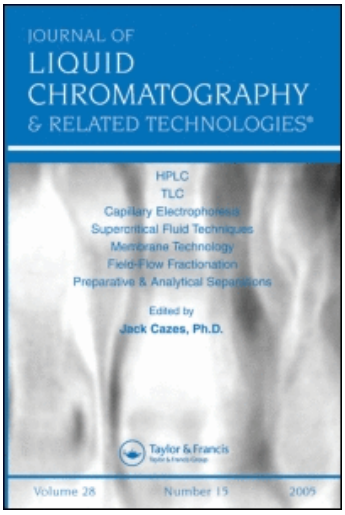

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STUDY AND OPTIMIZATION OF CITRUS FLAVANONE AND FLAVONES ELUCIDATION BY REVERSE PHASE HPLC WITH SEVERAL MOBILE PHASES: INFLUENCE OF THE STRUCTURAL CHARACTERISTICS

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ABSTRACT

A high-performance liquid chromatographic procedure for the analysis of the different flavonoids found in *Citrus aurantium* tissue extracts has been developed. It employs a C_{18} reverse phase column and an elution isocratic-gradient system with a mixture of water, methanol, acetonitrile and acetic acid. We make an exhaustive description of the optimisation process by studying the quantitative chromatographic parameters: k', w, α, N, *HETP* and R. The use of different extraction solvents for flavonoids in *Citrus aurantium* tissues was also studied, DMSO presenting the best results. Twelve compounds were detected, isolated and their spectral structures characterized.

INTRODUCTION

Citrus species are of great interest because they accumulate large amounts of flavanone glycosides in fruit and young vegetative tissues [1, 2, 3], whose aglycones are early intermediates in the flavonoid biosynthetic pathway [4]. Naringenin and hesperetin are widely found in *Citrus* species in their glycoside forms: naringin, neohesperidin (neohesperidosides), narirutin and hesperidin (rutinosides) [5, 6, 7] being the most common. *Citrus aurantium* is characterized by the accumulation of these four flavanones [5, 6, 8]. We recently demonstrated and quantified the glucosides of the flavanones naringin and hesperetin, prunin and hesperetin 7-*O*-glucoside, respectively in the same natural source (in press). The presence of the flavanones, neoeriocitrin [9] and poncirin [10], and the flavones rhoifolin [11] and neodiosmin [12] has also been described.

In recent years, high pressure liquid chromatography (HPLC) has become the method of choice for the separation of diverse natural flavonoids mixtures [13, 14, 15]. However, only very specific methods have been described for particular extracts of certaines plant materials, in which such a high number of flavonoids does not occur simultaneously.

The present work has three objectives: (I) to optimize the analytical technique used for the elucidation of all the above mentionated flavonoids. (II) To characterize the most suitable agent for the extraction of flavonoids from the plant material. (III) To isolate and analyse the flavonoids found in the variety of *Citrus aurantium* studied by using semipreparative liquid chromatography and to identify them by spectroscopic techniques and their quantification.

MATERIALS AND METHODS

Plant Material

Young leaves (10-130 mm in length) and immature fruits (3-61 mm diameter) were obtained from 5-year-old *Citrus aurantium* cv Sevillano trees, grown in greenhouses of the University of Murcia.

Chemicals

Naringin, hesperidin, prunin, hesperetin 7-*O*-glucóside, neohesperidin, neodiosmin, naringenin and hesperetin were obtained from Zoster S.A., Murcia, Spain. Neoeriocitrin, narirutin, rhoifolin and poncirin were obtained from Extrasynthese S.A, Genay, France. The structures of these flavonoids are shown in Table 1. Dimethylsulphoxide (DMSO) was used as solvent.

Extraction of Flavonoids

Leaves and fruits were collected, immediately dried at 50°C [16] and ground. The flavonoids were extracted with several solvents at different concentrations in order to establish the most suitable. The solvents used were: methanol, dioxane-methanol (1:1), 0.01 % sodium hidroxide (aq), 0.1 and 0.01 % potassium hidroxide (methanolic solution), pyridin, dimethylformamide and dimethylsulphoxide. The plant materials were extracted in three differents ratios: 2 mg/ml, 20 mg/ml and 200 mg/ml. The solutions were filtered through a 0.45 μm nylon membrane.

Chromatographic Analysis

For the elucidation of the flavonoids present in the *Citrus aurantium* leaf and fruit, we used a μBondapak C_{18} (250 x 4 mm ID) analytical column with an average particle size of 5 μm. The flow rate was 1 ml/min at room temperature.

HPLC analysis was performed using a Beckman liquid chromatograph with a Model 110B solvent-delivery module and a System Gold Module 168 diode array detector (Beckman Instruments, Inc, CA., USA). The absorbance change was monitored at 280 nm.

In order to carry out a quantitative study, the elucidation capacity of the different mobile phases was verified by determining the following parameters [17] for each flavonoid:

a) Retention time (t_{Ri}= experimental value)

b) Capacity factor $k' = (t_{Ri} - t_o)/ t_o$

t_o= mobile phase interstitial volume (void volume)/flow rate.

c) Selectivity factor $\alpha = (t_{Ri+1} - t_o)/(t_{Ri} - t_o)$

d) *Number of theoretical plates*: $N = 16(t_{Ri}/ w_i)^2$

w= width of the peak.

e) The *height equivalent to a theoretical plate (HETP)* $HETP = L/ N$

L= column length (mm).

f) Resolution

$$R = 1/4(N)^{1/2}((\alpha-1)/\alpha)(k'/(k'+1))$$

Isolation of Flavonoids

For the isolation of different flavonoids found in DMSO extracts of *Citrus aurantium* leaves and fruits the following semipreparative column was used: Nucleosil C_{18}, 5 μm (250 x 10 mm ID), eluted with water-acetic acid-methanol (75-0.5-25) and a flow rate of 3 ml/min. All flavonoids whose retention times were similar to the flavonoid standard were isolated by several chromatographs in this semipreparative column, and the

fractions were collected with a Pharmacia FRAC 100 (Pharmacia LKB Biotechnology, Uppsala, Sweden).

All the compounds isolated were identified by their melting points (Gallemkamp, England) and their ^{1}H NMR (200 MHz) and ^{13}C NMR (50 MHz) spectra (Bruker, Bremen, Germany) in hexadeutero dimethylsulphoxide (DMSO-d_6).

RESULTS AND DISCUSSION

Chromatographic Analysis Optimisation. A Structural Study of Flavonoid Elucidation.

HPLC elucidation of the flavonoids contained in the standard mixture (see Table 1) was optimized in a first step by using isocratic systems in which only the proportions of the components of the mobile phase were varied.

Mobile Phases with Water and Methanol.

This has traditionally been one of the most commonly used combinations for flavonoid elucidation [18, 19, 20].

An analysis of the variation in the k' values for the different flavonoids according to the percentage of methanol in the mobile phase reveals the influence of their structures on their separation capacity (Table 2). Three structural aspects can first be considered: a) glycosilation in position 7; b) the B-ring substitution pattern maintaining a similar type of 7-glycosilation and c) the relation between the flavanone structures and their corresponding flavones. In principle, it seems that any direct influence of the molecular weight on the order of elucidation can be discarded (Table 2).

a) Figure 1 shows variations in ln k' according to variations in the percentage of methanol in the mobile phase for the flavonoids hesperidin, neohesperidin and hesperetin 7-*O*-glucoside. An analysis of the data

TABLE 1

Flavonoid Structures in Standard Mixture.

	Radical Positions			
Flavonoids	5	7	3'	4'
Flavanones				
Neoeriocitrin	OH	O-NEO[a]	OH	OH
Naringin	OH	O-NEO[b]		OH
Narirutin	OH	O-RUT[b]		OH
Hesperidin	OH	O-RUT	OH	OCH_3
Neohesperidin	OH	O-NEO	OH	OCH_3
Poncirin	OH	O-NEO[b]		OCH_3
Prunin	OH	O-GLU[c]		OH
Hespt.7-*O*-glu.	OH	O-GLU	OH	OCH_3
Naringenin	OH	OH		OH
Hesperetin	OH	OH	OH	OCH_3
Flavones (double bond $C_2 = C_3$)				
Rhoifolin	OH	O-NEO		OH
Neodiosmin	OH	O-NEO	OH	OCH_3

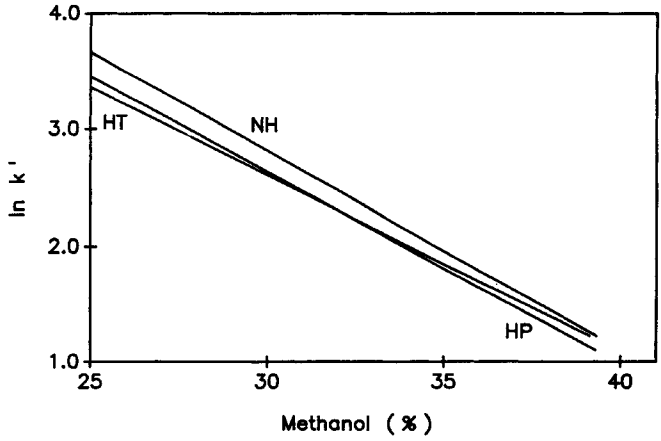

FIGURE 1. Variation of ln k' versus methanol percentage of mobile phases with 0.01M phosphoric acid-methanol. NH: Neohesperidin; HT: Hesperetin 7-*O*-glucoside; HP: Hesperidin.

TABLE 2

k' Values for Flavonoids of Standard Mixture Elucidated with Mobile Phases: Methanol-0.01 M Phosphoric acid.

MeOH (%)[a]	50	40	35	30	25
Flavonoid			k' values		
Neoeriocitrin	0.2	1.3	2.5	5.1	14.4
Prunin	0.8	2.3	4.1	7.8	20.3
Narirutin	0.8	2.1	4.1	7.8	22.0
Naringin	0.8	2.3	4.5	9.6	27.3
Hesp.7-*O*-glu.	1.4	3.4	5.5	12.0	32.8
Hesperidin	1.2	3.0	5.5	12.0	36.2
Neohesperidin	1.4	3.4	6.4	14.4	44.7
Rhoifolin	2.2	5.3	8.2	19.0	57.6
Neodiosmin	2.2	7.5	10.8	27.2	88.5
Naringenin	2.7	9.6	16.7	31.4	77.6
Poncirin	2.2	9.6	19.6	44.7	135.8
Hesperetin	3.4	13.4	23.1	47.1	124.3

[a]Methanol percentage of mobile phase.

corresponding to the flavonoids isonaringin (narirutin), naringin and naringenin 7-*O*-glucoside (prunin) shows similar results to those represented in this figure.

The glycosilation of a 7-OH group produces a much greater hydrophilic interaction with the mobile phase solvent, as has been described by other authors [21]. The hydrophilic character of the flavonoid molecule increases with the number of sugars in the side chair [22], however, it must be borne in mind that this hydrophilic character is not only marked by the number of sugars in the side chain but also by their nature. Thus, for a particular flavonoid k'-glucoside < k'-arabinoside < k'-rhamnoside [22].

TABLE 3

Variation of k' Values for 7-*O*-glycosides of Naringenin and Hesperetin with Reference to Methanol Percentage Changes in the Mobile Phase.

Methanol (%)	k' values
50-40	RUT[a] = GLU[b] = NEOH[c]
40-35	RUT < GLU = NEOH
35-25	RUT = GLU < NEOH
< 25	GLU < RUT < NEOH

[a] Rutinoside; [b]glucoside; [c]neohesperidoside

Table 3 shows how the k' value of glucosylated structures undergoes a greater relative change than the corresponding rhamnoglucosides. From Figure 1, it can be seen that hesperidin and neohesperidin show a similar decrease of their k' values as the percentage of methanol in the mobile phase increases. The k' of the rutinosides is always below that of their corresponding neohesperidosides. This clearly contradicts the experimental data obtained when the solubility of these compounds was analysed, as shown in Table 4, where it can be seen that the solubility of neohesperidin is always greater than that of hesperidin in all the mobile phases tested. It seems likely, then, that the cause of this alteration in the k' values of both glycosylated flavanones has a structural origin, which particulary affects the reverse phase-flavonoid interaction. The flavanones are stabilized by the formation of an intramolecular hydrogen bond between the *per*-hydroxyl group of C-5 and oxigen of 4-keto group [23]. This speacial structure between the A and C rings is planar except in the C_2- C_3 bond [24]. The

TABLE 4

Hesperidin and Neohesperidin Solubility (gr/l and Room Temperature) in Chromatographic Mobile Phases with Phosphoric Acid and Methanol.

Methanol (%)	Hesperidin	Neohesperidin
25	0.010	0.575
30	0.011	0.754
35	0.013	0.992
40	0.016	1.123
50	0.023	1.680

substitution of the hydroxyl group in position 7 by a glycosidic group (rhamnoglucoside) might affect this configuration. Given its special "lineal" type configuration (rhamnose-glucose bond type 1-6), the presence of a rutinoside type substitution would not affect the mentioned intramolecular hydrogen bond, thus maintaining the highly conjugated planar system of the aglycon portion of the flavanone [25]. However, the presence of 7-O-neohesperidoside substitution (rhamnose-glucose bond type 1-2) would affect the stability of the intramolecular hydrogen bond, and the neohesperidoside structures can be non planar to rutinosides [24]. These would certainly be a greater hydrophobic interaction between the neohesperidoside molecules and those of the stationary phase of the column which, in spite of the enormous difference in the solubility of the phases studied, would increase the k' value of these compounds compared with their respective rutinosides.

b) The different substitution in the B ring of these flavonoid structures strongly affects the order

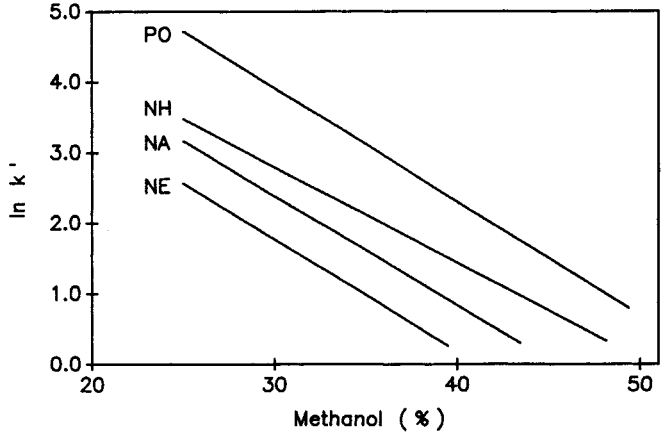

FIGURE 2. Variation of ln k' versus methanol percentage of mobile phases with 0.01M phosphoric acid-methanol. PO: Poncirin; NH: Neohesperidin; NA: Naringin; NE: Neoeriocitrin.

of these compounds elucidation. In Figure 2, it can be seen how this order and the consequent ln k' values are basically affected by the degree of polarity of these flavonoids. The enormous influence on the order of the chromatographic resolution, of the number of hydroxyl groups in the B ring of its structure is clear. It is interesting to note how the addition of a methoxyl group to the neoeriocitrin molecule (neohesperidin) decreases the polarity of the flavonoid. The influence of this effect is increased when the hydroxyl radical in the monohydroxylated molecule (naringin) is replaced by a methoxyl radical (poncirin).

c) The comparison between the flavanones naringin and neohesperidin with their flavones, rhoifolin and neodiosmin, respectively, reveals the effect of insaturation between positions 2 and 3 upon elution behavior. The flavones eluted much later than

respective flavanones, and their k' values are higher (see Table 2). One factor operating to make flavones much less polar than respective flavanones is a larger electron density on the oxigen atom of the 4-keto group of flavones resulting from resonance structures where the keto oxigen assumes a negative charge [21].

The larger electron density will make the hydrogen bond between the 5-hydroxyl group and the 4-keto group stronger and make both funcional groups appear less polar to the solvent. However, this may not be the only possible explanation for the greater polarity of flavanones over flavones. In flavanones, the 4-keto group may be out of the plane of the adjacent phloroglucinol ring, thus making a hydrogen bond with the *peri*-hydroxyl group weaker and exposing both functionalities to stronger interactions with the solvent. Flavones because of their total planarity, simply may be more difficult to solvate than the partially planar flavanones. The planar flavones would require a more ordered solvent structure and thus a larger entropy term in solvation than would flavanones [21].

Figure 3 shows ln k' versus variations in the methanol percentage of the mobile phase. A decrease in the k' value with increases in the methanol percentage results in a similar slope for the flavanones and their respective flavones.

Acid must be present in the mobile phase for reverse phase HPLC, and it has been included for sound reasons, such as the suppression of ionisable acidic groups, to decrease the tailing of peaks or for unspecified improvements in separation [21, 26].

In this paper we haved tested the influence of the degree of acidification of the mobile phase by observing variations of k' and w values in all the

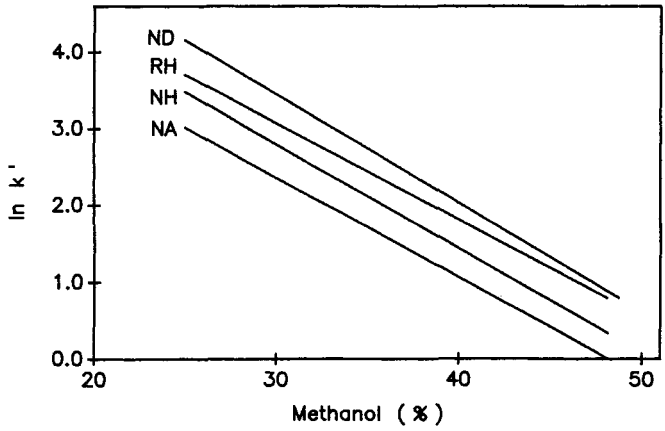

FIGURE 3. Variation of ln k' versus methanol percentage of mobile phases with 0.01M phosphoric acid-methanol. ND: Neodiosmin; RH: Rhoifolin; NH: Neohesperidin; NA: Naringin.

flavonoid studied (Table 5). Best results were obtained with 0.5 % acetic acid in the mobile phase (the pH of aqueous fraction was 2.46) as against the use of 0.01 M phosphoric acid (pH solution is 3.24). For this reason, acetic acid was used in subsequent experiments with new ternary and quaternary phases. Using acetic acid at concentrations in excess of 0.5 %, did not result in a better elucidation of the compounds under study and even produced new overlaps. In addition, the stability of silica-based bonded phase packings towards most solvents in the pH 2.0-7.5 range. Below pH 2.0, the silicon-carbon bond of the stationary phase is subject to nucleophilic attack [27].

Finally, the total resolution of all the flavonoids contained in the standard mixture was only attained in the analytical conditions studied when the percentage of methanol in the mobile phase was equal or less than

TABLE 5

k' and w Values of the Flavonoid Standard Mixture Elucidated with Mobile Phases containing 30 % of Methanol and different Acid Agents.

Mobile Phase	A[a]		B[b]		C[c]	
Flavonoid	k'	w	k'	w	k'	w
Neoeriocitrin	6.0	2.1	5.1	1.1	5.0	1.1
Prunin	8.2	2.1	7.8	1.8	7.8	1.8
Narirutin	8.2	2.1	7.8	1.1	7.8	1.8
Naringin	12.4	2.5	9.6	2.2	9.4	2.2
Hespt.7-*O*-glu.	12.6	3.1	12.0	2.9	11.8	2.3
Hesperidin	12.6	3.1	12.0	2.9	11.8	2.3
Neohesperidin	15.2	3.1	14.4	2.9	14.2	2.5
Rhoifolin	20.8	4.5	19.0	4.0	18.0	3.6
Neodiosmin	30.6	6.5	27.2	5.0	26.1	5.0
Naringenin	33.7	7.5	31.4	6.5	32.3	5.4
Poncirin	46.5	10.2	44.7	7.6	44.2	7.9
Hesperetin	49.6	12.3	47.1	10.4	47.0	7.9

[a]Methanol-water (30:70); [b]Methanol-0.01 M phosphoric acid (30:70); [c]Methanol-water-acetic acid (30:70:0.5).

25%. The high k' value for most of the flavonoids elucidated means that this technique needs further optimisation. However it does represent a valuable aid to semipreparative and preparative chromatography in the isolation of this type of flavonoids, because it adequately separates the different substances present and permits the easy and correct programming of the fraction collection systems.

Mobile Phases with Water and Acetonitrile.

Mobile phases containing acetonitrile have been widely used in the HPLC elucidation of flavonoid compounds of low polarity and high molecular weight,

such as flavone, flavanone aglycons and a large number of polymethoxylated flavonoid structures [20, 28, 29].

Using this type of mobile phase to elucidate the standard mixture considerably reduces the retention times of all the flavonoids contained in it, which produces a simultaneous overlapping of many and impedes accurate elucidation. This overlapping particularly affects the flavanone-glycosides and some of these are not resolved until the percentage of acetonitrile in the mobile phase drops below 25 %. Of note are the different results obtained for the separation of the 7-*O*-glycosylated derivatives of hesperetin and naringenin. In the former case, the flavonoids are not resolved, while in the case of naringenin, the three 7-*O*-derivatives are resolved. This must be related with the lower degree of polarity of the 3'-hydroxylated-4'-methoxylated B ring structures compared with those which are merely 4'-hydroxylated, since the former are more affected as regard, the diminution in k' values as the proportion of acetonitrile in the mobile phase increases.

When the percentage of acetonitrile in the mobile phase falls below 20 %, the glycosylated flavanones are better resolved, although still never completely, and the other chromatographic parameters are adversely affected. In particular w and k' values increase, which produces excessively high flavone and aglycone retention times. When the acetonitrile content of the mobile phase is 10 %, neoeriocitrin shows a retention time of 64 minutes, which explains why scant viability of this method.

The use of mobile phases containing acetonitrile does provide useful information for the elucidation of this and other flavonoid mixtures of a greater or

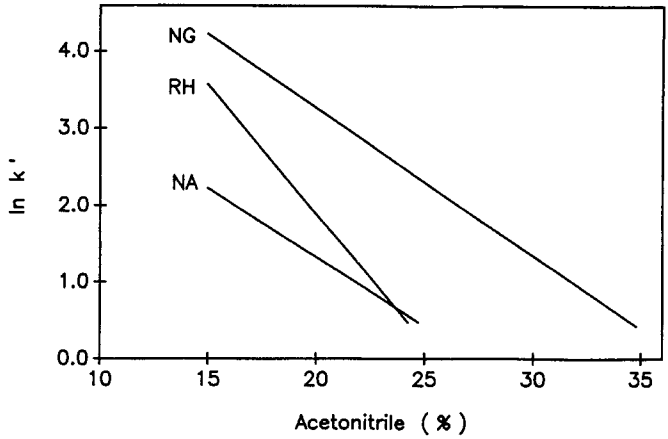

FIGURE 4. Variation of ln k' versus acetonitrile percentage of mobile phases with water-acetonitrile-acetic acid. NG: Naringenin; RH: Rhoifolin; NA: Naringin.

lesser complexity. In the first place there is a general decrease in the k' values of all the flavonoids when compared to phases containing a similar proportion of methanol. This pattern of behaviour is not the same for all the flavonoids, since the flavones, compounds with a very low polarity, show a much more pronounced decrease in k' than do the flavanone aglycones and 7-*O*-glycosides of flavanones, which both show a similar behaviour (Fig. 4). This general fall in k' values, the most pronounced characteristic of the use of these mobile phases, generally hinders the correct elucidation of the most polar flavonoids, glucosides and rhamnoglucosides although it does permit the good resolution of flavanone aglycones and the slightly polar flavanone poncirin, especially within the 20-25 % acetonitrile range. The considerable reduction in the w values of all flavonoids analysed is also of not.

Mobil Phases with Water, Methanol and Acetonitrile.

In order to attain a better elucidation of the different flavonoids studied by optimising the analytical parameters mentionated, mobile phases containing water, acetic acid, methanol and acetonitrile were used. Because of the strong influence of acetonitrile on the resolution of these compounds, increasing quantities of this solvent were introduced into the mobile phases containing water and methanol at the expense of the methanol proportion. This was does until the proportion of both in the mobile phase was identical, the water and acetic acid proportions remaining unaltered.

The mobile phases with less than 70 % water did not improve the elucidation of these compounds, despite the substitution of methanol by acetonitrile.

An analysis of the ln k' values against variations in acetonitrile, shows that the resolutions obtained with 70 and 75 % water both permit similar structural interpretations. For this reason, Figures 5, 6 and 7 depict variations in ln k' against increases in the proportion of acetonitrile with 70 % water in the mobile phase. The parameters providing most information for the elucidation of these compounds, k' and R, are shown in Table 6.

Figure 5 shows that, similar to that which occurred in phases containing only methanol and water, the k' values for a neohesperidoside are always higher than their rutinoside counterpart, whatever the composition of the mobile phase. This increases the influence of the esterospacial structure on the order of elucidation of these compounds compared with their solubility in this phases, which in phases with a light acetonitrile content favour the neohesperidosides. Increases in the percentage of acetonitrile affects rutinoside

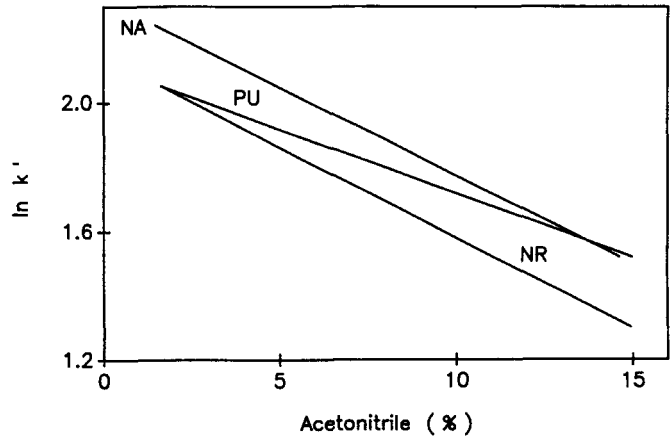

FIGURE 5. Variation of ln k' versus acetonitrile percentage of mobile phases: water-acetonitrile-methanol-acetic ac. NA: Naringin; PU: Prunin; NR: Narirutin.

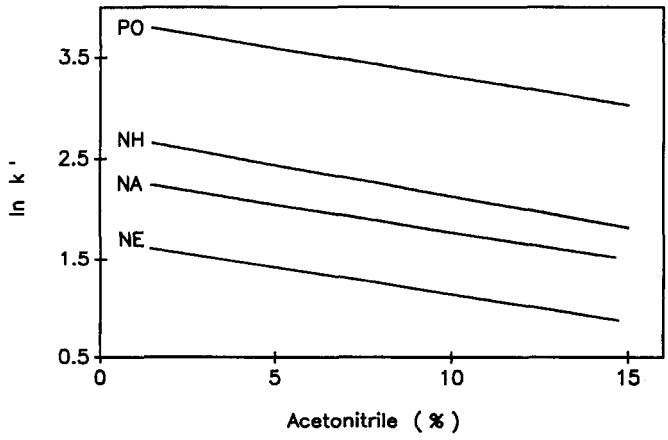

FIGURE 6. Variation of ln k' versus acetonitrile percentage of mobile phases:water-acetonitrile-methanol-acetic ac. PO: Poncirin; NH: Neohesperidin; NA: Naringin; NE: Neoeriocitrin.

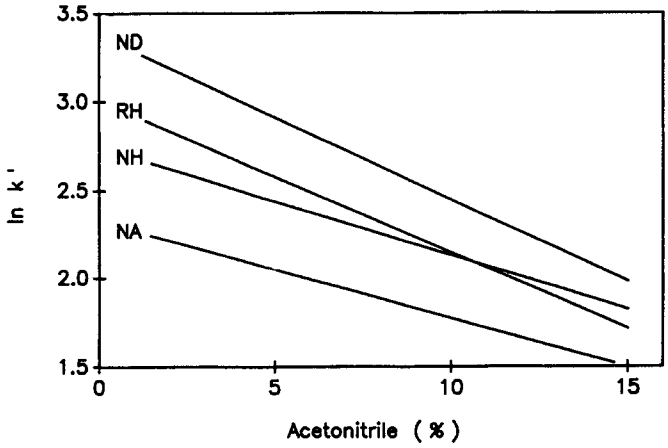

FIGURE 7. Variation of ln k' versus acetonitrile percentage of mobile phases: water-acetonitrile-methanol-acetic ac. ND: Neodiosmin; RH: Rhoifolin; NH: Neohesperidin; NA: Naringin.

TABLE 6

k' and R Values of the Flavonoid Standard Mixture Elucidated with Quaternary Mobile Phase containing 70 % Water.

Mobile Phase	A[a]		B[b]		C[d]	
Flavonoid	k'	R	k'	R	k'	R
Neoeriocitrin	4.9	3.7	2.8	2.8	2.4	2.8
Prunin	7.8	1.1	5.2	0.0	4.6	0.0
Narirutin	7.8	0.0	4.4	1.3	3.7	2.1
Naringin	9.3	1.9	5.2	1.1	4.6	0.7
Hespt.7-*O*-glu	11.2	1.5	7.9	0.0	6.1	0.0
Hesperidin	11.2	0.0	6.4	1.7	5.1	0.6
Neohesperidin	13.6	1.9	7.9	0.0	6.1	1.4
Rhoifolin	16.3	3.1	7.9	1.5	5.5	0.8
Neodiosmin	22.6	4.0	10.7	5.3	7.1	6.5
Naringenin	33.6	2.9	23.0	1.0	20.5	2.2
Poncirin	43.2	1.2	25.4	1.8	20.5	0.0
Hesperetin	46.6	1.2	32.1	1.2	27.7	2.2

[a]water-acetic acid-methanol-acetonitrile:(70:0.5:25:5).
[b]water-acetic acid-methanol-acetonitrile:(70:0.5:20:10).
[c]water-acetic acid-methanol-acetonitrile:(70:0.5:15:15).

(narirutin) and neohesperidoside (naringin) equally, while glucoside (prunin) shows a more pronounced decrease as the polarity of the mobile phase falls. In the 2-14 % range of acetonitrile concentration, prunin at first shows the lowest k' value and then the highest. This shows once again that the most esterospacial polar flavanones are less influenced as regards their decrease in k' values by increases in the proportion of acetonitrile.

Figure 6 confirms the close relationship between the k' value and the flavonoid polarity due to the B-ring substitution pattern, with an identical 7-O-glycosilation type. The k' values are inversely proportional to the polarity of the flavonoids elucidated. Variations in these values show similar slopes for the four neohesperidosides represented, which confirms that the B-ring substitution pattern does not modify the relationship between the k' values, whatever the mobile phase over the ranges studied.

Figure 7 illustrates how the substitution of methanol by acetonitrile in the mobile phase brings about a sharper drop in the k' value of the flavones than of their corresponding flavanones as a direct consequence of the decrease in polarity of the mobile phase. Thus, when the proportion of acetonitrile reaches or surpasses 11 %, rhoifolin, shows a k' value lower than that of neohesperidin. Such effects as this must he borne in mind when choosing solvent mixtures to elucidate extracts or solutions, whether known or not, of flavonoids and for the their isolation and identification.

Isocratic-Gradient Quaternary System.

From the results obtained in the analysis of the different mobile phases used in the optimisation of the

simultaneous elucidation of these twelve compounds, it is possible to design a system which permits the complete resolution of all the flavonoids found in the standard mixture in a much shorter time than hitherto possible, at the same time allowing the location and identification of these flavonoids in *Citrus aurantium* extracts.

Since experiments carried out with continuous lineal gradient systems, some of which are mentioned in the bibliography [13], did not permit the complete elucidation of all the flavonoids analysed, the system finally used was a combination of two isocratic regime periods and one intermediate lineal-gradient step: 1) water-acetic acid-methanol-acetonitrile (70:0.5:23:7) for 40 minutes, 2) lineal gradient to water-acetic acid-methanol-acetonitrile (55:0.5:25:20) in 15 minutes, and 3) isocratic water-acetic acid-methanol-acetonitrile (55:0.5:25:20) for 10 minutes and reequilibrate. An analysis of all the chromatographic parameters obtained with this system is to be found in Table 7.

Figure 8 shows the chromatogram of the flavonoids of the completely elucidated standard mixture.

Optimization of the Flavonoid Extraction Solvent in Plant Material.

In order to determine the most suitable solvent for flavonoid extraction in the *Citrus aurantium* tissues analysed (leaves and fruits), the extraction capacity of several solvents was studied (see Materials and Methods) in these tissues. In all cases, the extraction capacity of each solvent (in the above indicated concentrations) was estimated according to the extraction of the major flavonoids in *Citrus aurantium*, naringin and neohesperidin [1]. Three weight/volume

TABLE 7

Chromatographic Parameters of the Flavonoid Standard Mixture Elucidated with Mobile Phase: Water-Acetic Acid-Methanol-Acetonitrile in an Isocratic-Gradient System.

Flavonoid	k'	α	w	N	*HETP*	R
Neoericitrin	3.2	1.5	1.0	1644	0.15	2.7
Narirutin	5.0	1.1	1.6	1331	0.19	0.8
Prunin	5.6	1.1	1.9	1194	0.21	0.6
Naringin	6.1	1.2	2.1	1127	0.22	1.2
Hesperidin	7.3	1.1	2.3	1337	0.19	0.8
Hespt.7-*O*-glu	8.1	1.1	2.4	1438	0.17	0.7
Neohesperidin	8.8	1.1	2.7	1289	0.19	0.9
Rhoifolin	9.9	1.4	3.3	1113	0.22	2.0
Neodiosmin	13.5	1.5	4.1	1234	0.20	2.8
Naringenin	20.7	1.0	2.6	7123	0.04	0.6
Poncirin	21.3	1.1	1.8	15137	0.02	1.7
Hesperetin	22.6	1.1	1.7	20142	0.01	2.1

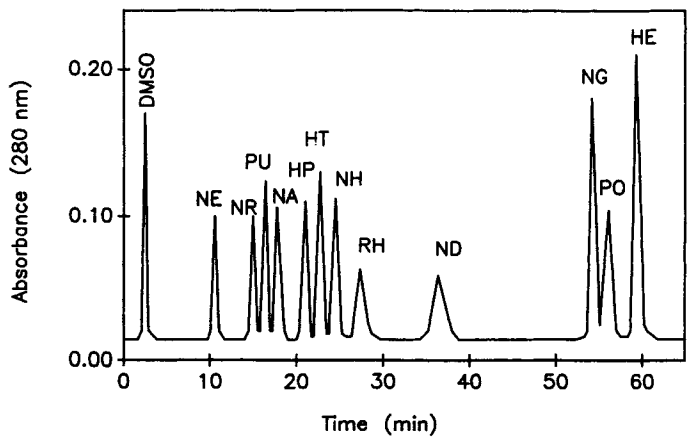

FIGURE 8. Chromatogram of flavonoid standard mixture elucidated by mean of isocratic-gradient system with mobile phase: water-acetonitrile-methanol-acetic acid. NE: Neoeriocitrin; NR: Narirutin; PU: Prunin; NA: Naringin; HP: Hesperidin; HT: Hesperetin 7-*O*-glucoside; NH: Neohesperidin; RH: Rhoifolin; ND: Neodiosmin; NG: Naringenin; PO: Poncirin; HE: Hesperetin.

TABLE 8

Relative Content of Naringin and Neohesperidin in *Citrus aurantium* Extracts using Different Solvents.

CONCENTRATION	2 mg/ml		20 mg/ml		200 mg/ml	
SOLVENTS	NAR[a]	NEOH[b]	NAR	NEOH	NAR	NEOH
Methanol	97	95	97	96	75	60
Diox/MeOH[c]	100[d]	100[d]	99	99	89	86
NaOH(aq)0.01%	86	87				
KOH(MeOH)0.01%	92	92	90	90	77	68
KOH(MeOH)0.1%	87	89				
Pyridin	66	71				
DMF[e]	96	95	96	94	95	85
DMSO[f]	100	100	100	100	100	100

[a]Naringin; [b]neohesperidin; [c]dioxane/methanol (1:1); [d]maximum signal obtained (chromatographic area) for naringin and neohesperidin; [e]dimethylformamide; [f]dimethylsulphoxide.

ratios were used in the extraction: 2, 20 and 200 mg/ml. See Table 8.

Solvents of an organic nature gave the best results for both flavonoids, except pyridin. The alkaline hydroxides commonly used for flavonoid solution gave clearly poorer results, basically because in these media the flavanones are in equilibrium with their respective chalcones, which are susceptible to rupture (forming phloroacetophenones).

The most effective solvent for flavonoid extraction at all concentrations was DMSO, not only in the case of naringin and neohesperidin but also all the other flavonoids in these extracts. For this reason DMSO was used as the extraction agent of the *Citrus aurantium* flavonoids.

Analysis, Isolation and Identification of Flavonoids in Citrus aurantium Leaves and Fruits.

After the complete resolution of all the flavonoids which, according to the bibliography, can be found in the tissues of *Citrus aurantium*, with the characteristic of having a hydroxil or methoxyl radical in one or more of the 5,7.3' and/or 4' position of their structure, the DMSO extracts of leaves and fruits of this plant material were analised in order to confirm the presence of these compounds and to isolate and identified them.

HPLC analysis of the extracts of young leaves and fruits of *Citrus aurantium* shows the presence of many flavonoid structures. Figure 9 shows a characteristic chromatogram of an extract from fruits (5-6 mm in diameter). The chromatogram of the leaf extracts showed the same peaks although with different relative proportions.

Peaks whose spectra are similar or identical to the flavanones or flavones referred to in this work (standard mixture) are numbered (1 to 12). In the chromatogram of Figure 9, compounds 1-12 present retention times in identical order to those of all flavonoids referred to in Table 7, and coincide with those shown by the other chromatographic systems: water-acetic acid-methanol (75:0.5:25) and similarity, these compounds (1-12) were isolated in order to confirm their identity.

These componds were isolated with a semipreparative column (see Materials and Methods) and 12 products were obtained. Their melting points and characteristic UV absorption spectra maxima are described in Table 9. The principal characteristics of both ^{1}H NMR and ^{13}C NMR spectra for compounds 1-12 are also described in Table 9, [12, 30, 31, 32, 33]. All these data can be used to

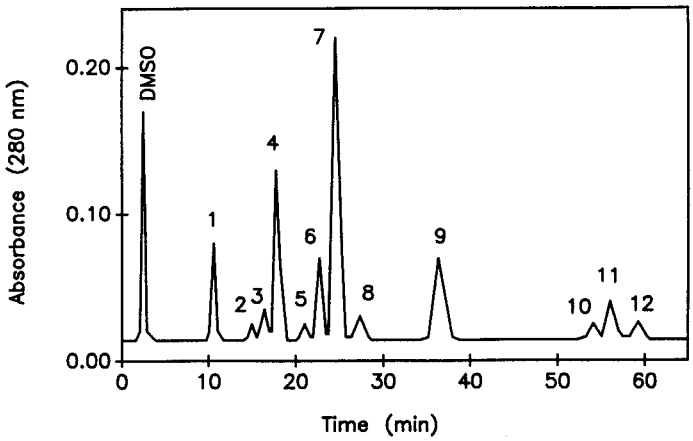

FIGURE 9. Characteristic flavonoid chromatogram of DMSO extracts of young leaves (20 mm in length) and immature fruits (5-6 mm diameter). Peaks whose spectra are similar or identical to flavanones and flavones are numbered (1 to 12).

TABLE 9

Melting Points, Spectral Characteristics and Flavonoid Structures of 12 Products Isolated by Semipreparative Chromatography from DMSO of *Citrus aurantium* Extracts.

P[a]	MP[b]	UV[c]	NMR SP[d]	GLY[h]	STRUCTURES
1	192	285,330	FAG[e]	7-NEO[i] [30, 31]	Neoeriocitrin
2	181	284,328	FAG	7-RUT[j] [30, 31]	Narirutin
3	156	283,330	FAG	7-GLU[k] [31, 32]	Prunin
4	172	284,328	FAG	7-NEO [30, 31]	Naringin
5	262	285,330	FAG	7-RUT [30, 31]	Hesperidin
6	243	284,330	FAG	7-GLU [31, 33]	Hespt.7-*O*-glu
7	245	285,330	FAG[f]	7-NEO [31, 33]	Neohesperidin
8	246	268,335	FOG[f]	7-NEO [30, 31]	Rhoifolin
9	268	253,268 344	FOG	7-NEO [12, 31]	Neodiosmin
10	252	289,326	FA[g]	[30, 31]	Naringenin
11	212	286,330	FAG	7-NEO [30, 31]	Poncirin
12	227	283,326	FA	[30, 31]	Hesperetin

confirm the nature of compounds 1-12, isolated from extracts of the young leaves and and immature fruits of *Citrus aurantium*.

In short, the present work shows that it is possible to determine simultaneously by means of HPLC the quantitative presence of a large number of flavonoids in *Citrus aurantium*, which until now have only been described independently or in closed structural groups.

This work not only intends to describe a specific methodology for the elucidation, isolation and identification of the flavonoids found in *Citrus aurantium* extracts, but also to suggest a way of varying this methodology for the study of flavonoids of a similar nature in other extracts.

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