Improvements in the Quantitation of Limonin in Citrus Juice by Reversed-Phase High-Performance Liquid Chromatography†

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A cyano stationary phase was found to have good selectivity for reversed-phase HPLC analysis of limonin in citrus juices. The cyano stationary phase is, however, susceptible to contamination by constituents contained in citrus juices. A method is described for solid-phase extraction (SPE) of limonin from citrus juices followed by reversed-phase analysis. The method has a rapid sample preparation, does not cause premature deterioration of the analytical column, and offers an improved separation of limonin from other constituents over previously described reversed-phase methods. The SPE procedure provided 95–108% recovery and 2–4-fold concentration of limonin in extracts for increased sensitivity with UV detection at 214 nm. Limonin is extracted into 70% methanol, and extracts were stable for 30 days if kept at −4 °C. Juices analyzed for limonin included white and pink grapefruit varieties, navel, Valencia, Hamlin, and sour oranges.

Limonin, a triterpenoid dilactone, can cause a bitterness problem in juice processed from some citrus varieties when harvested early. A rapid and reliable method for the quantitation of limonin in citrus juices is important to processors interested in monitoring juice bitterness. Many methods for the analysis of limonin in citrus juices have been developed. The normal-phase HPLC method for analysis of limonin in citrus juices by Rouseff and Fisher (1980) is accurate but requires lengthy chloroform extraction of the juice. A rapid method for analysis of limonin by enzyme-linked immunoassay has also been developed (Jourdan et al., 1984) and made into a commercially available kit (Idetek, San Bruno, CA). The method has advantages in that average analysis time is rapid and equipment costs are less than those for HPLC equipment. However, a collaborative study done by Widmer and Rouseff (1991) using the commercial kits indicated there was a problem with reproducibility.

More recently, several reversed-phase HPLC analytical methods for limonin analysis were developed (Shaw and Wilson, 1984, 1988; Shaw, 1986; Van Beek and Blaakmeer, 1989). Either a C-8 or a C-18 column was used for analysis with rapid solid-phase extraction (SPE) to separate limonin from interfering components. Many solvent combinations and strengths for the analytical mobile phase were investigated with good results obtained for navel orange juices. However, grapefruit juice extracts required relatively long analysis times or were susceptible to interferences at low limonin concentrations. Shaw (1986) also reported the stability of limonin in acetonitrile such that samples must be analyzed the same day they are prepared.

Van Beek and Blaakmeer (1989) investigated using C-2, C-8, C-18, CN, cyclohexyl, and phenyl SPE columns to extract limonin from citrus juices, seeking an improvement in selectivity between limonin and interfering components in grapefruit. Extracts from the different SPE column types reportedly showed few selectivity differences among column types when analyzed. The only difference reported was in elution strength of wash solvent and limonin eluent required; acetonitrile–water combinations reportedly gave better results for the SPE than methanol–water combinations.

The purpose of this study was to develop improvements in the rapid analysis of citrus juices for limonin using SPE and reversed-phase HPLC. Because of the extensive work already done with C-18 reversed-phase columns, a CN column under reversed-phase conditions was chosen for analysis. Rouseff and Fisher (1980) reported on the rapid and irreversible loss in performance obtained when a CN column was used to analyze chloroform extracts (evaporated and redissolved in mobile phase) of citrus juices for limonin. A method using SPE was developed to concentrate limonin in extracts for improved sensitivity and eliminate components that caused premature column deterioration or interferences in analysis. The previously developed SPE procedures did not concentrate limonin in the extracts over amounts found in juice.

EXPERIMENTAL PROCEDURES

Juice Samples. Processed grapefruit juice samples were received from several Florida processors through the single-strength juice survey program conducted by the Florida Department of Citrus. Untreated and debittered navel orange juices were obtained from Sunkist Growers, Inc. (Ontario, CA). Valencia orange juice was received from Citrus World, Inc. (Lake Wales, FL). Juice was also hand squeezed from Hamlin sweet oranges and sour oranges (*Citrus aurantium* L.) obtained from the Citrus Budwood Registry, Winter Haven, FL.

Reagents and Standards. Water was purified by the Milli-Q (Millipore, Bedford, MA) system. All other solvents used for extraction and analyses were of HPLC grade (Fisher Scientific, Fairlawn, NJ). Solvents for HPLC analyses were filtered through a 0.45-μm filter and sparged with helium prior to use. Limonin was obtained from defatted grapefruit seeds by extraction with methylene chloride. The crude limonin, obtained on removal of the methylene chloride, was purified to mp 295–298 °C by washing the crystals with hot (75 °C) 2-propanol, redissolving the dried crystals in a minimum of methylene chloride, and recrystallizing by adding an equal volume of 2-propanol.

Stock 500 and 100 ppm limonin standards were prepared by weighing 0.0500 and 0.0100 g of limonin, respectively, into 100-mL volumetric flasks. Limonin was dissolved in 2 mL of acetonitrile, made up to volume with methanol, and stored at 4 °C. Stock standard solutions were stable for 3 months. Working standards of 1, 5, 10, 20, and 50 ppm limonin were prepared by dilution of stock with mobile phase or 0.1% aqueous acetic acid.

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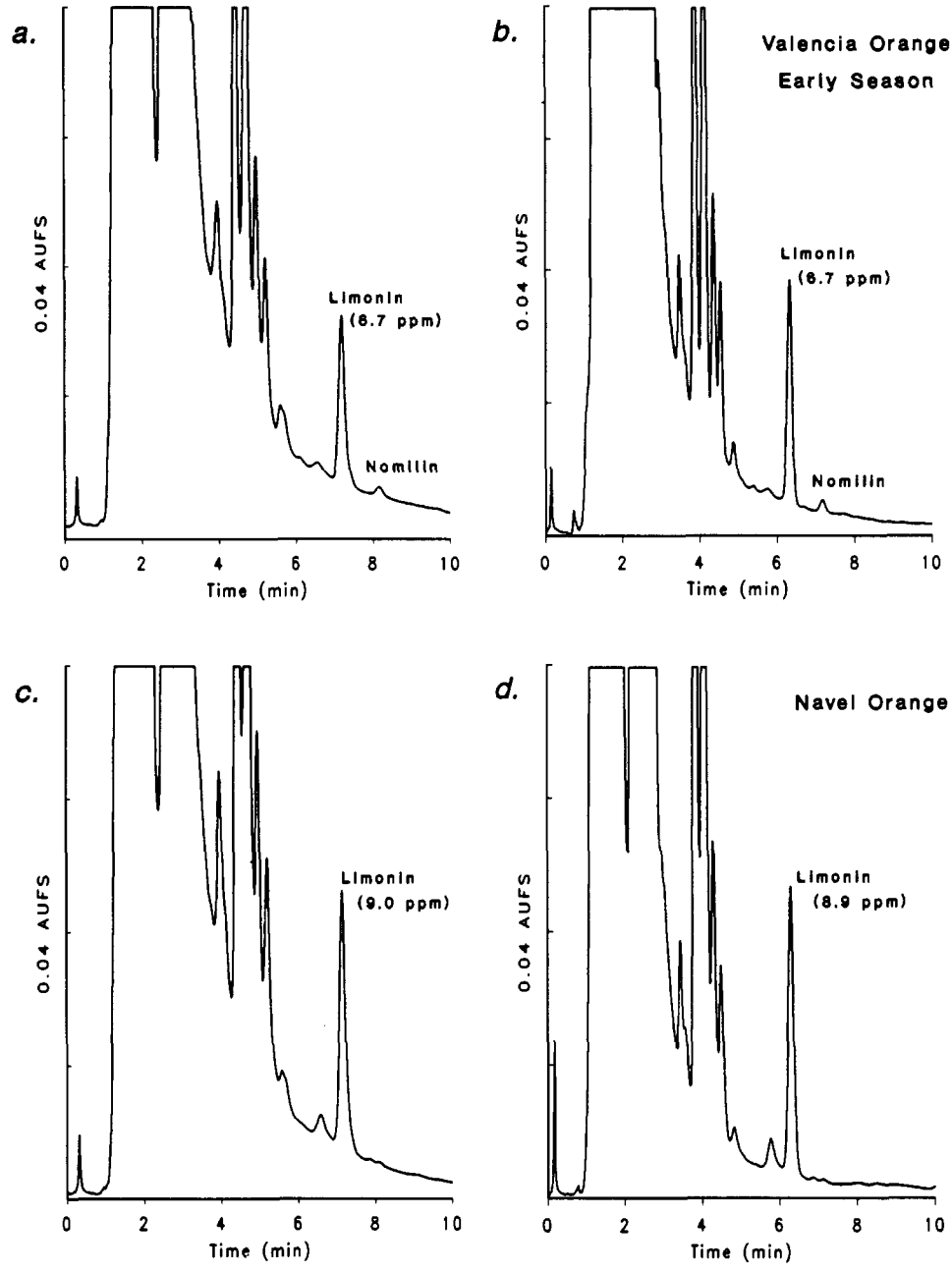

Figure 1. HPLC traces of SPE extracts: Valencia orange (a) with precolumn, (b) without precolumn; navel orange (c) with precolumn, (d) without precolumn. Other conditions are given in the text.

A stock model juice solution was prepared by weighing 15.0 g of fructose, 15.0 g of glucose, 30.0 g of sucrose, 7.5 g of citric acid, and 0.75 g of citrus pectin into 750 mL of distilled water. The contents were stirred with heating until dissolved and allowed to cool. Spiked model and grapefruit juices (5.66 ppm of natural limonin) were then prepared in an identical manner to contain 0.5, 5, 10, and 20 ppm added limonin.

Sample Preparation. All samples were centrifuged at 12100*g* (10 000 rpm) for 10 min. Measured with a volumetric pipet, 10 mL of the supernatant juice was used for analyses. For normal-phase analysis, juice was extracted three times with chloroform as described by Rouseff and Fisher (1980). For reverse-phase analyses, samples were extracted by using C-18 Sep-Pak SPE cartridges (Waters Associates, Milford, MA) attached to 10-mL syringes. Sep-Paks were conditioned with 3 mL of methanol followed by 5 mL of water. Ten milliliters of juice was then slowly eluted through the Sep-Pak. During the above steps care was taken not to allow the liquid level to fall below the packing. Elution of the juice was followed with a 6-mL 30% (v/v) aqueous methanol wash. The wash was flushed out of the Sep-Pak with air by using a syringe plunger. (Do not dry column packing, just remove liquid from between particles.) Measured with a volumetric pipet, 2.5 mL of 70% (v/v) aqueous methanol was used to slowly elute the limonin. All of the 70% methanol eluent was collected into a 4-mL sample bottle or a 5-mL volumetric flask by flushing the Sep-Pak with air. When 5-mL volumetric flasks were used, samples were made to volume with 70% methanol. The eluent, with limonin, was then filtered through a 0.2-μm Anotop filter (Alltech Associates).

HPLC Conditions. A Perkin-Elmer (Norwalk, CT) Series 4 pumping system, Waters (Bedford, MA) 712 Wisp automatic sample injector, Kratos (Ramsey, NJ) URA 200 column heater, either a Kratos 570 variable-wavelength detector set at 210 nm or a Waters 440 UV detector with extended wavelength module for detection at 214 nm, and a Spectra Physics (San Jose, CA) 4270 integrator connected to a Winner Model 319 data station were used. For some analyses, two UV detectors were connected in series with multiple detection at 214 and 254 nm. Detector response at 254 nm would be an indication of interference with limonin detection at 214 nm. A sample injection volume of 20 μL was used for both reversed- and normal-phase analyses. The integrator attenuation was set to 0.06 AUFS. All extracts were analyzed with duplicate injections.

For reversed-phase analysis a binary mobile phase consisting

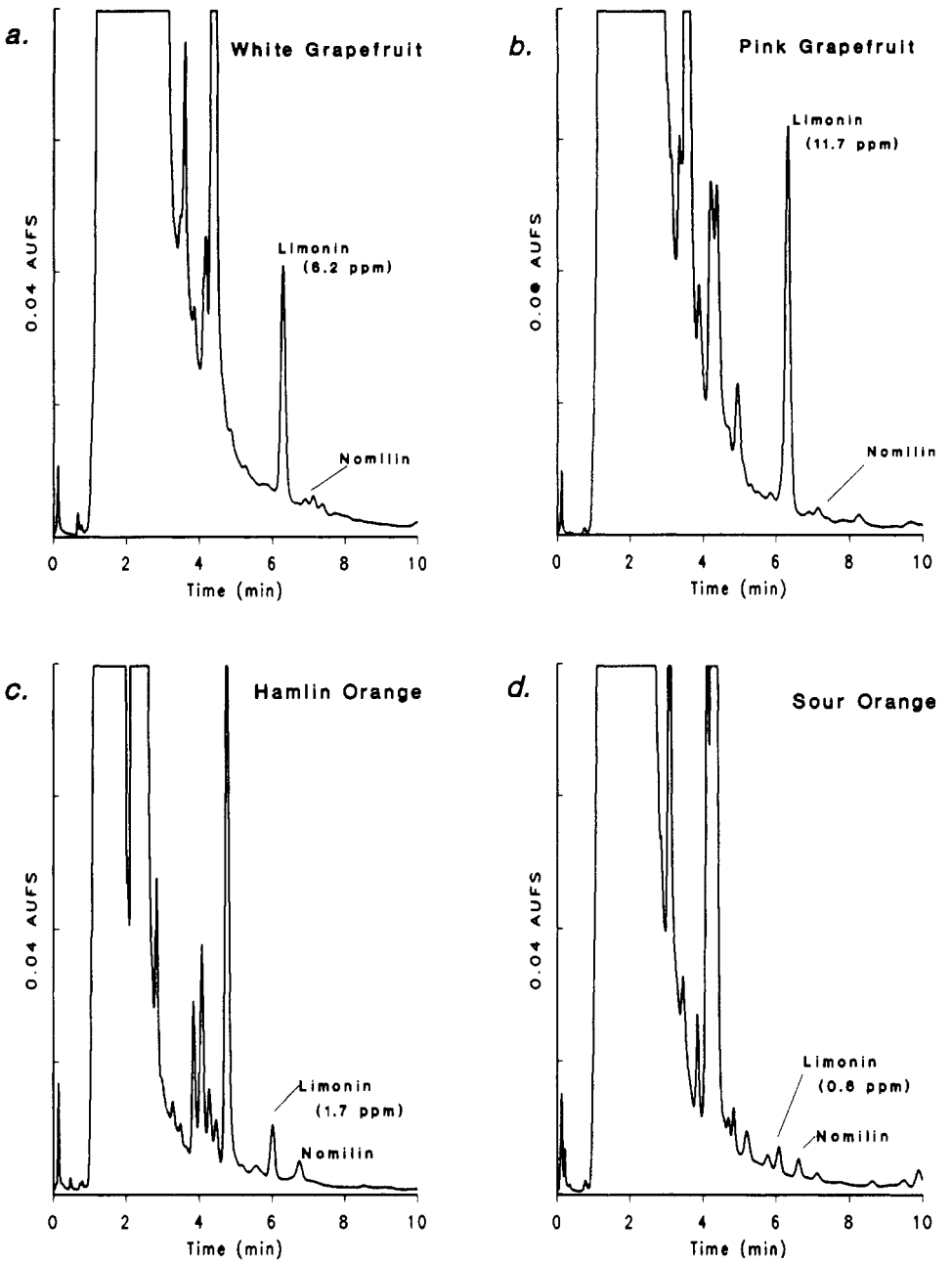

Figure 2. HPLC traces of SPE extracts using no precolumn from (a) white grapefruit, (b) pink grapefruit, (c) Hamlin orange, and (d) sour orange. Conditions are given in the text.

of acetonitrile/water (38:62) was used with a flow rate of 1.5 mL/min. A Supelco 5-μm CN analytical column (4.6 mm × 25 cm) preceded by a 0.45-μm in-line filter was heated to 30 °C. For some analyses a Brownlee 4.6 mm × 3 cm CN guard column was placed between the filter and the analytical column. Column back pressure was 1100 psi (1500 psi with precolumn). Following a set of analyses, which ranged from 10 to 50 injections, the HPLC system was washed with 30 mL of acetonitrile to clean the column of retained components. Best results were obtained if the column was also washed with 10 mL of acetonitrile and allowed to equilibrate with mobile phase just prior to analysis. When this procedure was followed, integrity of the column was maintained.

Normal-phase analyses were performed as described by Rouseff and Fisher (1980) using a ternary mobile phase of 2-propanol/heptane/methanol (48:44:8) at a flow rate of 1.0 mL/min. A Supelco 5-μm CN 4.6 mm × 25 cm analytical column was heated to 40 °C and was preceded by a 0.45-μm in-line filter. Following analyses, the column was washed with methanol to elute retained components.

RESULTS AND DISCUSSION

As mentioned under Experimental Procedures, a precolumn was used for some analyses. Use of a precolumn is always advisable when practical to protect the more expensive analytical column. The first precolumn did allow adequate separations to be performed. However, use of the precolumn proved to be unnecessary. After approximately 500 injections, the column efficiency for limonin had only decreased slightly (N = 10 050 vs 9200). When the precolumn was changed as a precaution, the new precolumn caused severe peak tailing, and the analytical column alone was used for all subsequent analyses. Use of other types or manufacturers of precolumns may yield better results. The analytical column alone provided separations with increased efficiency (N = 12 000) and less peak tailing. Tracings in parts a and c of Figure 1 show separations of Valencia and navel juice extracts, respectively, with the good precolumn preceding the analytical column; parts b and d were separations performed with the analytical column alone. Figure 2 shows additional tracings of juice extracts (analytical column only) from white grapefruit, pink grapefruit, Hamlin orange, and sour orange varieties. Analysis of limonin performed on the CN column in reversed-phase mode using

Table I. Recovery of Limonin from Model Juices and Grapefruit Juices Using Disposable C-18 SPE Columns[a]

	limonin, ppm							
			solid-phase extraction (n = 2)					
	direct[b] (n = 4)		2.5 mL			5 mL		
sample	mean	SD	mean	SD	% rec	mean	SD	% rec
model juice								
1	0.46	0.014	0.44	0.013	94.6			
2	1.89	0.015	1.86	0.001	98.4			
3	4.74	0.042	5.13	0.024	108.5			
4	9.70	0.086	10.20	0.049	105.3	9.69	0.092	99.9
5	19.81	0.096	20.30	0.008	102.5	19.81	0.028	97.1
grapefruit								
1	(not spiked)		5.68	0.249		5.67	0.113	
2	(+0.46 ppm)		6.11	0.040	99.5			
3	(+4.74 ppm)		11.29	0.008	108.3	10.73	0.237	102.9
4	(+9.70 ppm)		16.00	0.018	104.0	15.60	0.133	101.4
5	(+19.81 ppm)		25.56	0.129	100.3	24.42	0.252	95.8

[a] Amounts in original juices, extracts eluted into vials, and extracts eluted into volumetric flasks are compared. [b] Values determined by direct injection and analysis of model juices.

Table II. Reproducibility of Limonin Analysis Using SPE and CN Column under Reversed-Phase Conditions

	limonin,[a] ppm, for extract no.				statistics		
	1	2	3	4	mean	SD	% RSD[b]
navel	9.03	8.92	8.80	9.05	8.95	0.11	1.2 (n = 8)
navel (debittered)	2.75	2.72	2.69	2.63	2.70	0.05	1.8 (n = 8)
grapefruit 05	12.48	12.38	13.69	12.82	12.84	0.55	4.3 (n = 8)
grapefruit 41	6.94	7.11	7.58		7.21	0.30	4.2 (n = 6)
grapefruit 42	5.74	5.72	6.30		5.92	0.29	4.8 (n = 6)
Valencia (early)	6.66	6.41	6.74		6.56	0.19	2.9 (n = 6)
Valencia (late)	1.73	2.08	2.26		2.02	0.24	11.8 (n = 6)

[a] Values for each extract are an average of two injections. [b] Percent relative standard deviation.

the new SPE procedure showed an improved separation of limonin from other components over separations reported by previous investigators (Shaw and Wilson, 1984, 1988; Shaw, 1986; Van Beek and Blaakmeer, 1989) using a C-18 or C-8 column. Other citrus juices are currently under investigation with the new method.

Nomilin, another bitter component with a structure similar to that of limonin and known to be present in some citrus fruits, was well separated from limonin. The peak corresponding to nomilin, identified by comparing the retention time with that of a standard, is indicated in Figures 1 and 2. While recovery of nomilin from juice using SPE was not determined, it appears quantitation of nomilin should be possible. Nomilin recovery and quantitation using the SPE procedure are currently being investigated.

Stationary-Phase Extraction. In preliminary experiments, results reported by Van Beek and Blaakmeer (1989) were confirmed; acetonitrile was found to elute limonin from the SPE columns more efficiently (less required) than methanol. However, acetonitrile extracts had a detrimental effect on the CN stationary phase similar to that reported by Rouseff and Fisher (1980). When 100% acetonitrile was used to elute limonin from the SPE columns, extracts caused deterioration of the CN stationary phase exhibited by a decrease in limonin retention. Washing with 100% acetonitrile did not completely restore the column. When 2.5 mL of 70% aqueous methanol was used as eluant, extracts had no adverse effect on the CN column, yet solvent strength was sufficient for quantitative recovery (as shown in Table I). The slightly larger volume of 70% methanol (compared to acetonitrile) eluent required to quantitatively recover limonin does not appreciably increase the limit of detection. With the 4-fold concentration of limonin on extraction, the minimum amount of limonin in juice that can be quantitated reliably is 0.2 ppm or less.

The volume of the 30% methanol wash, when varied from 5 to 10 mL, resulted in a slight reduction in recovery with little improvement in sample cleanup for the larger wash volumes. Composition of the methanol wash and limonin eluent was varied from 0 to 40% aqueous methanol and from 50 to 100% aqueous methanol, respectively. Using water as a wash failed to eliminate an interfering peak, and sample stability was poor. Solvent washes stronger than 30% methanol resulted in reduced limonin recovery, particularly when limonin concentrations were above 10 ppm. Methanol alone as a limonin eluent caused interferences to elute from the solid-phase adsorbent along with limonin.

Recovery. Limonin recovery rates from the SPE procedure were determined by addition of known amounts of limonin to model and grapefruit juices. Previous investigation (Rouseff and Fisher, 1980), confirmed by the author, found that limonin was not lost in the centrifugation process. Exact amounts added to the juices were determined by direct injections (n = 4) of spiked model juices; results are listed in Table I. With grapefruit juice treated identically with model juice, amounts added should be identical. Variations in recovery were reduced when the limonin fraction was eluted into a 5-mL volumetric flask and made to volume, most likely due to increased precision in the final extract volume. Detector response was linear ($y = 1549.81x + 149.44$; $r = 0.99998$) up to 50 ppm of limonin. This corresponds to 12.5 or 25 ppm of limonin in juice depending on the concentration factor. When high limonin concentrations are expected, 5 mL of juice should be used for extraction.

Precision. Method reproducibility is shown in Table II. Juices were extracted in triplicate or quadruplicate, with duplicate injections of each extract. Replicate extractions had a relative standard deviation (RSD) of 5% or less for all but one juice. One juice with a limonin concentration of 2 ppm had a RSD of 12% for three

Table III. HPLC Results of $CHCl_3$ Extracts vs SPE Extracts

sample	limonin, ppm $CHCl_3$	limonin, ppm SPE	ratio[a]
grapefruit 01	19.41	18.38	1.06
grapefruit 05	14.51	12.84	1.13
grapefruit 31 (pink)	5.52	5.62	0.98
grapefruit 37	8.54	7.63	1.12
grapefruit 41	8.08	7.21	1.12
grapefruit 42	6.70	5.69	1.13
grapefruit 43 (pink)	7.16	7.32	0.98
grapefruit 44	7.36	7.14	1.03
grapefruit 48	6.43	6.07	1.06
grapefruit 962	3.42	3.58	0.96
grapefruit 964	5.68	5.89	0.96
grapefruit 965 (pink)	6.58	6.60	1.00
grapefruit 966	6.55	6.52	1.00
grapefruit 967	4.88	5.34	0.91
grapefruit 968	2.48	2.59	0.96
grapefruit 969	4.58	4.81	0.95
grapefruit 970	7.40	7.29	1.02
grapefruit 971	3.75	3.46	1.08
grapefruit 972	5.22	4.81	1.08
grapefruit 973	6.60	6.21	1.06
grapefruit 974 (pink)	4.04	3.51	1.15
bitter navel	9.83	8.95	1.10
debittered navel	2.88	2.66	1.08
Valencia (early)	6.86	6.56	1.05
Valencia (late)	2.43	2.02	1.20

[a] Ratio of results from $CHCl_3$ extracts/SPE extracts.

extracts. The RSD between injections (n = 2) for any extract was 2.0% or less with few exceptions and was never greater than 4.5%. Comparison of results from reversed-phase analysis of solid-phase extracts with those from the normal-phase analysis of chloroform extracts (Table III) shows generally good agreement.

For the reversed-phase method developed, the good agreement obtained when results are compared with those from chloroform extracts, an absence of shoulders (Figures 1 and 2) on the limonin peak, and the lack of detector response at 254 nm in the area where limonin elutes indicate the limonin peak is free of interferences.

Sample Stability. Finally, solid-phase extracts obtained by using the new method are more stable than SPE extracts in acetonitrile. Shaw and Wilson (1984) reported the stability of limonin in acetonitrile to be less than 24 h. Table IV shows solid-phase extracts in 70% methanol are stable for 30 days when stored at −4 °C. It is always recommended that samples be analyzed with minimum delay following preparation. However, should a problem arise preventing immediate analysis, it is reassuring to know the sample matrix is stable and can be stored for later analysis.

CONCLUSIONS

The CN stationary phase was found to have good selectivity for limonin analysis. The CN stationary phase is, however, susceptible to contamination by some constituents present in citrus juices. By use of SPE with disposable C-18 columns, it was possible to remove column-contaminating constituents from citrus extracts. The SPE and HPLC procedure developed offers rapid sample preparation and an improved separation of limonin from other constituents over previously described reversed-phase methods. The method was reproducible and gave quantitative recovery of limonin, and the 2–4-fold concentration of limonin in extracts provided improved sensitivity in analysis. Methanol extracts were stable for 30 days when kept at −4 °C, considerably more stable than previously reported (Shaw, 1986) for acetonitrile extracts of limonin.

Table IV. Sample Stability of Limonin in 70% Methanol SPE Extracts

	limonin, ppm (n = 2), for sample aged 0 days	30 days	ratio[a]
grapefruit 05	13.52	13.23	0.98
grapefruit 37	7.89	8.02	1.02
grapefruit 41	7.49	7.49	1.00
grapefruit 42	6.21	6.36	1.02
grapefruit 972	4.94	5.13	1.04
grapefruit 974	3.51	3.69	1.05
grapefruit 975	3.78	4.04	1.07
grapefruit 976	7.58	7.76	1.02
bitter navel	8.74	8.99	1.03
debittered navel	2.59	2.67	1.03
Valencia (early)	6.02	6.23	1.03
Valencia (late)	2.26	2.25	1.00

[a] Ratio of results from extracts analyzed fresh/30 days at −4 °C.

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