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DETERMINATION OF LIMONIN IN GRAPEFRUIT JUICE AND OTHER CITRUS JUICES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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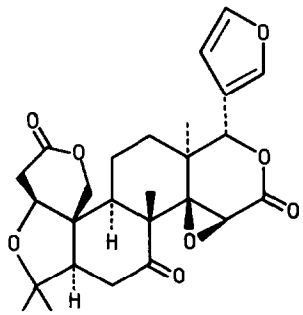
SUMMARY

A method has been developed for the quantitation of the bitter component limonin in grapefruit juice and other citrus juices. The sample clean-up consisted of centrifugation, filtration and a selective, rapid and reproducible purification with a C_2 solid-phase extraction column. The limonin concentration was determined by high-performance liquid chromatography on a C_{18} column with UV detection at 210 nm. A linear response was obtained from 0.0 to 45 ppm limonin. The minimum detectable amount was 2 ng. The minimum concentration which was detected without concentration with good precision was 0.1 ppm. The method was also used for the determination of limonin in different types of oranges, including navel oranges, mandarins, lemons, limes, pomelos and uglis.

INTRODUCTION

Limonoids, a group of oxygenated tetranortriterpenoids, are of general occurrence in the genus *Citrus*. Two of these compounds, limonin (**1**) and nomilin (**2**), have a very bitter taste which is still detectable in concentrations of 2–5 ppm[1]. As they occur in the economically important oranges and grapefruits their exact concentration is of much interest to the citrus industry. Too high a concentration of limonin causes excessive bitterness which is not acceptable to the consumer.



Limonin (**1**)

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Of nomilin and limonin the latter is the more important because of its more widespread occurrence and its usually higher concentration. Limonin occurs in two forms: the bitter dilactone **1** and the non-bitter monolactone **3**. The monolactone, which is stored separately in carpellary membrane tissues in the intact fruit[2], is however converted into the bitter dilactone within hours once it comes in contact with the acidic juice during processing[1,3]. This explains the delayed bitterness which is sometimes observed in processed citrus juices, especially in early season grapefruits and navel oranges. Recently several investigations have been carried out on the reduction of the concentrations of bitter principles in juices of early season oranges and grapefruits[4–8]. To check the effect of the various techniques it is necessary to be able to measure the concentrations before and after treatment.

Due to the interest of the citrus industry in the limonin concentration a large number of quantitations have been published. Limonin has been determined by thin-layer chromatography (TLC)[9,10], chemical derivatization followed by spectrometry[11,12] or fluorimetry[13], gas–liquid chromatography (GLC)[14], high-performance liquid chromatography (HPLC)[15–20], radioimmunoassay (RIA)[21] and enzyme immunoassay (EIA)[22,23]. Two articles have summarized and discussed the different methods for the determination of limonin[1,24].

Several HPLC determinations have been published using either reversed (C_{18}, C_8, CN) or straight-phase (CN) materials. The major problem of the HPLC determinations is the absence of any selective chromophore in limonin ($\lambda_{max.}$ 207 nm) and the low concentration in the citrus juices (1–20 ppm). The sample clean-up has to be very thorough. This results in time-consuming partitions, usually with chloroform. The actual HPLC run takes from 10 to 20 min. A recently published method uses small solid-phase extraction columns instead of partitions, which resulted in a considerable time gain[18,25]. This advantage was however partially offset by the diminished quality of the resulting chromatograms. Especially in grapefruit juice extracts, the limonin is only a minor peak when compared with several impurities. Also the analysis time was much longer due to the long retention time of some major impurities when compared with for instance the straight-phase system of Rouseff and Fisher[17].

In this article we report on an improved method for the determination of limonin in various citrus juices using solid-phase extraction columns with a selective purification step and a C_{18} column with UV detection.

EXPERIMENTAL

Solvents

The acetonitrile, methanol and tetrahydrofuran (THF) used were of HPLC quality. Water was doubly distilled in an all-glass apparatus. All solvents were filtered (0.45 μm) and ultrasonically degassed before use.

Standards

The limonin used for recovery experiments and calibration graphs was supplied by Dr. R. L. Rouseff. Its purity was checked by HPLC in various solvents, TLC and 300-MHz ^{1}H NMR. The limonin and nomilin used for comparison of retention times were supplied by Dr. J. H. Tatum. A purified extract of grapefruit seeds was also used for this purpose. This extract was prepared by crushing several seeds, extracting them

in acetonitrile for 1 h with ultrasonic agitation, diluting the crude extract in four parts of water and purifying the turbid solution over a C_2 column as described below for the HPLC samples.

Plant materials

All fruits were obtained locally from Dutch greengrocers or supermarkets. When known, the type and the country of origin are given in the text.

Instrumentation

All separations were carried out isocratically at room temperature (20°C) with a Gilson 303 HPLC pump, equipped with a Rheodyne Model 7125 injector with a 20-μl or a 100-μl loop. The detection was carried out at 210 nm with a Gilson 116 variable-wavelength detector. Integration was done with a Shimadzu CR3A integrator. The column (150 mm × 4.6 mm) and guard (15 mm × 4.6 mm) were manufactured by Rainin-U.S.A. and packed with 5-μm C_{18} material (Microsorb, Cat. No. C_{18} 80-215-C5).

Chromatographic conditions

Three different solvent systems were used: (1) acetonitrile–methanol–water (28.5:13:58.5), flow-rate 2.0 ml/min (standard solvent). (2) acetonitrile–methanol–water (31.8:22.7:45.5), flow-rate 1.0 ml/min; (3) methanol–water (65:35), flow-rate 1.0 ml/min.

Identification and purity of peaks

Limonin and nomilin were identified in the chromatogram by comparing the retention times of the various peaks with those of authentic reference samples.

The identity and purity of limonin and the two compounds corresponding to the adjacent peaks in the HPLC chromatogram of grapefruit juice with solvent system 1 was also checked by recording UV, mass and 300-MHz ^{1}H NMR spectra of collected HPLC fractions. For this purpose 600 ml of grapefruit juice were processed on a larger scale but in exactly the same way as described below for the quantitation of limonin (column 25 cm × 1 cm, 5-μm C_{18}). The purity and identity of the limonin fraction was additionally investigated by HPLC with solvent system 3 and TLC (ready-made silica gel 60F_{254} plates, Merck, Cat. No. 5719). The following two TLC systems were used in saturated chambers: (1) toluene–ethanol–water–acetic acid (76:18:5.7:0.5) (limonin, R_F 0.21; nomilin, R_F 0.18); (2) 100% ethyl acetate (limonin, R_F 0.61; nomilin, R_F 0.48). Detection on the TLC plates was carried out by viewing under UV light at 254 and 366 nm followed by spraying with 5% *p*-dimethylaminobenzaldehyde in ethanol and placing the plates in a warm hydrogen chloride atmosphere[26]. The purity of the limonin peak in the chromatograms of citrus juices other than grapefruit juice was checked by recording the chromatogram both at 210 and 254 nm. When only a baseline was observed at the retention time of limonin in the chromatogram at 254 nm the limonin peak was considered pure.

Extraction and purification procedure

A grapefruit or other citrus fruit was cut in two and immediately extracted by hand on a lemon squeezer. The juice was centrifuged for 30 min in an Heraeus labofuge

I at 2100 *g* and the supernatant filtered over a filter paper (Schut, V259, diameter 5.5 cm for Büchner).

A Bond Elut® (Analytichem International) 500-mg C_2 solid-phase extraction column was washed with 4 ml acetonitrile and equilibrated with 4 ml acetonitrile–water (2:98). Next 2.00 ml of the clear, filtered juice were introduced on the column, the column was washed with 4 ml acetonitrile–water (30:70) and finally the limonin was eluted with 2 ml acetonitrile–water (60:40). This fraction was collected in a 2-ml volumetric flask, and water was added to 2.00 ml. The column was never allowed to run dry. The flow-rate through the column was 2.5 ml/min with the wash solvent and 4.7 ml/min with the extraction solvent (constant vacuum). The limonin-containing solution in the volumetric flask was directly injected into the HPLC column.

To determine the concentration of both limonin and its precursor limonin monolactone (total limonin), the pressed juice was heated at 80°C for 15 min after centrifugation.

Calibration graph and reproducibility of integration

A calibration graph for limonin was prepared by injecting 20 μl of ten known concentrations (0.000, 0.430, 1.09, 3.12, 5.45, 7.84, 11.36, 18.18, 27.27 and 45.45 ppm) and plotting the integration value from 0.0 to 45 ppm against the concentration. A linear detector response from 0.0 to 45 ppm limonin was observed ($r = 0.99993$). All injections were done five times. The reproducibility of the integration process was determined for five standard solutions. Each solution was injected five times and standard deviations were calculated. The results are given in Table I.

TABLE I

STANDARD DEVIATIONS OF INTEGRATION RESULTS OF LIMONIN SOLUTIONS

For conditions see Experimental. $n = 5$.

Limonin concentration (ppm)	*Standard deviation (ppm)*	*Relative standard deviation (%)*
1.160	0.045	3.91
3.136	0.079	2.52
5.454	0.083	1.52
12.35	0.113	0.92
24.96	0.293	1.17

Recovery experiments

Known amounts of limonin in water were submitted to the purification procedure and the relative recovery determined. Each fraction was tested five times on the C_2 column. The resulting extracts were each injected four times for HPLC analysis. Four different concentrations (1.160, 3.136, 12.35 and 24.96 ppm) were investigated. The results are given in Table II.

Standard addition experiments

To grapefruit juice with a known limonin concentration (determined on the same day using the same procedure), various but exactly known quantities of limonin were

TABLE II

RESULTS OF RECOVERY EXPERIMENTS

For conditions see Experimental. $n = 5$.

Initial limonin concentration (ppm)	*Concentration after C_2 column (ppm)*	*Recovery (%)*	*Standard deviation (ppm)*	*Relative standard deviation (%)*
1.16	1.11	95.5	0.026	2.24
3.14	3.12	99.4	0.075	2.40
12.35	12.02	97.3	0.137	1.11
24.96	25.19	100.9	0.362	1.45

added. The resulting enriched solutions were processed according to the described procedure. The limonin concentration of the grapefruit juice, the limonin-containing solution and the spiked grapefruit juice were each purified five times over the C_2 column. Each of the resulting fifteen extracts was injected four times for HPLC analysis. The results are given in Table III.

Quantitation

The citrus fruits to be investigated were worked up in triplicate according to the above purification procedure. Each resulting extract was injected in triplicate for HPLC. The limonin concentration of the juice was calculated by comparing the integration values with the calibration graph. The results are given in Table IV.

TABLE III

RESULTS OF STANDARD ADDITION EXPERIMENTS

For conditions see Experimental. $n = 5$.

Grapefruit sample no.	*Limonin concentration (ppm)*	*Limonin added (ppm)*	*Expected concentration (ppm)*	*Concentration found (ppm)*	*Recovery (%)*	*Stand. dev. (ppm)*	*Rel. stand. dev. (%)*
1	6.25	10.33	16.58	16.50	99.5	0.09	0.54
2	8.95	5.53	14.48	14.14	97.7	0.17	1.17
3	8.96	10.78	19.74	19.35	98.0	0.27	1.37

RESULTS AND DISCUSSION

Our goal was to develop a simple, rapid, sensitive and accurate determination of limonin in grapefruit and preferably other citrus juices. As the final separation and quantitation technique, reversed-phase HPLC (standard C_{18}) with UV detection was chosen. Many laboratories have HPLC apparatus and such columns available for other analysis so additional costs remain limited to solvents. As UV detectors have become much more sensitive in the last 5 years, the difference in sensitivity between HPLC and the immuno techniques has become very small. A simple and rapid method means no tedious and time-consuming partitions but the use of rapid and reproducible

TABLE IV

LIMONIN CONCENTRATIONS OF VARIOUS CITRUS FRUITS

For conditions see Experimental. n.d. = Not determined.

Fruit	*Type or brand*	*Country of origin*	*Limonin concentration (ppm)*	
			Before heating	*After heating*
Grapefruit	Yellow (Jaffa)	Israel	10.4	n.d.
Grapefruit	Red (Pride)	U.S.A.	0.60	1.17
Orange	Navel	Spain	0.78	5.61
Orange	Grandiosa	Spain	0.66	2.15
Mandarin	Unknown	Argentina	0.74	3.53
Lemon	Unknown	Spain	4.77	4.77
Lime	Unknown	Brazil	20.8	n.d.
Pomelo	Jaffa	Israel	33.1	n.d.
Ugli	Unknown	Jamaica	0.5	1.21

solid-phase precolumns. Several different RP precolumns were investigated, *i.e.*, C_{18}, C_8, C_2, cyclohexyl, phenyl and CN, to determine whether they showed any selective retention of the limonoids or the major impurities (coumarins, flavonoids) present in grapefruits. Surprisingly this was not the case; only the elution strength of the washing and extraction solvents varied.

The composition of the washing and extraction solvent had however a big influence on the purity of the final extract. Acetonitrile–water mixtures gave much better results than methanol–water mixtures. Finally a C_2 column was selected with acetonitrile–water (30:70) as the washing solvent and acetonitrile–water (60:40) as the extraction solvent. The extracts were initially investigated with several different HPLC solvents on a C_{18} column: acetonitrile–water mixtures, methanol–water mixtures and methanol–acetonitrile–water (21.5:26.5:52), according to Shaw[25] the optimum solvent for the determination of limonin in citrus juices. However, none of these solvents showed good results with our purified extract due to either coelution of limonin with impurities, large negative peaks or very late eluting impurities. Because of the low absorption maximum of limonin at 207 nm, only methanol, acetonitrile and water can be used for the HPLC solvent. Many different HPLC solvents consisting of these three solvents were subsequently tried. Finding the optimum solvent was greatly hampered by the fact that water was not the non-selective solvent which it is supposed to be in RP-HPLC. When the water content was changed not only the retention time changed but also the elution order of limonin and two of the most disturbing remaining impurities in the purified sample changed.

Finally two solvents were found to be suitable for the analysis of limonin. The first, acetonitrile–methanol–water (28.5:13:58.5), can be used for all citrus juices investigated by us and is the standard solvent used for all the analyses described in this publication. For a characteristic chromatogram of a purified extract of a yellow grapefruit see Fig. 1a. Due to the high water content the k' value is high, 12.5. For reasonable analysis times (*ca.* 10 min per experiment) the flow-rate has therefore to be quite high, 2 ml/min. If necessary the solvent consumption can be reduced by 80% without loss of resolution, speed or sensitivity by using a 2.0 mm I.D. instead of a 4.6

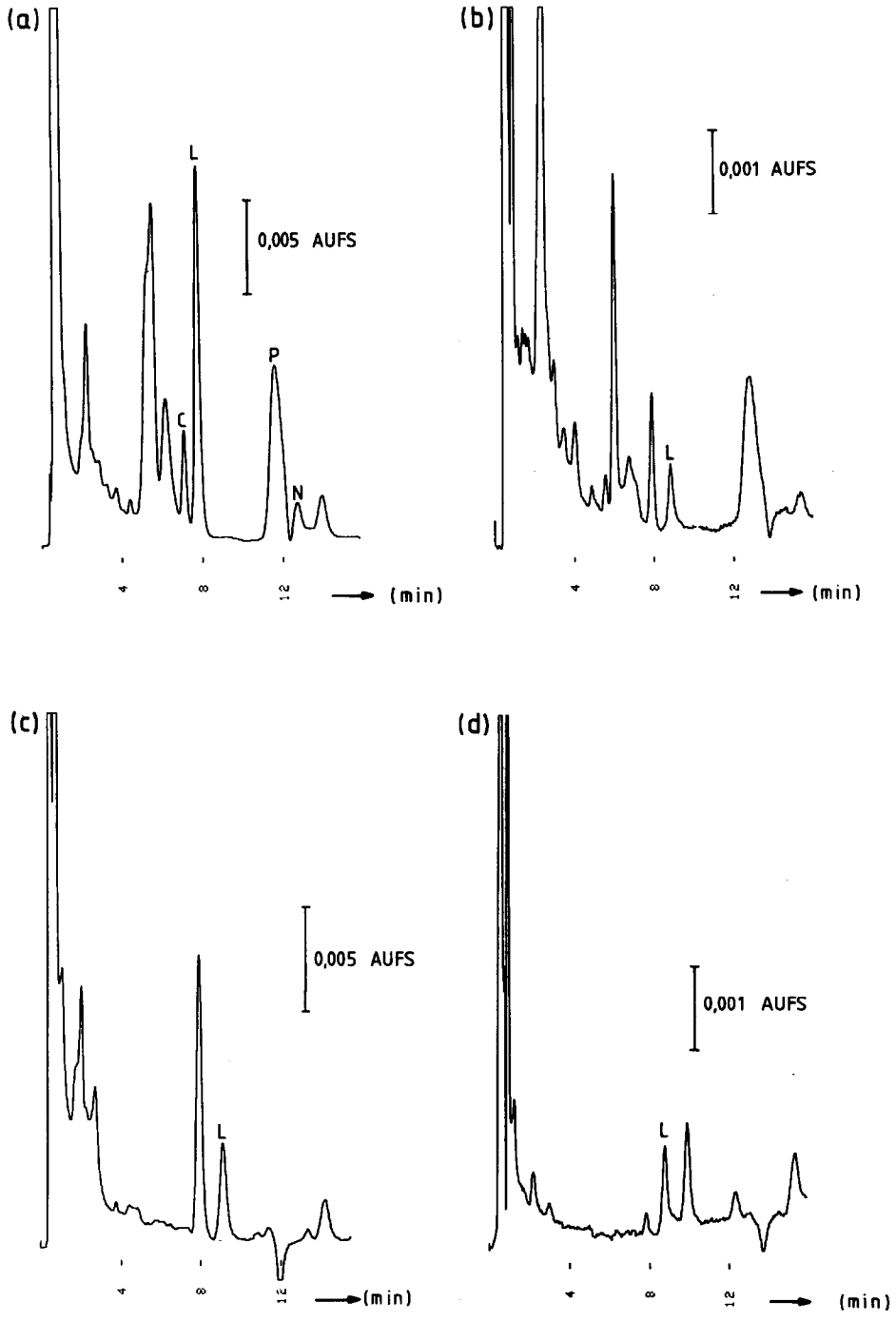

Fig. 1.

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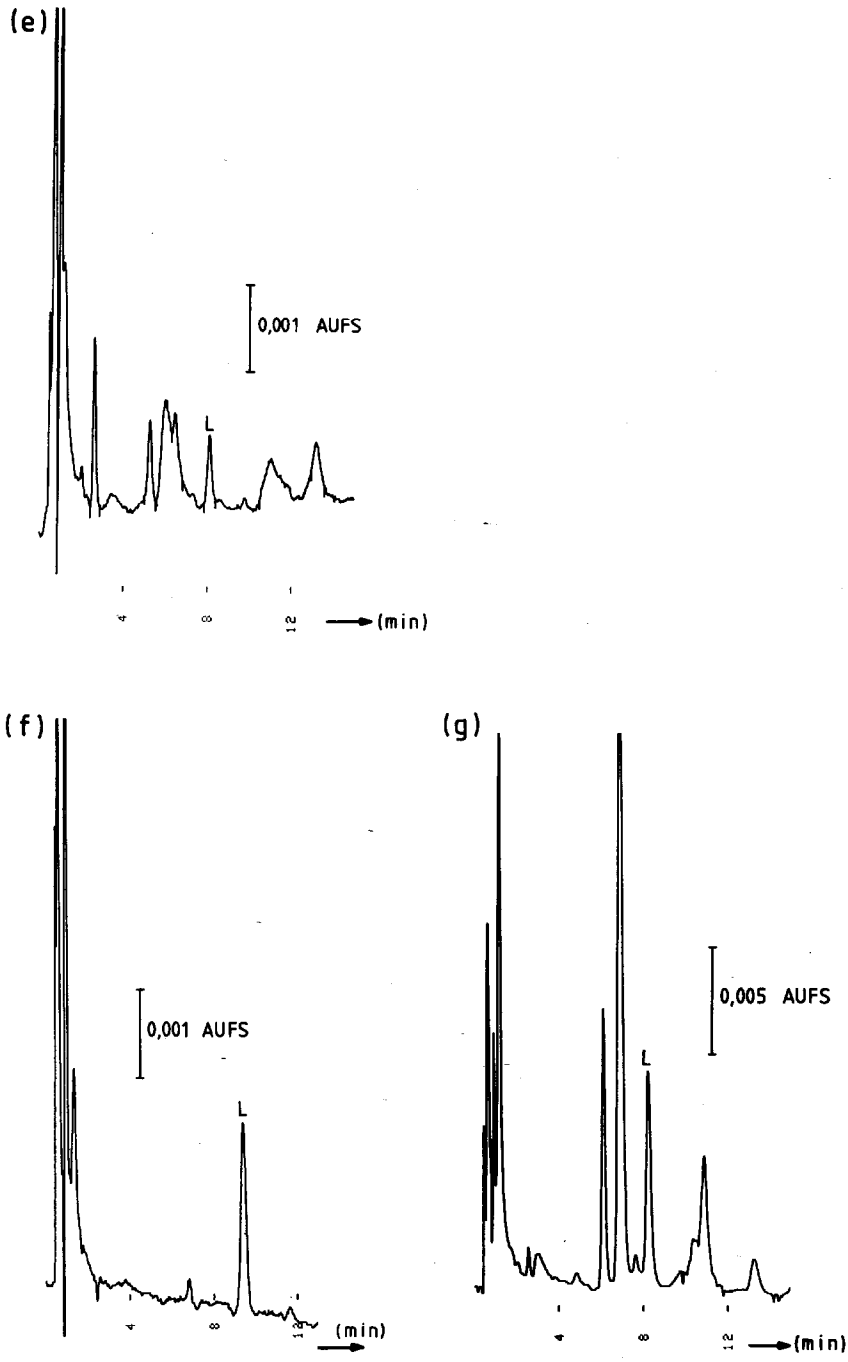
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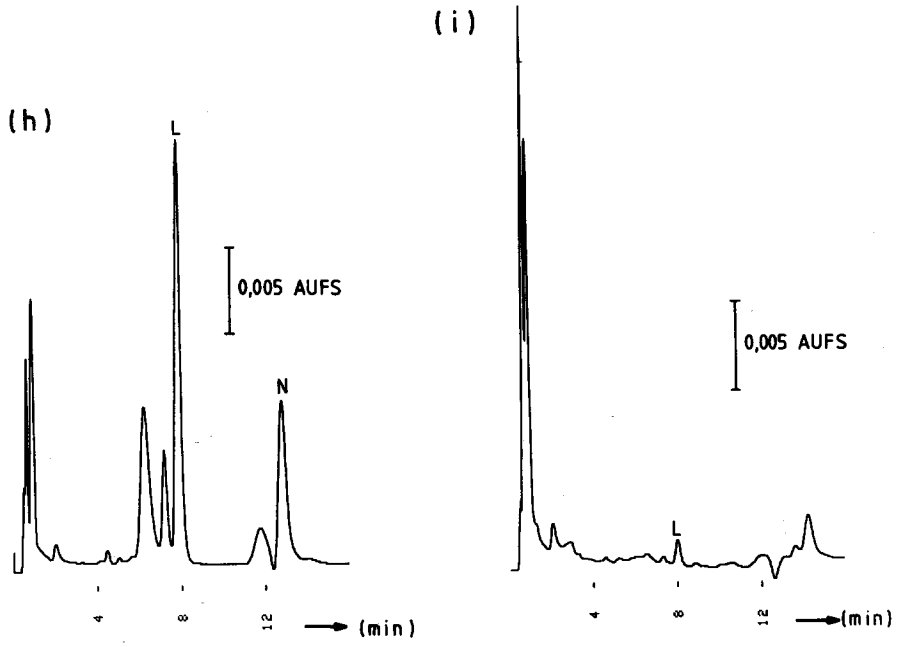

Fig. 1. HPLC traces of purified extracts of various citrus juices. Solvent 1, flow-rate 2.0 ml/min. C = isoaurapten; L = limonin; N = nomilin; P = psoralen. (a) Yellow grapefruit, unheated, 100-μl loop; (b) red grapefruit, heated, 20-μl loop; (c) navel orange, heated, 100-μl loop; (d) Grandiosa orange, heated, 20-μl loop; (e) mandarin, unheated, 20-μl loop; (f) lemon, unheated, 20-μl loop; (g) lime, unheated, 20-μl loop; (h) pomelo, unheated, 100-μl loop; (i) ugli, unheated, 100-μl loop.

mm I.D. column. In grapefruit juice, nomilin (k' = 21.2) cannot be determined simultaneously because it almost coelutes with a major impurity.

The other useful solvent was acetonitrile–methanol–water (31.8:22.7:45.5). Limonin has a much lower k' value (3.5) in this solvent resulting in shorter analysis times with a 50% reduction in solvent consumption. Also nomilin can be determined simultaneously (k' = 5.2). A disadvantage of this solvent system is that the limonin peak is very close to two other peaks in the chromatogram of grapefruit juice (see Fig. 2). When the limonin concentration is relatively low this results in less accurate quantitations. In many cases however this solvent may be of much benefit.

The following amounts of time are necessary for the determination of one juice sample: extraction, 3 min; centrifugation and filtration, 32 min; purification, 5 min and one actual HPLC run, 10–15 min depending on the time of injection. Centrifugation can be carried out for many samples at the same time and thus the centrifugation time per sample will be small. Purification can be done manually for 12 to 20 samples at the same time. Automation is nowadays possible, *e.g.*, with the Gilson ASPEC system. HPLC can be done manually or with an autosampler. With an autosampler it should be possible to analyse routinely 100 juice samples per day.

Detection was carried by UV at 210 nm. Although the reported maximum for limonin is at 207 nm no difference in integration values for a standard solution of

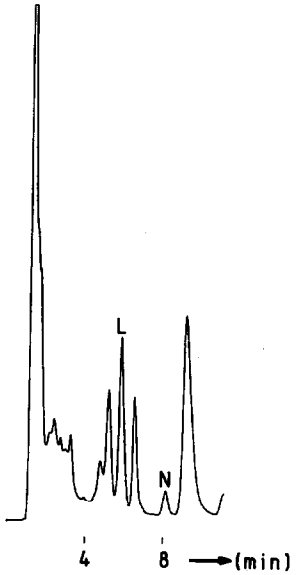

Fig. 2. HPLC trace of a purified yellow grapefruit juice extract. Solvent system 2, flow-rate 1.0 ml/min, 20-μl loop. L = Limonin; N = nomilin.

limonin was found between a detection wavelength of 207 or 210 nm. At 210 nm problems with solvent absorption will be less.

The purity of the limonin peak was not only assessed by comparing the retention time with a reference and by recording the chromatogram at 254 nm, but also by recording mass and 300-MHz ^{1}H NMR spectra off-line. For this purpose, 600 ml of grapefruit juice were worked up in the same manner as for an analytical sample. The resulting extract was concentrated and separated into the individual components by means of semipreparative HPLC with solvent 1. According to the NMR and mass spectra, the limonin fraction consisted solely of limonin. The purity was additionally investigated by analytical TLC in two solvent systems and using a spray reagent selective for limonoids, and by HPLC in solvent system 3. Again no other compounds were detected.

Fractions corresponding to the peaks marked C and P in Fig. 1a were also collected and investigated by UV, mass and NMR spectroscopy. Peak C was identified as 7-methoxy-8-(3-methyl-2-butenyloxy)coumarin (**4**) (synonym isoaurapten). Peak P was identified as 5-[(6,7-dihydroxy-3,7-dimethyl-2-octenyl)oxy]psoralen (**5**). A minor component present in the preparative juice sample which eluted just before **4** was identified as 7-[(6,7-dihydroxy-3,7-dimethyl-2-octenyl)oxy]coumarin (**6**) (synonym marmin).

After determining the major experimental conditions, the method was investigated with respect to its reproducibility and recovery. The relative standard deviations of the detector response for standard solutions of limonin are given in Table I. Next the reproducibility and the recovery of the purification procedure were determined. Results are given in Table II. Standard addition experiments in which known amounts of limonin were added to known quantities of limonin in grapefruit juice were also

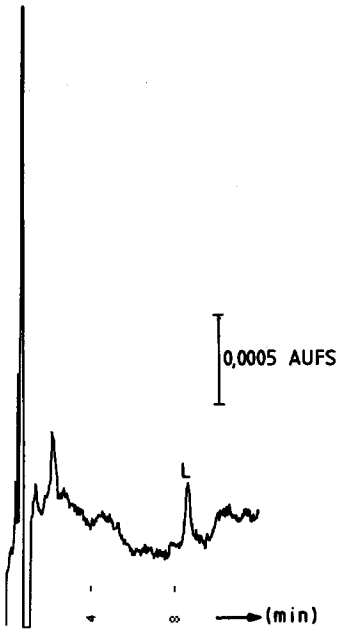

Fig. 3. HPLC trace of a 0.1-ppm standard solution of limonin. Solvent system 1, flow-rate 2.0 ml/min, 100-μl loop. L = Limonin.

performed. The results are similar to those obtained in the recovery experiments and are presented in Table III. Limonin concentrations below 1 ppm were determined with adequate precision.

The minimum detectable quantity of limonin was approximately 2 ng (limonin peak twice as big as the short term noise amplitude, 20-μl loop). This value resulted in a minimum detectable concentration of 0.1 ppm for qualitative purposes and of 0.5 ppm for quantitative purposes (20-μl loop). With the 100-μl loop these values were 0.03 and 0.1 ppm respectively. A chromatogram of a 0.1-ppm standard solution of limonin is given in Fig. 3 (100-μl loop).

In order to find out whether this sample clean-up is also applicable to other citrus juices apart from yellow grapefruit juice, it was tested on the following fruits: red grapefruit (Pride, Florida), navel orange (Spain, Genesis), orange (Spain, Grandiosa), mandarin (Argentina), lemon (Spain), lime (Brazil), pomelo (Israel, Jaffa) and ugli (Jamaica). The resulting chromatograms are presented in Fig. 1b–1i. In all cases the proposed procedure was used. Limonin concentrations were calculated with the calibration graph and are given in Table IV. The limonin concentration of some juices was determined both before and after heating. The peak purity of limonin was determined by recording a chromatogram at 210 and 254 nm. In all instances only a baseline was observed at 254 nm at the retention time of limonin.

CONCLUSION

Solid-phase extraction columns in combination with standard C_{18} HPLC

columns can be used with good results for the quantitation of the bitter compound limonin in various citrus juices. The present method combines speed with acceptable resolution and sensitivity. The purification, injection and analysis procedure is suitable for automation.

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