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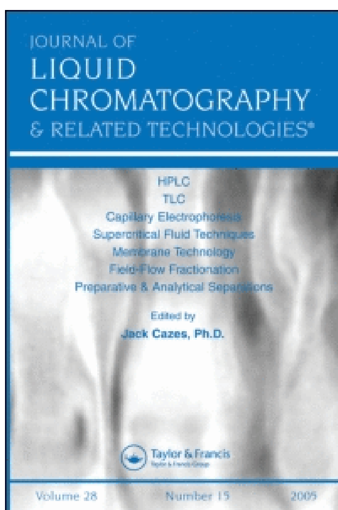

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Elucidation by Reverse Phase HPLC of Some Citrus Flavanones and Their Respective Dihydrochalcones: Structural Study

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ELUCIDATION BY REVERSE PHASE HPLC OF SOME CITRUS FLAVANONES AND THEIR RESPECTIVE DIHYDROCHALCONES: STRUCTURAL STUDY

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ABSTRACT

A high-performance liquid chromatographic method for the elucidation of the principal *Citrus* flavanone glycosides in biosynthesis and their respective dihydrochalcones has been developed. A C_{18} reverse phase column and an elution isocratic-gradient system is used with a mixture of water, acetonitrile and acetic acid. We describe the optimization process by studying the influence of the structural characteristics of these compounds on the quantitative chromatographic parameters: k', w and R.

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INTRODUCTION

Flavonoids are secondary metabolites widely distributed through the plant kingdom, to which many roles have been attributed [1]. *Citrus* species are of great interest because accumulate high amounts of flavanone glycosides in their fruit and young tissues [2, 3, 4]. *Citrus aurantium* has the widest variety of flavanones of all the citric species of the spanish levante. Among these of great importance from a biosynthetic point of view are: naringin, hesperetin 7-*O*-glucoside, neohesperidin and hesperidin [3, 4].

The key enzyme in flavonoid biosynthesis is chalcone synthase, which catalyses the condensation between the ester CoA of *p*-coumaric acid and 3 molecules of malonylCoA, forming the structure known as chalcone, which makes up the basic skeleton of all known groups of flavonoids [5]. These chalcones are very unstable compounds and until now it has been impossible to isolate and quantify them in the proportions in which they are found during flavonoid biosynthesis in plants.

Some authors consides that chalcones cycle spontaneously as a consequence of a chemical equilibrium

with their corresponding flavanones, an equilibrium displaced towards the flavanones. However, without denying the existence of this displaced equilibrium, other authors have suggested that specific enzymes are responsible for chalcone cyclation, the so-called chalcone-cyclases or chalcone isomerases [6, 7, 8, 9]. Whatever the case, the instability of the chalcone type intermediaries makes it impossible to quantify them in a stationary state.

The selective catalytic hydrogenation of the double bond between the carbons in the α and β positions with the carbonile group of the chalcone makes it possible to obtain the corresponding dihydrochalcones. These are very stable compounds in the biosynthetic conditions of the plant and, by quantifying them, it is possible to indirectly quantify the chalcones in the stationary state of the chalcone structures.

As a previous step to developing this technique on extracts of *Citrus* tissue, the present work attempts to optimize an analytical method using HPLC which would permit a fast and accurate elucidation of the above mentioned flavanones and their respective dihydrochalcones.

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MATERIALS AND METHODS

Chemicals

Naringin (N), hesperidin (H), hesperetin 7-*O*-glucoside (H7), neohesperidin (NH), naringin dihydrochalcone (NDC), hesperedin dihydrochalcone (HDC), hesperetin 7-*O*-glucoside dihydrochalcone (H7DC) and neohesperidin dihydrochalcone (NHDC) were obtained from Zoster S.A., Murcia, Spain. Dimethylsulphoxide (DMSO) was used as solvent [10], and the solution was filtered through a 0.45 μm nylon membrane.

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Chromatographic Analysis

For the elucidation of flavanones and dihydrochalcones we used a μBondapak C_{18} (250 x 4 mm ID) analytical column with an average particle size of 5 μm. Several solvents were used and these are described in Results. In all the solvent systems 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 a quantitative study, the elucidation capacity of the different mobile phases used was verified by determining the following parameters [11] for each flavonoid in each of the said phases:

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) Width of the peak (w).

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

N = number of theoretical plates.

α = selectivity factor.

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RESULTS AND DISCUSSION

Chromatographic Analysis Optimisation. Preparation of Flavonoid Mixture.

The structural study and the optimization of the procedure for analysing the flavanones and their respective dihydrochalcones mentioned in Materials and Methods (Chemicals) was realized by using a mixture of these substances in DMSO. This mixture contained (in 20

ml DMSO): naringin (N), 13 mg; hesperidin (H), 6 mg; hesperetin 7-*O*-glucoside (H7), 3 mg; neohesperidin (NH), 7 mg; naringin dihydrochalcone (NDC), 2 mg; hesperidin dihydrochalcone (HDC), 1 mg; hesperetin 7-*O*-glucoside dihydrochalcone (H7DC), 4 mg and neohesperidin dihydrochalcone (NHDC), 20 mg.

In a first step, HPLC elucidation was optimized by using isocratic systems in which only the proportions of the mobile phase components were varied [10]. Acetic acid (0.5 % v/v) was included in all mobile phases for to improve separation [10, 12, 13].

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Mobile Phases with Water and Methanol.

Figure 1, shows how the order and the corresponding ln k' values of these flavonoids are affected by their degree of polarity according to the different substitution in the B ring [10]. The presence of the methoxyl group in position 4' also means that the 3'-hydroxy-4'-methoxy flavonoids (NH and NHDC) are less polar that the respective 4'-hydroxy flavonoids (N and NDC). An increase in the percentage of methanol in the mobile phase (Fig. 1) similarly affects the four structures represented, both flavanones and both

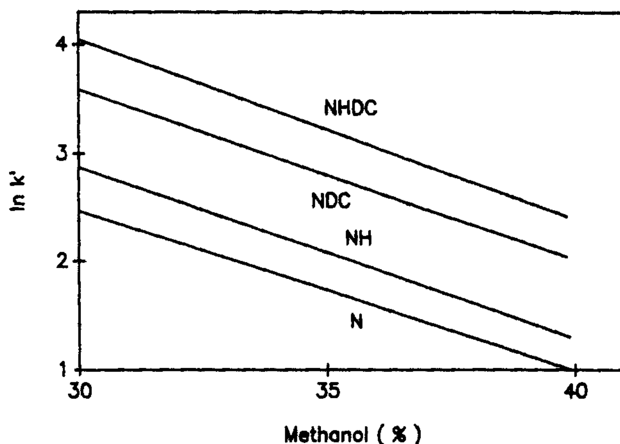

FIGURE 1. Variation of ln k' versus methanol percentage of mobile phases with water-acetic acid-methanol. N: Naringin; NH: Neohesperidin; NDC: Naringin dihydrochalcone; NHDC: Neohesperidin dihydrochalcone.

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dihydrochalcones showing similar slopes for decreases in ln k' values. The dihydrochalcones eluted much later than the respective flavanones and so their k' values are higher.

Figure 2 shows variations in ln k' according to variations in the percentage of methanol in the mobile phase for the flavonoids hesperidin, neohesperidin, hesperetin 7-*O*-glucoside and their respective dihydrochalcones. The k' value of glucosylated structures undergoes a greater relative change than that

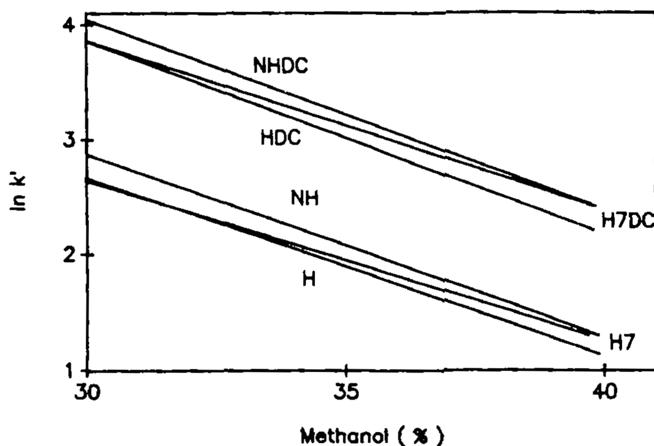

FIGURE 2. Variation of ln k' versus methanol percentage of mobile phases with water-acetic acid-methanol. H: Hesperidin; H7: Hesperetin 7-*O*-glucoside; NH: Neohesperidin; HDC: Hesperidin dihydrichalcone; H7DC: Hesperetin 7-*O*-glucoside dihydrochalcone; NHDC: Neohesperidin dihydrochalcone.

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of the rhamnoglucosides; H7DC and H7, show a lower slope for the decrease of their ln k' values; these values are identical to the values of their rutinosides with a 30 and 32 methanol percentage, respectively, and are identical to those of their neohesperidosides with a 40 and 41 methanol percentage, respectively.

Furthermore, it can be seen that the k' values of hesperidin and neohesperidin show a similar decrease as the percentage of methanol in the mobile phase

increases. The k' of the rutinosides is always below that of their corresponding neohesperidosides. The presence of 7-*O*-neohesperidoside substitution would affect the stability of the intramolecular hydrogen bond and the neohesperidoside structures would not be planar to rutinosides [14]. There would certainly be a greater hydrophobic interaction between the neohesperidoside molecules and those of the stationary phase of the column, which increase the k' value of these compounds compared with their respective rutinosides [10].

The k' values of the flavanones is always below that of their corresponding dihydrochalcones. This clearly contradicts the experimental data obtained when the solubility of these compounds was analysed. NH shows a solubility (at room temperature) of 0.03 g/l in water, and 1.6-1.7 g/l in water-ethanol (vol 1:1), whereas NHDC shows a solubility of 0.4 g/l in water and 120-130 g/l in water-ethanol (vol 1:1). The cause of this alteration in the k' values of both flavonoid types with respect to the solubility data probably has a structural origin (Fig. 3).

For example, the molecular structure and absolute configuration of neohesperidin dihydrochalcone have been

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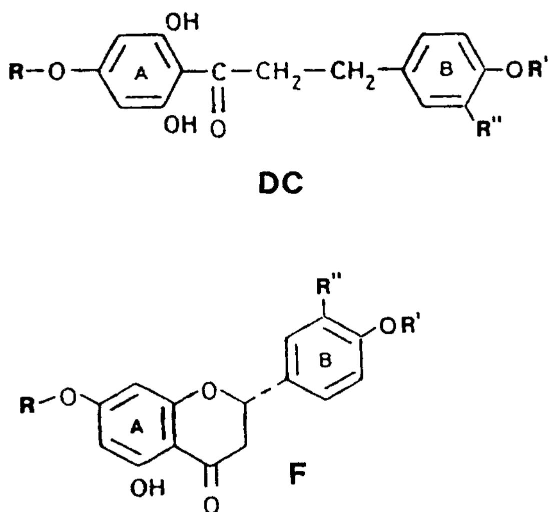

FIGURE 3. Basic structures of flavanones (F) and dihydrochalcones (DC). R: neohesperidosyl, rutinosyl or glucosyl; R': H or methyl; R'': H or hydroxyl.

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determined by single-crystal X-ray analysis. The crystal structure consists of two crystallographically independent molecules which differ mainly in the orientation of the isovanillyl B-ring. The molecules are linked together by an intricate arrangement of intermolecular hydrogen bonds formed between the host and solvent water molecules [15].

The presence of this intrincate system of intermolecular hydrogen bonds, which is not found in the flavanones and which results in a voluminous mode of

molecular packing, particulary affects the reverse phase-dihydrochalcone interaction, increasing the k' values of these flavonoids.

The high values obtained for the parameters k' and w (between 7 and 1 min) in the water-methanol phases, which permitted the resolution of all the compounds in the mixture, together with the overlapping effects between glucosides and rhamnoglucosides by the different variations in ln k' according to changes in the percentage of methanol in the mobile phases studied, support the use of mobile phases with water and acetonitrile, as in previous studies [10].

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Mobile Phases with Water and Acetonitrile.

Acetic acid (0.5 % v/v) was included in all the mobile phases used. This type of mobile phase with 25 % acetonitrile considerably reduces the retention times of all the flavonoids contained in the standard mixture, which produces a simultaneous overlapping of many and impedes accurate elucidation. When the percentage of acetonitrile in the mobile phase falls below 20 %, flavanones and dihydrochalcones are better resolved, but the other chromatographic parameters are adversely

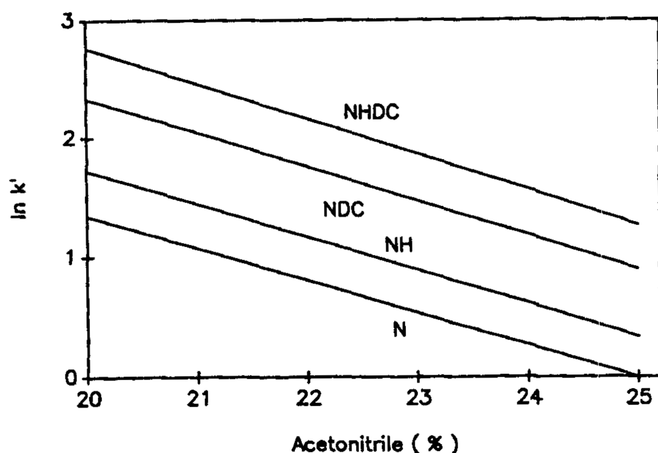

FIGURE 4. Variation of ln k' versus acetonitrile percentage of mobile phases with water-acetic acid-acetonitrile. N: Naringin; NH: Neohesperidin; NDC: Naringin dihydrochalcone; NHDC: Neohesperidin dihydrochalcone.

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affected, particularly the w and k' values which increase with respect to those the 20-25 % acetonitrile volume percentage range.

Figure 4 shows how the form of substitution in the B ring when the same type of glycosylation is maintained in the 7 position, does not affect chromatographic resolution, similar to what happens in the water-methanol phases. N, NH, NDC and NHDC show similar slopes for the decrease in ln k' values when the percentage of acetonitrile in the mobile phases increases.

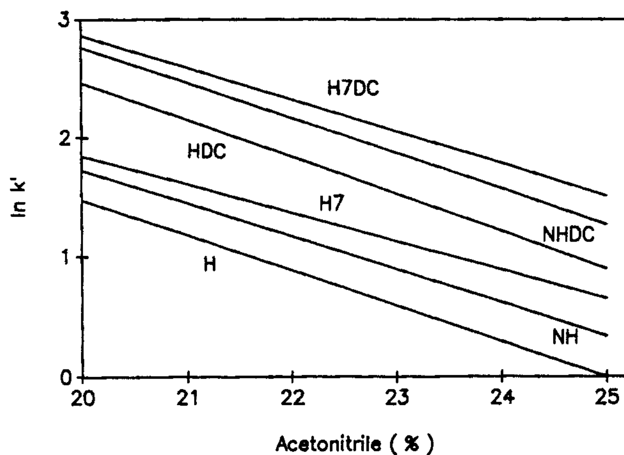

FIGURE 5. Variation of ln k' versus acetonitrile percentage of mobile phases with water-acetic acid-acetonitrile. H: Hesperidin; H7: Hesperetin 7-*O*-glucoside; NH: Neohesperidin; HDC: Hesperidin dihydrichalcone; H7DC: Hesperetin 7-*O*-glucoside dihydrochalcone; NHDC: Neohesperidin dihydrochalcone.

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The flavonoids H7 and H7DC show a different behaviour to that described in the water-methanol phases since throughout the range considered (20-25 % acetonitrile), the k' values for both compounds were better than those of their rhamnoglucosylated flavanones and dihydrochalcones, respectively. The glucosides, rutinosides and neohesperidosides show similar slopes for the decrease in their k' values when the percentage of acetonitrile in the mobile phases varies, both for flavanones and dihydrochalcones. This fact served for

TABLE 1

Chromatographic Parameters of the Flavonoid Standard Mixture of Flavanones and Dihydrochalcones (DC) with Mobile Phase: Water-Acetic Acid-Acetonitrile (Gradient System from 20 % to 25 % Acetonitrile percentage, for 7 min).

Flavonoid	k'	α	w	R
Flavanones				
Naringin	3.04	1.10	0.37	1.10
Hesperidin	3.34	1.17	0.40	1.87
Neohesperidin	3.92	1.13	0.40	1.64
Hespt.7-*O*-glu.	4.41	1.26	0.45	3.06
Dihydrochalcones				
NaringinDC.	5.57	1.05	0.40	1.03
HesperidinDC.	5.86	1.15	0.52	2.22
NeohesperidinDC.	6.75	1.12	0.48	2.17
Hespt.7-*O*-gluDC.	7.53	1.08	0.57	1.51

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considering the mobile phases containing water and acetonitrile as the most suitable for obtaining the simultaneous resolution of both types of flavonoid compound.

To improve the k' values while maintaining the w values close to those obtained using mobile phases with 25 % acetonitrile (below 0.7 min in all cases) and

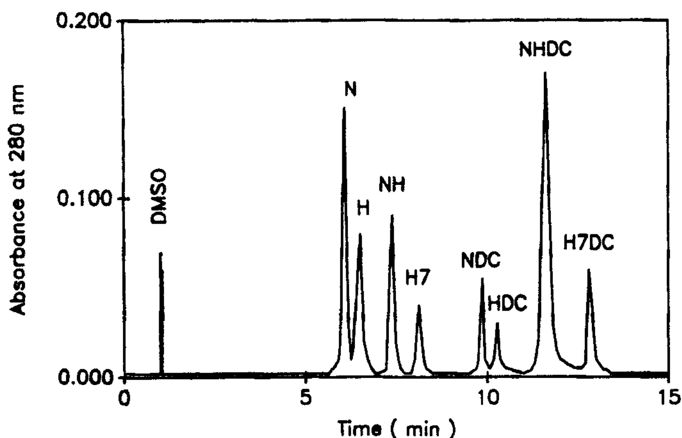

FIGURE 6. Chromatogram of flavanone and dihydrochalcone standard mixture elucidated by means of a gradient-isocratic system with mobile phase: water-acetic acid-acetonitrile. N: Naringin; H: Hesperidin; NH: Neohesperidin; H7: Hesperetin 7-*O*-glucoside; NDC: Naringin dihydrochalcone; HDC: Hesperidin dihydrichalcone; NHDC: neohesperidin dihydrochalcone; H7DC: Hesperetin 7-*O*-glucoside dihydrochalcone.

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keeping the values of R similar to those obtained using 20 % (above 1.0 min in all cases), a lineal gradient system was designed which increased the percentage of acetonitrile of the mobile phase from 20 (80:0.5:20 water-acetic acid-acetonitrile) to 25 % (75: 0.5: 25 water-acetic acid-acetonitrile) in a given time, maintaining this composition until all the flavonoids in the mixture were resolved; this gradient change of

acetonitrile percentage shows an optimum time value of 7 min. An analysis of several chromatographic parameters obtained with this system is to be found in Table 1. Figure 6 shows the chromatogram of the flavonoids of the completely elucidated standard mixture.

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