.g. thymol), nutritional value (e.g. antiscorbutic effects), stability (e.g. flavanols), therapeutic value (e.g. nobiletin), and toxicity (e.g. rutin). Changes in these compounds during processing and storage is also important to the quality and safety of commercial food products.

Reversed-phase HPLC with ultraviolet and visible absorbance detection is a common technique for the study of these analytes. Analysis of specific phenolic compounds and profiles of particular classes have been used to examine both source and process-related variability, for example in the detection of fruit juice adulteration [1,2], and in the study of process-related effects on quality [3]. Quantitation and characterization, however, can often be difficult due to sample complexity or if studying these substances at trace levels. HPLC with amperometric electrochemical detection (ED) provides selectivity and sensitivity well suited to the analysis of many of these components in complex matrices [4]. Differences in electroactive substituents on analogous structures can lead to characteristic differences in their voltammetric behavior.

* Corresponding author.

0021-9673/93/$06.00

These properties are thus a basis for selectivity and provide a useful source of qualitative information [5,6].

In our studies we have used a coulometric type flow-through carbon graphite working electrode [7]. This form of highly efficient ED provides mass conversion (oxidation or reduction) of each analyte at its maximum potential. The use of several coulometric sensors in series (coulometric array), maintained at different potentials, provides resolution of co-eluting compounds whose oxidation or reduction potentials differ by as little as 60 mV [8]. This largely extends the resolution capabilities of conventional HPLC–amperometric ED. Also, since the response profile across several electrodes is representative of an analyte's voltammetric properties, useful qualitative information is obtained for several analytes from a single injection. This HPLC coulometric array technique has previously been used for identification and quantitation of phenolic compounds in lemon juice, plant extracts and alcoholic beverages [9] and for the study of process-related changes in reducing substances present in beer [10]. The intent of this paper is to show the applicability of this technique to the study of electroactive substances in fruit juice beverages emphasizing the selective and qualitative characteristics of the array. Analysis of these multi-component HPLC-ED profiles for differentiation of various juice beverages is also addressed.

EXPERIMENTAL

Sample treatment

The samples which were analyzed (e.g. apple, orange and grapefruit juice) included: concentrates, fresh squeezed, juice from concentrate, and retail products. Juice from specific varietals and authentic juice samples were generously supplied by the Citrus Research and Education Center, Florida Department of Citrus. Beverage samples were stored at -20°C until preparation on the day of analysis. Concentrates were diluted with deionized water to 11.8" Brix (percentage by mass of apparent solids in a sugar solution determined by the Brix spindle hydrometer). An aliquot *(ca. 0.5* ml) was centrifuged (10 000 g, 4°C, 5 min) and the supernatant was passed through a 0.22-μm filter (Millipore, Bedford, MA, USA) by centrifugation (conditions as above). A 20-μl volume of filtrate was used for analysis by HPLC with coulometric array detection.

Standards

A standard mixture representative of several classes of endogenous electroactive compounds was made using commercially available chemicals. All powders were obtained from Sigma (St. Louis, MO, USA) with the exception of narirutin and tangeritin (Atomergic Chemetals, Farmingdale, NY, USA). Compounds were first dissolved individually at 0.1-1.0 mg/ml in 50% (v/v) aqueous ethanol and stored at -20°C. Aliquots were combined and diluted in 1% (v/v) aqueous methanol to comprise the working standard.

HPLC

Samples were analyzed with a Coulochem Electrode Array System (CEAS, Model 5500; ESA, Bedford, MA, USA) consisting of a Model 460 autoinjector, two Model 420 dual-piston pumps, an M800 high-pressure gradient mixer, and four coulometric array cell modules (each with four working electrodes). A stainless steel 150 mm × 4.6 mm I.D. column, packed with 5-μm M.S. Gel C_{18} (Niko Bioscience, Tokyo, Japan) was used. The column and detector array were housed in a temperature-regulated compartment maintained at 35 ± 0.1°C. System control, data acquisition, and analysis were performed with the CEAS software on an Epson 386 computer. LOTUS 123 (Lotus, Cambridge, MA, USA) and Einsight (Infometrics, Seattle, WA, USA) software were used for data base management and statistical analysis, respectively.

For binary gradient elution, mobile phase A consisted of 0.1 *M* monobasic sodium phosphate containing 10 mg/l sodium dodecyl sulfate (SDS) and adjusted to pH 3.35 with phosphoric acid. Mobile phase B consisted of acetonitrile-0.1 *M* monobasic sodium phosphate containing 50 mg/l SDS-methanol (60:30:10, v/v/v). The pH of this mixture was adjusted to 3.45 with phosphoric

acid. The total flow-rate was maintained at 1.0 ml/min throughout the run. The gradient cycle consisted of an initial 10-min isocratic segment (6% B), a 20-min linear gradient (+1.2% B/min), a 10-min linear gradient (+7% B/min), and a 5-min isocratic segment (100% B) before returning to initial conditions. Samples were injected precisely at 9.0 min after return to initial conditions. The 16 detector array was set from 0 to 900 mV in increments of 60 mV *versus* palladium reference electrodes.

RESULTS AND DISCUSSION

The chromatographic conditions were adapted from HPLC absorbance methods described by Kirksey et al. [11] and Rouseff [2] for analysis of a variety of flavonoid compounds (e.g. flavanone glycosides, anthocyanins). SDS was included as an ion-pairing reagent for retention of amines. The detectors were configured in an oxidative array to allow voltammetric resolution of compounds based on ease of oxidation across incrementally increasing anodic potentials. Fig. 1 shows a 15-channel chromatogram (channel 16 not shown for clarity) of a n-component external standard with representative compounds from structural classes including amino acids, coumarins, hydroxybenzoic and cinnamic acids, simple phenolics, purines, in addition to several classes of flavonoids. Analytes were resolved in two dimensions -chromatographic and voltammetric. Each analyte had a specific retention time and a response "signature" across the array. With 60-mV increments between sensors, the majority of response for a single oxidation wave typically occurred across three adjacent sensors. The highest responding sensor was defined as the dominant channel for each analyte while the leading and following channels refer to the adjacent upstream and downstream sensors. Peak comparison by retention time and voltammetric response, measured as ratios between three sensors, was used to increase the confidence in matching sample peaks and external standard peaks.

Table I shows the retention time and dominant oxidation potential for each of these standard compounds. Resolution with the detector array was based on ease of oxidation and may be related to differences in structure where availability of electrons and the capacity for charge stabilization differ (e.g. charge delocalization, electron-donating properties). For particular aromatic substituents, a consistent voltammetric relationship was evident both between analyte classes and among analogues. Compounds having catechol groups (caffeic acid, catechin, chlorogenic acid) all responded at lower channels (60

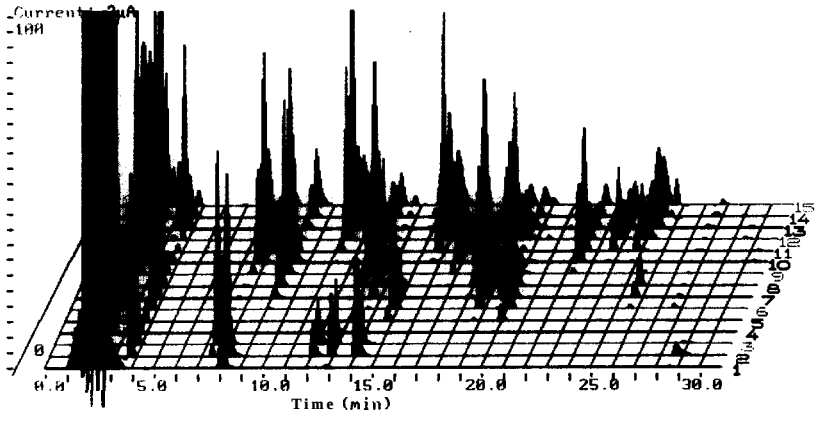


Fig. 1. Fifteen-channel chromatogram of a 27-component standard, 20 μl of a 1 μg/ml solution. Compounds were resolved on the basis of retention time in combination with voltammetric response behavior across the array. Sensor potentials were 0 mV (channel 1) to 840 mV (channel 15) with 60-mV increments (Pd reference). All channels are displayed at 2 μA full scale. See Experimental for further details on analytical conditions and Table I for compound listing, retention times, and dominant oxidation potential.

TABLE I

SUMMARY OF STANDARD MIXTURE

Peak No.	Compound	Retention time (min)	Dominant oxidation potential (mV)	Detection limit (ng/ml)	r [b]
i	Ascorbate	1.86	240	200	0.9983
2	Guanine	2.06	540	2	0.9992
3	Tyrosine	2.50	540	1	0.9999
4	Guanosine	3.31	780	2	0.9992
5	Gallic acid	3.65	60	1	0.9992
6	Protocatechuic acid	7.45	120	1	0.9999
7	4-Aminobenzoic acid	7.70	600	2	0.9987
8	Gentisic acid	8.13	60	1	0.9999
9	Tryptophan	9.14	540	2	0.9998
10	4-Hydroxybenzoic acid	11.32	720	1	0.9999
11	Chlorogenic acid	12.12	120	1	0.9939
12	Catechin	12.96	120	1	0.9997
13	4-Hydroxyphenylacetate	130.8	540	1	0.9998
14	Vanillic acid	13.70	420	1	0.9999
15	Caffeic acid	14.12	60	1	0.9967
16	Syringic acid	14.88	300	1	0.9999
17	4-Hydroxycoumarin	15.79	660	3	0.9989
18	Coumaric acid	18.15	540	2	0.9939
19	Syringaldehyde	18.84	360	1	0.9998
20	Umbelliferone	19.09	660	5	0.9995
21	Eugenol	19.70	420	1	0.9992
22	Ferulic acid	20.14	300	1	0.9997
23	Scopoletin	22.53	600	2	0.9998
24	Narirutin	23.98	660	2	0.9996
25	Naringin	24.98	660	3	0.9994
26	7-Methoxycoumarin	25.27	780	25	0.9477
27	Hesperidin	25.83	360	3	0.9993

[a] Signal-to-noise ratio >5, 20 μl injection.
[b] Correlation coefficient obtained from duplicate injections of 1, 2, 10, 20, 50, 100, 250, 500 and 1000 ng/ml standard mixtures.

mV dominant). Differences of greater than 100 mV in dominant oxidation potential were evident among simple phenolics, hydroxycinnamic acids, as well as flavonoids. Within each group, the oxidation potentials followed the order: catechol < methoxycatechol < monohydroxyl < methoxyl. The 60-mV array therefore provided good resolution of analytes on the basis of these substituents. Fig. 2 shows a smaller segment of the standard chromatogram where combined chromatographic and voltammetric resolution are evident for coumaric acid (monohydroxyl), eugenol (methoxycatechol), ferulic acid (methoxycatechol), syringaldehyde (4-hydroxyl, 3,5-dimethoxyl) and umbelliferone (monohydroxyl). Resolution of eugenol and ferulic acid is attributable to resonance delocalization promoting a lower oxidation potential for ferulic acid.

While the flavonoids represent a very diverse class of endogenous plant metabolites, a major aspect of this diversity relates to the pattern of aromatic substituents (e.g. alcohol, alkoxyl, carbohydrate) [12]. The flavonoid backbone contains two benzene rings linked by a 3-carbon chain. With several possible oxidizable moieties present (e.g. aromatic alcohol and alkoxyl) the voltammetric behavior can be characteristically complex. The voltammetric properties of catechin, hesperidin, and naringin are shown in the hydrodynamic voltammograms (HDV) in Fig. 3.

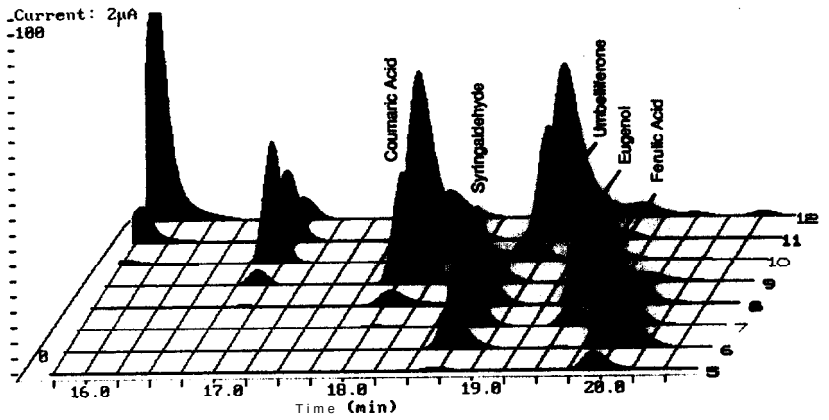
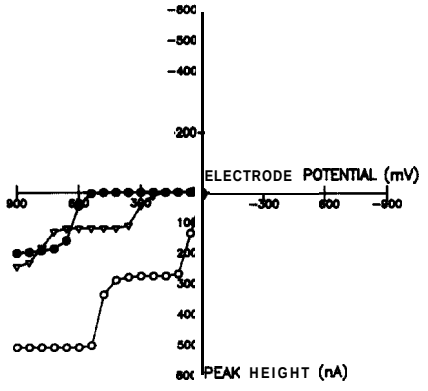
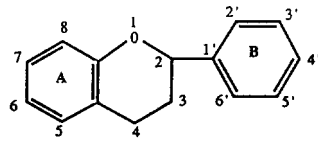


Fig. 2. A smaller time-potential segment of Fig. 1 is shown above. An increasing array of coulometric detectors is used to resolve standard components (20 μl of a 1 μg/ml solution). Detector potentials are 240, 300, 360, 420, 480, 540, 600 and 660 mV (Pd reference). Full scale amplification is 2 μA. gee Experimental for further details on analytical conditions.





	9	4	5	6	7	3'	4'	5'
Catechin	OH	H	OH	H	OH	OH	OH	H
Naringin	H	=O	OH	H	Rh,Gl	H	OH	H
Hesperidin	H	=O	OH	H	Rh,Gl	OH	OCH3	H

Rh = Rhamnose, Gl = Glucose

Fig. 3. Summation of peak heights (current) generated across a coulometric array [detector potentials: O-900 mV, 60-mV increments (Pd reference)] is plotted as a function of electrode potential. Data were obtained under binary gradient conditions as described in Experimental. The characteristic voltammetric behavior of these substituted flavonoids may be used qualitatively (structural identification and peak purity). 0 = Catechin; ● = naringin; V = hesperidin.

The data for these HDVs were generated instantaneously by the array and are represented by plotting the cumulative current across the 16 sensors. The catechol group on the B ring of catechin oxidized at the low potential sensors *(0* to 120 mV) with voltammetric response behavior analogous to the simple catechol, caffeic acid. The lower oxidation wave of hesperidin may be attributed to the methoxycatechol group on the B ring similar to the simple methoxycatechol compound, eugenol. The voltammetric response of naringin (single B ring hydroxyl) likewise, resembled p-coumaric acid. The second oxidation waves of catechin and hesperidin are likely related to the A ring substituents. Qualitative examination of these "signatures" provided a means of examining peak purity for known compounds as well as an indication of the possible substitution pattern for unknowns.

Juice analysis

A 16-channel chromatogram of an orange juice filtrate is shown in Fig. 4. More than 300 electroactive components were typically resolved in this sample type. Automatic gain ranging with the CEAS allowed measurement of components over a wide range of concentrations (low ng/ml to high μg/ml). The limits of detection using a 20-μl injection volume were typically in the low

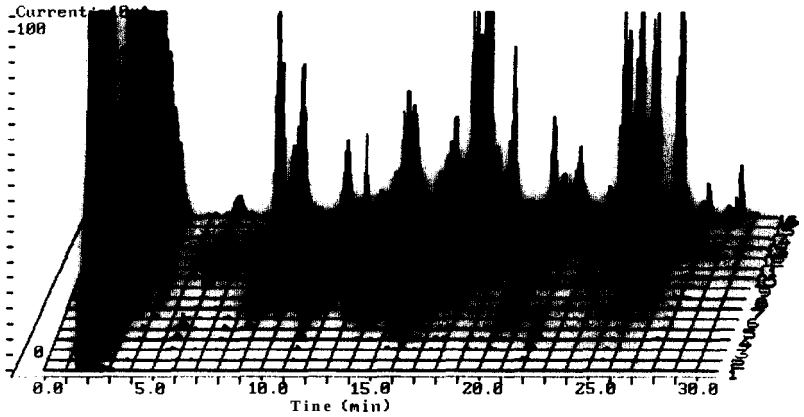


Fig. 4. Sixteen-channel chromatogram of a filtered orange juice from concentrate. Detector potentials were: O-900 mV, 60-mV increments (Pd reference). Full scale amplification is 10 μA. Single-strength orange juice was passed through a 0.22-μm filter and 20 μl were injected. See Experimental for further details on analytical conditions.

ng/ml range with a linear response range of at least three orders of magnitude (see Table I). Levels of some of the electroactive components found in orange juice are presented in Table II.

In addition to quantitation using available external standards, we have examined the relative concentrations (calculated by peak height) of unidentified components among samples. Relative levels were determined by using a sample pool (e.g. authentic orange juice) as an external standard to generate normalized data for a large number of analytes in all samples. An estimation of the intra-assay variability in retention time, response ratios, and resultant quantitative value was made by examination of twenty-five unidentified analytes in ten replicate injections of an orange juice sample. Analytes were chosen to be representative of a wide range of meth-

TABLE II

LEVELS (IN μg/ml) OF SOME ELECTROACTIVE COMPONENTS IN ORANGE JUICE

External standard quantitation.

Compound	Varieties[a]				Mean	S.D.[b]
	Blend	Navel	Hamlin	Valencia		
Ascorbate	276	274	271	270	252	(67.6)
Cysteine	10.0	8.84	13.6	12.3	12.7	(2.53)
Hesperidin	165	219	128	145	76.4	(37.4)
Methionine	11.6	34.3	21.9	17.6	25.2	(29.5)
Narirutin	13.4	54.2	26.3	29.2	32.0	(11.7)
Tryptophan	6.30	3.02	4.26	4.00	5.35	(1.86)
Tyrosine	23.5	9.28	20.3	21.3	29.0	(8.74)
Naringin	0.04	0.17	0.39	0.31	0.79	(2.05)

[a] Navel, Hamlin and Valencia are cultivars of orange (*Citrus sinensis*). "Blend" refers to an adulterated orange juice sample of unspecified composition.

[b] Mean (standard deviation) of 41 commercial samples.

odological parameters: chromatographic (retention time: 3.19-25.4 min), electrochemical (dominant oxidation potential: 60-900 mV), and response (peak height: 0.01-20 μA). The relative standard deviation (R.S.D.) in retention time for each of these 25 analytes ranged from 0.26 to 0.69% (mean = 0.40%) indicating precise gradient control. Variability in peak height at the dominant sensor for each analyte ranged from 0.49 to 4.74% (R.S.D., mean = 1.74%). For 23 analytes, the R.S.D. in response ratio between the dominant and leading sensors ranged from 0.36 to 5.35% (mean = 1.58%). For one analyte, no response ratio was obtained due to insufficient signal at the leading sensor. Another analyte co-eluted with a lower oxidizing compound and high variability (33.5% R.S.D. for the ratio) was evident at the leading sensor. In both cases the variability in response ratios between the dominant and following sensors were low (0.59 and 2.38% R.S.D.). While the response at the following sensor was insufficient to allow ratio determinations in 3 of the 25 analytes good precision was obtained in these cases for the dominant/leading ratios. Variability in response ratios (dominant/following) for the remaining 22 analytes ranged from 0.40 to 7.51% (R.S.D., mean = 2.31%). Based on these performance data, matching criteria of ±2% for retention time and ±10% for response ratios were used. More than 150 components in a juice sample were typically within these criteria. External standard quantitation was performed using either the sum of the peak heights across three sensors when the response ratios were within these criteria, otherwise at the dominant sensor only. The variability in concentration, normalized to an authentic juice sample, for these 25 analytes ranged from 0.53 to 3.04% (R.S.D., mean = 1.11%).

In the investigation for orange juice adulteration we have examined these multivariate data for: endogenous markers of authenticity, peaks introduced by common adulterants (e.g. grapefruit, peel, pulpwash), and general dilution of multiple components. We have used this information along with pattern recognition analysis for differentiation of various juice beverages. An example of this approach is shown in Fig. 5. Here the results from principal components

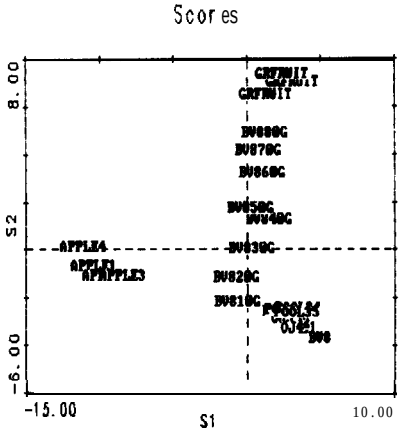


Fig. 5. Plot of vectors 1 (S1) and 2 (S2) from principal components analysis of a 50-variable data base. Grapefruit, orange, and apple juice samples along with various mixtures are differentiated through pattern recognition analysis. Sample key: APPLE l-4 = different retail brands of apple juice; GRFRUIT= different retail brands of grapefruit juice; BV8 = pure Valencia *(Citrus sinensis cv Valencia)* orange juice from Florida; BV810G–80G = 10–80% grapefruit juice content in BV8, respectively. The cluster in the lower right quadrant is solely comprised of 17 orange juice samples as follows: BV8, 4 different retail brands of orange juice, 11 orange juice samples from Mexico and a pool of these 16 orange juice samples. Mexican samples included the following cultivars: Criolla, Hamlin, and Valencia as well as early-mid varieties (made from a mixture of early maturing and mid-season oranges). These varieties were obtained from Puebla, Tabasco, Tamaulipas, Veracruz and Yucatan regions.

analysis (PCA) [2,13] of data from 4 retail brands of apple juice, 17 samples of orange juice, 3 retail brands of grapefruit juice, as well as mixtures of grapefruit and orange juice are graphically represented. These results obtained from a 50 variable database demonstrate the utility of this technique for the differentiation of juice beverages. Clear separation between each fruit juice [orange (lower right quadrant), grapefruit (top right cluster), and apple (left cluster)] is evident. The mixtures (BV810G–BV880G) represent 10–80% grapefruit in orange respectively, and show a consistent progression of points between the respective groups of pure orange and grapefruit. Since several brands, sources, and varieties were included in this group of samples, differentiation in this instance appears to be quite specific to the fruit species.

Future reports will include studies of varietal and source differentiation as well as the detection of adulteration and percent juice analyses.

CONCLUSIONS

Coulometric array detection allows increased resolution for HPLC analysis of phenolic and flavonoid compounds based on differences in their voltammetric properties. Detailed voltammetric data across a wide potential range may be generated on-line from a single injection and with good precision. The basis for differences in ease of oxidation within and among compound classes corresponds to the patterns of aromatic substituents. Comparison of the electrochemical "signature" across the array may be used qualitatively to examine resolution and for some degree of structural characterization. Multivariate data from complex juice matrices can be obtained without extraction and with good precision. Differences in the patterns of endogenous electroactive components can be used to differentiate apple, orange, grapefruit, as well as orange-grapefruit mixtures. This technique may be a useful tool in such areas as adulteration detection, varietal and source classification, the study of process-related variability, and the analysis of percent juice in mixtures.

REFERENCES

1 G.A. Perfetti, F.L. Joe, T. Fazio and S.W. Page, *J. Assoc. Off. Anal. Chem.*, 71 (1988) 469.
2 R.L. Rouseff, in S. Nagy, J.A. Attaway and M.E. Rhodes (Editors), *Adulteration of Fruit Juice Beverages*, Marcel Dekker, New York, 1988, p. 49.
3 G.A. Spanos and R.E. Wrolstad, *J. Agric. Food Chem., 38* (1990) *817.*
4 P.T. Kissinger, *Anal. Chem., 49* (1977) 447a.
5 S.M. Lunte, *J. Chromatogr., 384* (1987) 371.
6 0. Friedrich and G. Sontag, *Fresenius' Z. Anal. Chem., 334* (1989) 59.
7 R.W. Andrews, C. Shubert and J. Morrison, *Am. Lab., Oct.* (1982) 140.
8 W.R. Matson, P.J. Langlais, L. Volicer, P.H. Gamache, E. Bird and K.A. Mark, *Clin. Chem.*, 30 (1984) 1477.
9 G. Achilli, G.P. Cellerino, P.H. Gamache and G.M. d'Eril, *J. Chromatogr.*, in press.
10 M. Uchida, Y. Kataoka and M. Ono, presented at the *2nd Brewing Congress of the Americas Meeting, St. Louts, MO, 1992.*
11 S.T. Kirksey, J.O. Schwartz and R.L. Wade, presented at the *2nd Annual Fruit Juice Authenticity Workshop, Herndon, VA, 1989.*
12 T. Robinson, *The Organic Constituents of Higher Plants: Their Chemistry and Interrelationships*, Cordus Press, N. Amherst, MA, 5th ed., 1983, pp. 54–200.
13 J. Arunachahun and S. Gangadharan, *Anal. Chim. Acta*, *157 (1984) 245.*