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DISTRIBUTION OF LIMONOIDS IN CITRUS SEEDS

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Key Word Index—*Citrus* species; Rutaceae; limonoids; limonin; nomilin; obacunone; deacetylnomilin; ichangin; concentration profiles; species identification.

Abstract—Absolute concentrations of the major limonoids in the seeds of 18 cultivars representing eight species in the subgenus *Citrus* have been determined. This study represents the first attempt to determine and employ limonoid concentration profiles to differentiate *Citrus* species. Limonin, nomilin, obacunone and deacetylnomilin were found in varying amounts in all eight species. Limonin was usually the dominant limonoid, comprising 42–89% of the total limonoids. The percentage of nomilin ranged from 8.9 to 48% of the total limonoids and in a few cases was as great or greater than limonin. The relative nomilin concentration and the nomilin–limonin ratio were the most useful parameters in distinguishing the various citrus species. Analysis of the minor limonoids, such as obacunone and deacetylnomilin, assisted in the positive identification of each species. The limonoid concentration profile of each species was unique. Each variety within a species had similar amounts of, as well as showed characteristic ratios among, the various limonoids. Cultivars that varied appreciably from their respective intra-species limonoid pattern were probable hybrids. Their possible parentage is discussed.

INTRODUCTION

The distribution and concentration of limonoids in citrus fruit is of considerable interest to the citrus industry because of the bitterness imparted to juice by some of these compounds. Limonoids such as limonin are intensely bitter, whereas others such as deoxylimonin and obacunone are tasteless. In fruit, highest limonoid concentrations are found in seeds, and it has been proposed that seeds act as a limonin storage site [1]. In an earlier chemotaxonomic study of citrus limonoids, Dreyer [2] used TLC with Ehrlich's reagent to separate and identify limonin, obacunone and deacetylnomilin in the seeds of 26 *Citrus* species and hybrids. Since only the presence or absence of three limonoids was reported, it was not possible to differentiate between the various species. Dreyer concluded that limonoids were of little value as taxonomic markers at the species level but might have some use at the generic level. Hasegawa *et al.* [3] using TLC, published a brief report on the relative proportions of four neutral and three acidic limonoids of six citrus species. No attempt was made to differentiate citrus species.

With the advent of HPLC, limonoids could be separated and accurately quantified. A recent HPLC procedure [4] showed that the major citrus limonoids could be determined with excellent precision and high recoveries. Thus, the purpose of this study was to re-evaluate the use of limonoids as taxonomic markers using a more sensitive technique capable of differentiating, as well as quantifying, a number of citrus limonoids.

RESULTS AND DISCUSSION

Comparison with previous chemotaxonomic studies

In a study of the limonoid distribution in the seeds of *Citrus* species, Dreyer [2] found limonin in each of the 26 species and hybrids studied. He estimated limonin to be present in greater quantity than either obacunone or deacetylnomilin in all except two cases. The quantitative data in Table 1 support this observation. Nomilin was not determined in Dreyer's study because it could not be routinely resolved from limonin. In the present study, nomilin was found to be the second most predominate limonoid; its concentration rivaled that of limonin in several cases (see Table 1). Furthermore, the nomilin concentration proved to be highly instrumental in differentiating the various citrus species.

In reporting either the presence or absence of limonin, obacunone or deacetylnomilin, Dreyer found few differences between the various species. He did report that *C. medica* and *C. limon* contained limonin and obacunone but lacked deacetylnomilin. In our study, each of the three limonoids plus nomilin were found, although the concentration of deacetylnomilin was low (5–19 ppm). Dreyer also reported that *C. aurantium* contained limonin and deacetylnomilin but no obacunone. Again all three limonoids plus nomilin were found by our HPLC analysis (Table 1).

The differences between these two studies may be due to the differences in the relative sensitivities of the two analytical methods used. The TLC method employed by Dreyer was probably not sufficiently sensitive to detect the low levels of deacetylnomilin found in *C. medica* and *C. limon*. In addition, Dreyer may have missed obacunone in *C. aurantium* because he used hexane to remove the seed oils prior to extraction and analysis of the limonoids, and failed to analyse the hexane extract. It has been recently shown [4] that as much as 60% of the obacunone may be lost in the hexane fraction. Thus, the concentration of obacunone may have been reduced below the detection limit for his TLC method.

Dreyer found few qualitative differences in the limonoid composition of any of the citrus species or hybrids studied and concluded that limonoids were of little use as

Table 1. Limonoid distribution in the seeds of the subgenus *Citrus*

Species	% Total limonoids					Total limonoids (ppm)
	Deacetyl-nomilin	Obacunone	Nomilin	Limonin	Nomilin limonin	
C. medica						
Etrog citron	0.3	22.6	38.7	38.3	1.01	1527
C. limonin						
Lisbon lemon	0.6	0.6	10.3	88.5	0.12	2479
Kusner lemon	0.9	0.8	12.2	85.7	0.14	2166
Malta lemon	0.4	4.9	48.4	46.2	1.05	1443
C. limettioides Tan.						
Columbia sweet lime	15.8	0.8	16.6	66.5	0.25	2162
C. aurantium						
Common sour orange	38.5	4.0	9.3	48.2	0.19	2605
C. sinensis						
Valencia orange	6.5	0.4	9.5	83.7	0.11	2136
Ruby blood orange	6.3	0.5	12.3	80.9	0.15	2193
Queen orange	10.3	0.5	14.3	74.9	0.19	1731
Jaffa orange	21.0	0.6	15.7	62.6	0.25	2242
C. reticulata						
Dancy tangerine	0.2	1.5	27.6	70.4	0.39	3125
Ponkan mandarin	4.0	1.4	31.0	63.2	0.49	3178
C. grandis						
Thong Dee pummelo	1.0	2.2	44.8	51.8	0.86	1994
Southwickii pummelo	trace	0.3	47.9	49.1	0.97	1128
C. paradisi						
Duncan grapefruit	6.7	1.9	34.4	57.0	0.60	2379
Marsh grapefruit	14.0	1.4	31.7	52.9	0.60	3891
Mott grapefruit	0.8	2.1	34.3	62.8	0.55	2745
Royal grapefruit	1.0	2.5	29.4	67.0	0.44	2101

chemotaxonomic markers. We have found the four major limonoids: limonin, nomilin, obacunone and deacetylnomilin to be ubiquitous among citrus species. Whereas these limonoids are of little use as qualitative chemotaxonomic markers, their concentrations vary widely enough so that the quantitative pattern of these limonoids might be used to distinguish the various citrus species.

Limonoid concentration patterns

Table 1 shows that each species has a characteristic limonoid concentration pattern, and that both total and individual limonoid concentrations are reasonably consistent within each species. (The botanical classifications employed in this study are largely those of Swingle [5].) Among the 17 cultivars in Table 1, there are a few whose limonoid concentration pattern differ appreciably from other members of the same species. These exceptions are probably the result of hybridization and will be discussed later.

The relative distribution (concentration profile) and total limonoid content are different for each of the eight species shown in Table 1. The chromatograms of four species with similar limonoid patterns are shown in Fig. 1. These examples represent the most difficult limonoid patterns to differentiate. However, it can be seen from a careful examination that there are distinct differences in the limonoid pattern of each example. Limonin is the dominant peak in each chromatogram and it alone is of little value in differentiating individual species. The principle differentiating features are the ratio of nomilin–limonin and the relative proportions of deacetylnomilin and obacunone. For example, Duncan grapefruit (Fig. 1, trace B) and Ponkan mandarin (Fig. 1, trace D) would be classified as cultivars manifesting intermediate nomilin-limonin ratios, whereas Lisbon lemon (Fig. 1, trace A) and Valencia orange (Fig. 1, trace C) would be low ratio cultivars because the nomilin peak is relatively small in the latter two species (see also Table 1). Lemons and sweet oranges can be further differentiated by an extremely small deactylnomilin peak in lemons (trace A), whereas it is unusually large in the sweet oranges (trace C). *C. reticulata* and *C. paradisi* chromatograms can be most easily differentiated in that *C. reticulata* chromatograms usually contain several minor peaks that elute after limonin, whereas *C. paradisi* chromatograms do not show any peaks after limonin.

In order to develop a characteristic limonoid profile for each species, each limonoid is normalized to limonin. This is accomplished by dividing each limonoid concentration by the corresponding limonin concentration, and multiplying by 100. Thus, each value in the limonin column is 100. The average values shown in Table 2 will be used to define the limonoid pattern for each of the eight species. By using these data it can be seen that each species has a unique and characteristic limonoid concentration pattern that can be used to differentiate it from other citrus species.

Distinguishing characteristics of individual species

Since the ratio of nomilin–limonin varies considerably, citrus species can be divided into one of the three groups

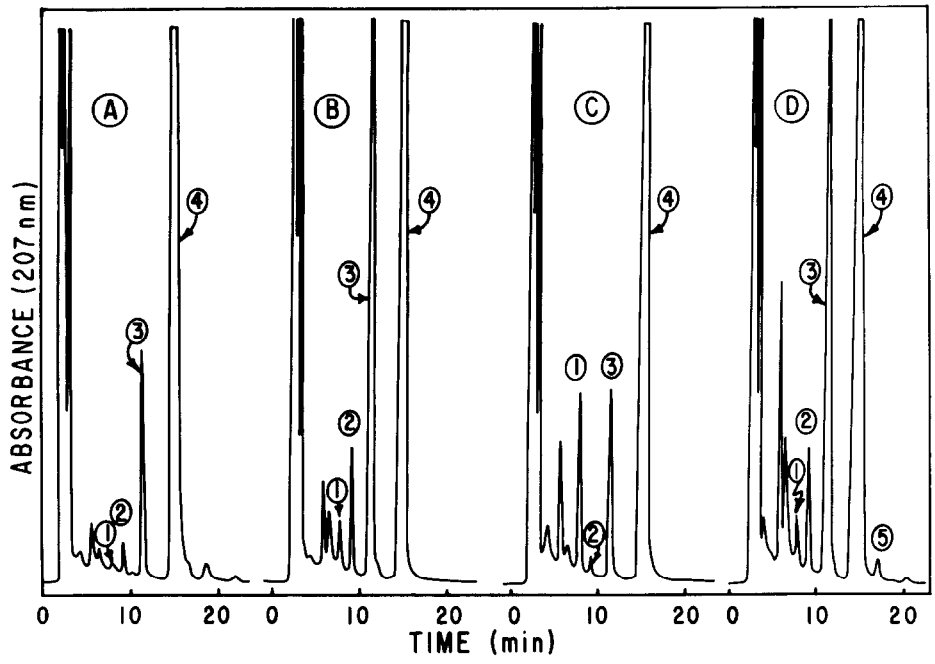

Fig. 1. Representative chromatograms of chloroform extracts from *C. limon*, *C. paradisi*, *C. sinensis* and *C. reticulata* seeds. A, Lisbon lemon; B, Duncan grapefruit; C, Valencia orange; D, Ponkan mandarin; 1, deacetylnomilin; 2, obacunone; 3, nomilin; 4, limonin; 5, deoxylimonin. Chromatographic conditions in text.

based on a high, intermediate or low nomilin–limonin ratio. The high group (1.1–0.9) consists of *C. medica* and *C. grandis*. Intermediate ratios (0.6–0.4) are characteristic of *C. paradisi* and *C. reticulata*. The low ratio group (less than 0.3) is the largest and consists of the four remaining species (Table 1).

C. medica *L.* Citrons are readily distinguished from other species because of their high nomilin–limonin ratio (due primarily to a low limonin content), unusually high proportion of obacunone and practically no deacetylnomilin. The normalized limonoid concentration profile is 1:59:101:100 (deacetylnomilin:obacunone:nomilin:limonin). This is clearly a unique concentration profile. Citrons also had an unusually low amount of total limonoids (Table 1) due to unusually low limonin concentrations. Seed limonin concentrations usually range from 1200 to 2200 ppm, however, the limonin concentration in *C. medica* is 585 ppm. *C. grandis* is the only other species with limonin concentrations less than 1200 ppm.

C. grandis (*L.*) *Osbeck.* Pummelo or shaddock is another species showing a high nomilin–limonin ratio. It has many unique morphological features in addition to an unusual limonoid concentration profile (2:5:92:100). The trace of deacetylnomilin (Table 1) cannot be accurately quantitated because of an unresolved interfering peak. Both pummelo and citrons have high nomilin–limonin ratios and little deacetylnomilin, but the

Table 2. Average limonoid concentration ratios

Species	Ratio (liminoid–limonin × 100)			
	Deacetylnomilin	Obacunone	Nomilin	Limonin
C. medica	1	59	101	100
*C. limon**	0.8	0.8	13	100
C. limettioides	24	1	25	100
C. aurantium	80†	8	19	100
C. sinensis	10	0.6	15	100
C. reticulata	3	2	44	100
C. grandis	2	5	92	100
C. paradisi‡	19	3	60	100
C. paradisi§	1	4	49	100

* Malta lemon not included.
† Contains ichangin.
‡ Averages of Duncan and Marsh only.
§ Averages of Mott and Royal only.

two can be readily distinguished because the percentage of obacunone in pummelo is only 2.2–0.3% whereas it is 21.4% in citron.

It is interesting to note that Barrett and Rhodes [6] consider both *C. medica* and *C. grandis* as true citrus species. However, except for obacunone, they have very similar concentration profiles and the total amount of limonoids is low in both. Both limonoid totals are low because of the unusually low limonin concentrations. This similarity suggests that the two species might be ancestrally linked.

C. paradisi *Macf.* Species status granted to grapefuit is a rather tenuous one. Given its uncertain parentage, limited history and close physical resemblances to the pummelo there are some [5] who question the species status of this citrus biotype. The work of Albach and Redman [7] support the concept that grapefruit is probably of hybrid origin with similarities with the pummelo. But while it may have many physical similarities with the pummelo, the limonoid contents and concentration profiles differ considerably. As a group, the total limonoid content of grapefruit is twice as great as pummelo (Table 1) and the distribution pattern is markedly different. There are about equal portions of limonin and nomilin in the pummelo, whereas in grapefruit there is approximately twice as much limonin as nomilin. Differences in the absolute concentrations of limonin and nomilin are even more striking. Grapefruit has two to four times as much limonin and twice as much nomilin as the pummelo.

Duncan and Marsh grapefruit are the principal commercial cultivars. The Duncan variety is much seedier than Marsh and has a lower limonin content. The total limonoid content of the Marsh grapefruit is about 63% greater than the Duncan and the greatest of any cultivar analysed. The limonin content of 'seedless' (low seed) cultivars is known to be greater than similar seedy cultivars. It has been proposed [1] that the seeds act as a limonin storage site for fruit. Thus, where just a few seeds exist the relative limonin concentration in each seed will be higher than if there were many seeds. This same trend appears to hold true for the other limonoids as well. However, it is interesting to note from Table 2 that even though the concentrations of limonoids is much greater the relative distribution of limonoids in the two cultivars is almost identical.

Mott and Royal grapefruit are representative of nonbitter grapefruit. They have been so designated because of the lack of the bitter naringin, the primary flavanone neohesperidoside in grapefruit and pummelo. Since their limonoid concentration pattern is slightly different from typical grapefruit they appear to constitute a group of uncharacterized biotypes in which flavour and the distinctive bitterness of grapefruit is lacking. The lack of bitterness and a flavour somewhat suggestive of the orange have caused some [8] to suggest that these cultivars are orange grapefruit hybrids. The lower nomilin–limonin ratio and lower proportions of limonin are what one might expect from the introduction of orange parentage. However, the concentration of obacunone would be expected to be considerably lower in a orange–grapefruit hybrid than in a typical grapefruit. There were no significant differences in the absolute concentrations of obacunone in any of the grapefruit cultivars and, if anything, the relative concentrations of obacunone were slightly greater in the nonbitter cultivars. In addition, the proportion and absolute concentrations of deacetylnomilin were considerably lower than in the Duncan and Marsh grapefruit or any of the sweet oranges. Therefore, the limonoid concentration profile would not support the suggestion that the nonbitter grapefruit are orange–grapefruit hybrids. In comparing the average limonoid concentration profile of the nonbitter grapefruit with those of the other species in Table 2, it appears that this group shares more in common with *C. reticulata* than with *C. sinensis*. Thus, the nonbitter grapefruit may be the results of a cross with *C. reticulata*.

C. reticulata *Blanco.* Mandarins (tangerines) have intermediate nomilin–limonin ratios. This ratio can be deceiving because the nomilin concentrations of *C. reticulata* (*ca* 900 ppm) is considerably greater than that found in such high momilin–limonin ratio species as *C. medica* (600 ppm). Some of the highest nomilin concentrations are found in *C. reticulata*. However, the nomilin–limonin ratio has only intermediate values because of unusually high concentrations of limonin as well.

This species has been crossed and backcrossed with many different cultivars, thus it is difficult to determine a true mandarin from appearances alone. Both Dancy tangerine and Ponkan mandarin are good representatives of *C. reticulata* because their origins are fairly well established.

Mandarins can be distinguished from other citrus species in a number of ways. As a group *C. reticulata* (and many of their hybrids) has the greatest absolute concentrations of nomilin, limonin and total limonoids (See Table 1). As seen in Table 2, their limonoid concentration pattern (3:2:44:100) is considerably different from all other species except for the nonbitter grapefruit. The amount of deacetylnomilin is highly variable in mandarins and the concentration of obacunone is similar to that of the nonbitter grapefruit. However, mandarins have greater absolute amounts of limonin and total limonoids that the nonbitter grapefruit. In addition, *C. reticulata* shows several minor peaks that elute after limonin (see Fig. 1), whereas no peaks can be seen after limonin for the nonbitter grapefruit. It is not known at this time if these late eluting peaks are minor limonoids.

C. limon (*L.*) *Burm. f.* Lemons generally have a nomilin–limonin ratio less than 0.2 and are one of the four species possessing low nomilin–limonin ratios. The Lisbon and Kusner lemons are good examples of this species. The Lisbon lemon is of Portuguese origin and is morphologically indistinguishable from the Kusner lemon of Russia [6]. The distribution of the major limonoids in these two cultivars is almost identical.

This species has a limonoid concentration pattern of 0.8:0.8:13:100. Therefore, *C. limon* can be distinguished from any of the other three low nomilin limonin ratio species by its unusually low concentrations of both deacetylnomilin and obacunone. No other species in Table 2 has a similar distribution of these seed limonoids.

The limonoid concentration profile of the Malta lemon from northern India is considerably different from the other two lemon cultivars. The differences are so profound that it is doubtful whether this cultivar can be classed as *C. limon*. Not only was the total limonoid content 37% lower but it had over twice as much nomilin and only about one-third as much limonin as the other two cultivars. The unusually high nomilin limonin ratio (1.05, Table 1)

suggests similarities to the citron and pummelo. Recent studies by Malik *et al.* [9] and by Barrett and Rhodes [6] suggest that the lemon is a unique, introgressed trihybrid of lime, citron and unidentified parentage. We speculate that because of the distribution and total amount of limonoids, and the nomilin–limonin ratio (Table 1), Malta lemon appears to contain a high proportion of citron genes. The unusually high concentration of obacunone is another strong argument in favor of citron parentage as citrons contain a high proportion of this limonoid, whereas the pummelo contains a rather low level.

C. limettioides *Tan.* Sweet lime is another species with a low nomilin–limonin ratio. It is represented by the Columbia sweet lime which is the best known clonal selection of the Indian sweet lime. However, while Tanaka has given *C. limettioides* species status neither Swingle [5] nor Barrett and Rhodes [6] concur. This *Citrus* biotype probably arose from a cross of *C. aurantiofolia* (acid lime) by *C. sinensis* (sweet orange) [6].

The normalized limonoid pattern of the sweet lime (24:1:25:100) resembles that of the sweet orange (10:0.6:15:100) in that both have about equal amounts of deacetylnomilin and nomilin and very little obacunone. Whereas limonoid patterns of the two species may be similar, chromatograms of sweet limes are readily distinguished from those of sweet oranges by the presence of minor peaks that elute after limonin. These late eluting peaks are totally absent in the sweet orange.

The limonoid pattern of sweet limes is distinctly different from most of the sweet orange cultivars in Table 1 in that the percentage of deacetylnomilin is appreciably greater, the percentage of limonin is distinctly lower and the nomilin–limonin ratio is higher. However, the limonoid pattern of the Jaffa orange is very similar to that of the Columbia sweet lime. Not only is the distribution of the limonoids almost identical but they also have the same amount of total limonoids. The closeness of both the qualitative and quantitative data suggest that these two cultivars may share a common genetic ancestor since the limonoid pattern of the Jaffa orange is considerably different from the other sweet oranges.

C. aurantium *L.* Of the eight species shown in Table 2, sour oranges have one of the most distinct limonoid concentration profiles (80:9:19:100). The most distinctive feature of this profile is the unusually high proportion of deacetylnomilin. *C. aurantium* has about twice the absolute concentration (Table 1) and three times the relative proportion (Table 2) of deacetylnomilin as the next most concentrated species. Another highly distinctive feature of sour orange seeds is that they contain ichangin (an uncommon limonoid). It should be pointed out that the retention times of ichangin and deacetylnomilin are very similar and although they may be distinguished as separate peaks, they are difficult to quantitate accurately. Therefore the deacetylnomilin concentration in Table 1 represents the sum of both limonoids. However, this does not represent a serious error as the peak height of deacetylnomilin is *ca* 10 times greater than that of ichangin, and they both have similar absorptivities at 207 nm.

Sour oranges have low nomilin–limonin ratios (0.19) due to distinctively low concentrations of nomilin (240 ppm). Only a few lemon and sweet orange cultivars have similarly low nomilin concentrations (see Table 1). Sour orange seeds also have unusually high concentrations of obacunone (102 ppm). Only *C. medica* has a greater obacunone concentration. Therefore sour orange seeds may be characterized by the presence of ichangin, distinctively high deacetylnomilin and obacunone concentrations, unusually low nomilin concentrations and low nomilin–limonin ratios.

C. sinensis (*L.*) *Osbeck.* Sweet oranges are the dominant citrus fruit in the world. Many cultivars have been developed for specific climatic conditions; thus, *C. sinensis* represents a rather diverse group. Of the four cultivars evaluated in this study the Valencia, Queen and Jaffa are common (blond) sweet oranges, whereas the Ruby blood orange is a pigmented sweet orange. Their limonoid concentration profiles are remarkably similar for such a divergent group. They can be differentiated from other citrus species by their high proportion of limonin, their low nomilin–limonin ratio, their relatively high concentration of deacetylnomilin and by their total lack of minor chromatographic peaks of greater polarity than limonin (see Tables 1 and 2 and Fig. 1).

There is surprisingly little difference between the limonoid concentration profiles of the pigmented and non-pigmented varieties. The greatest discrepancy within *C. sinensis* was with the Jaffa orange which had much higher amounts of deacetylnomilin (471 ppm compared to 139–178 ppm for the other three cultivars). Jaffa orange seeds also contained slightly higher concentrations of nomilin, and since the concentration of limonin was not appreciably different, it also had a higher nomilin–limonin ratio (0.25 vs 0.11–0.19). Other limonoids such as obacunone and limonin fit the general *C. sinensis* pattern very closely. The limonoid concentration profile of the Jaffa orange more nearly matches that of the Columbia sweet lime than the other three cultivars of *C. sinensis*. This strongly suggests that these two cultivars may be related. Furthermore, the unusually high concentration of deacetylnomilin suggests that the Jaffa orange may be the result of a cross with some high deacetylnomilin cultivar such as the common sour orange.

Seasonal, maturity and cultural variables

Preliminary data from two seasons have shown absolute and total limonoid concentrations will vary from season to season. However, for the species evaluated, the relative concentrations of seed limonoids in mature fruit remain surprisingly consistent. Fruit maturity may affect limonoid concentration ratios. Seeds from highly immature and over-mature fruit can have different limonoid concentration profiles from that of legally mature fruit. However, once the fruit reaches legal maturity there is relatively little change in limonoid concentration ratios. The changes in limonoid concentrations which occur during the time fruit is legally mature is small compared to the large differences in limonoid concentration profiles which exist between the species. Cultural variables, such as rootstock selection, do not appear to have a significant effect on seed limonoid concentration ratios. However, only two species (*C. sinensis* and *C. paradisi*) were evaluated on two different rootstocks in this study.

EXPERIMENTAL

Materials. High-purity Li Chrosolv (E. Merck, Darmstadt, Germany) grade heptane, *iso*PrOH and MeOH were used to prepare the chromatographic mobile phase. Li Chrosolv grade

hexane and $CHCl_3$ were used in extracting limonoids from the seeds.

Standard limonin was obtained from Dr. James Fisher, Florida Department of Citrus, Lake Alfred, FL. It had been extracted from ground, defatted grapefruit seeds by a method similar to that used by Emerson [10] and purified by repeatedly redissolving it in CH_2Cl_2, final mp 295–299° (with decomp.). Purified nomilin, deoxylimonin and obacunone were obtained from Dr. Shin Hasegawa, Western Regional USDA Laboratory, Pasadena, CA. Chromatographic analysis of these standards indicated that they could be used without further purification. Samples of deacetylnomilin and ichangin were supplied by Dr. Ray Bennett also of the Western Regional USDA Laboratory. Each limonoid standard was dissolved directly into MeCN and then diluted with mobile phase as they did not readily dissolve directly into the mobile phase. All standards were refrigerated when not in use.

Apparatus. The HPLC system consisted of a Waters Associates (Milford, MA) M-6000 pump with a WISP programmable sample injector. A Tracor (Austin, TX) model 970A variable wavelength UV–VIS spectrophotometer equipped with an 8-μl flow cell was used for the detection of limonoids. The wavelength was set at 207 nm, the UV absorption maximum for limonin. Chromatograms were recorded and peak areas determined by integration using a Spectra-Physics (Santa Clara, CA) SP-4000 chromatographic data system. Samples were redissolved and solvents degassed with the aid of a Bronson (Shelton, CT) model B 220 ultrasonic bath. A DuPont (Wilmington, DE) Sorvall grinder was used to grind the seeds.

Chromatographic conditions. Citrus limonoids were separated using the procedure of Rouseff [4]. Mobile phase, heptane–*iso*-PrOH–MeOH (1:12:2) at 1.0 ml/min; Zorbax CN column (25 cm × 4.6 mm) (Dupont) heated at 40°; typical column head pressures under these conditions were 54 kg/cm^2. Solvents were thoroughly degassed by applying aspirator vacuum and sonication for *ca* 3–5 min.

Sample preparation. Air-dried citrus seeds (20 g) were ground in 100 ml hexane at high speed for 5 min. The ground seed–hexane slurry was placed into a filter paper thimble set in a Soxhlet apparatus. An additional 100 ml hexane was added and the seeds were extracted for 20 min after the hexane began to boil. The hexane extract was reduced in vol. (rotary evaporator) and quantitatively transferred to a 5-ml volumetric flask. Mobile phase solvent was used to rinse the round-bottomed flask and dilute to vol. in the volumetric flask. After mixing, 2–4 ml of the hexane extract was filtered with a 1.2 μm filter and stored in a septum sealed vial.

Seed residues were then extracted in the same Soxhlet with *ca* 300 ml $CHCl_3$ for 40 min after boiling began. After cooling the $CHCl_3$ extract was reduced to dryness using a rotary evaporator and redissolved in exactly 5.00 ml MeCN. 2 ml MeCN soln was pipetted into a 5-ml volumetric flask and diluted with mobile phase. After mixing, 2–4 ml of the soln was filtered with a 1.2-μm filter and stored in a septum sealed vial.

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