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# Dihydrochalcones from apple juices and jams

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The occurrence of two dihydrochalcone glycosides has been demonstrated in Golden Delicious apple juices and jams by HPLC analysis. These substances have been isolated by a combination of Amberlite XAD-2 and reversed-phase liquid chromatography and by semipreparative HPLC. They have been completely identified as 2',4',6',4-tetrahydroxydihydrochalcone-2'-*O*-β-D-glucopyranoside (phloridzin) and 2',4',6',4-tetrahydroxy-dihydrochalcone-2'-*O*-(6''-β-D-xylopyranosyl)-β-D-glucopyranoside by spectroscopic methods.

## INTRODUCTION

The occurrence of hydrochalcone glycosides (phloretin glucoside and phloretin xyloglucoside) in apple juice has been known for a long time (Johnson *et al.*, 1968). In fact, these substances are characteristic of apple since they have not been detected in any other fruit (Herrmann, 1990), and therefore their analysis is useful in food authenticity studies. Dihydrochalcones are also important since they oxidize rather easily (Dziedzic *et al.*, 1985), and their oxidation contributes to apple juice browning (Burda *et al.*, 1990; Spanos *et al.*, 1990; Oszmianski & Lee, 1991). In spite of the importance of these substances and the large number of articles in which they are mentioned, the structure of the xyloglucoside has never been completely established. In a recent paper, the occurrence of phloretin xyloglucoside in apple was demonstrated by FAB MS (Oleszek *et al.*, 1988), but the site of sugar attachment to the aglycone moiety and the mode of the sugar linkage was never shown. In addition, the occurrence of phloretin xylogalactoside in Golden Delicious apples had recently been tentatively reported (Burda *et al.*, 1990).

As part of our research programme to isolate and identify flavonoids from juices and jams for authenticity analysis purposes, the aim of the present work is the isolation and complete characterization of the dihydrochalcones present in apple-derived food.

## MATERIALS AND METHODS

### Materials

Experimental apple juices (A and B) were obtained with mature Golden Delicious and Starking apples. The whole fruits (c. 3 kg) were liquefied and filtered to obtain 2 l of juice. In addition, three commercial apple juices (Golden Delicious) (C–E) manufactured in Spain by three different companies, one apple jam (50% Golden Delicious fruit) manufactured in Spain (F), one apple compote (90% Golden Delicious fruit) manufactured in Germany (G) and one apple jelly (45% Golden Delicious fruit) manufactured in France (H), were analysed.

### HPLC analysis of dihydrochalcones

Experimental and commercial apple juices (A–E) (1 l) were directly extracted with *n*-buthanol (200 ml). Apple jams (F–H) (200g) were extracted by stirring with methanol–water (1 : 1 v/v) (400 ml) for 18 h at room temperature 20–25°C and filtered. The filtrate was concentrated under reduced pressure until all the methanol had been removed and the dihydrochalcones present in the remaining aqueous extract were then extracted with *n*-butanol (200 ml). Butanol extracts were taken to dryness under reduced pressure and the residues redissolved in acid water (pH 2 with HCl). These aqueous extracts were then filtered through an Amberlite XAD-2 column (40 cm × 2 cm) (Fluka 20–50 mesh). Phenolic compounds remained in the column while sugars, pectins and other polar compounds eluted with the aqueous solvent. The column was then washed with acid water (c. 100 ml), and neutral distilled water (300 ml) successively. The phenolic compound fraction was then eluted with methanol (c. 300 ml), and concentrated under reduced pressure (García-Viguera, 1991).

Phenolic compound fractions were redissolved in methanol (2 ml) and 10 μl of these were analysed by HPLC with a reversed-phase column Lichrochart 100 RP-8 (10 cm × 0·4 cm, 5 μm particle size), using as solvents, water-formic acid (95 : 5) (solvent A) and methanol (solvent B). Elution was performed using a

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gradient starting with 5% solvent B to reach 30% B at 20 min, 50% B at 25 min, and 80% B at 35 min. The solvent flow rate was 1 ml/min and detection was achieved at 280 nm. All HPLC analyses were replicated, the mean values being reported. Reproducibility was *c.* ±6%. Dihydrochalcones were quantitated as phloridzin (García-Vigueara, 1991).

### Preparative isolation of dihydrochalcones

Ten litres of commercial Golden Delicious apple juice were filtered through an Amberlite XAD-2 column (40 cm × 2 cm) (Fluka 20—50 mesh) to adsorb phenolic compounds (including dihydrochalcones). No dihydrochalcone eluted with the aqueous eluate as revealed by the HPLC analysis.

The phenolic fraction was then eluted with methanol (500 ml), concentrated under reduced pressure (40°C), redissolved in water and extracted with *n*-butanol. This butanol extract, containing the dihydrochalcones, was taken to dryness under reduced pressure (60°C) (2·05 g), and fractions of 250 mg of this concentrate were dissolved in 1 ml methanol–water (1 : 1, v/v) and fractionated by Low Pressure Liquid Chromatography (Lobar column RP-8, 30 cm × 2·5 cm Merck) with methanol–water (1 : 1, v/v) with a flow rate of 5 ml/min and detection at 300 nm. Two fractions containing dihydrochalcones were detected by HPLC analysis.

Two dihydrochalchones (10 mg of 1 and 12 mg of 2) were then purified from both fractions by semipreparative HPLC using a column Spherisorb ODS (25 cm × 0·7 cm and 5 μm particle size) with methanol–water (33 : 66, v/v) and a flow rate of 3 ml/min. Detection was achieved at 280 nm. The purity of the isolated compounds was tested by analytical HPLC coupled with a diode array detector. The isolated compounds were freeze-dried and stored under $N_2$ to avoid oxidation.

### Identification of dihydrochalcones

Acid hydrolysis of the isolated dihydrochalcones was achieved as reported previously (Harborne, 1973). The UV-Vis analysis was achieved by classical methods (Mabry *et al.*, 1970) with the isolated dihydrochalcones dissolved in methanol, and spectra were recorded before and after the addition of the classical shift reagents. Dihydrochalcone **1** (nm): MeOH 283, 330 sh (shoulder); +NaOMe: 323; +$AlCl_3$: 362, 312, 280 sh; +$AlCl_3$+HCl: 361, 309, 279; +NaOAc: 323, 283; +NaOAc+$H_3BO_3$: 335, 282. Dihydrochalcone **2** showed the same UV spectra as compound **1**. $^1H$ NMR spectra were recorded in $d_6$-DMSO at 20°C in a Brücker AMX-600 spectrometer using saturation of the HOD solvent signal. Phase sensitive COSY and NOESY (350 ms mixing time) were recorded using the standard pulse sequences (Homans, 1990). Assignment of all the protons was performed with the combined use of 1-D and 2-D COSY spectra.

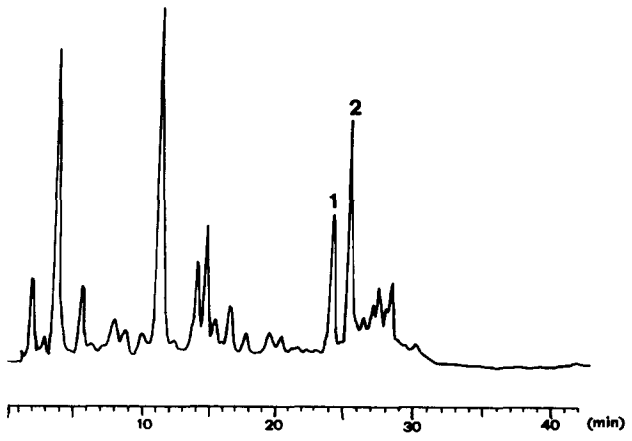


**Fig. 1.** HPLC analysis of Golden Delicious apple juice dihydrochalcones.

## RESULTS AND DISCUSSION

### Dihydrochalcone analysis in apple juices and jams

The dihydrochalcone glycosides present in the available juices and jams, were HPLC analysed to assess which product should be used for the preparative isolation of these substances. These analyses showed that two dihydrochalcones were present in all the analysed juices and jams, as evidenced by their characteristic UV spectra (Grayer, 1989) recorded with a diode array detector (Fig. 1). In Table 1, the milligrams of phloridzin (**2**) and phloretin xyloglucoside (**1**) per litre of juice or kilogram of jam are shown. The values obtained are similar to those reported for Granny Smith apple juices (Spanos *et al.*, 1990). However, this is the first report on dihydrochalcone composition of apple jams. The significant differences observed in the amount of dihydrochalcones present in the analysed jams (F—H) could be explained by the differences in the fruit content (see Materials section), in addition to the influence of the manufacturing process. All the analysed samples contained both dihydrochalcone glycosides in sufficient amounts to be quantitated by HPLC analysis. These results show that the percentage of compound **1**, was

**Table 1. HPLC analysis of dihydrochalcones in different apple juices and jams**

| | Compound 1[a] | | Compound 2 (Phloridzin) | |
|---|---|---|---|---|
| *Juices (mg/litre)* | | | | |
| A (Exp.) | 3·64 | (40·1) | 5·43 | (53·5) |
| B (Exp.) | 3·78 | (50·9) | 3·64 | (49·1) |
| C (Com.) | 2·09 | (46·5) | 2·66 | (53·5) |
| D (Com.) | 2·34 | (42·0) | 3·26 | (58·0) |
| E (Com.) | 2·21 | (45·4) | 2·66 | (54·6) |
| *Jams (mg/kg)* | | | | |
| A (Jam) | 1·73 | (42·7) | 2·32 | (57·3) |
| B (Compote) | 5·18 | (35·8) | 9·16 | (64·2) |
| C (Jelly) | 0·42 | (29·6) | 1·00 | (70·4) |

[a] Quantitated as phloridzin (parentheses show percentage of each dihydrochalcone).

similar to that of compound **2** in the juices obtained from Golden Delicious apple, while it was present in a lesser percentage in the analysed jams; for this reason apple juice was selected as the source for the preparative isolation of these dihydrochalcones.

### Dihydrochalcones characterization

Compounds **1** and **2** were isolated as described in the Materials and Methods part. Acid hydrolysis showed that compound **1** contained both glucose and xylose and compound **2** only glucose. Both substances yielded phloretin as an aglycone. These results suggested that compound **2** should be phloridzin (phloretin-2'-glucoside) previously described in apple, and this was confirmed by co-chromatography (TLC and HPLC) with an authentic marker (Sigma). The UV study of the isolated dihydrochalcones (Mabry *et al.*, 1970) suggested that both compounds had the same substitution pattern (phloretin with sugars linked to the hydroxyl at the 2' position) and therefore that xylose and glucose should be linked, in compound **1**, in a disaccharide. The sugar sequence in compound **1** as well as the interglycosidic linkage were established by a comparative study of the $^1$H NMR spectra of compounds **1** and **2** (Table 2), including the COSY and NOESY experiments. This analysis confirmed the substitution pattern of the aglycone (Grayer, 1989), and the monoglycosidic and diglycosidic nature of compounds **2** and **1** respectively. The COSY experiment allowed the assignment of the different chemical shifts of the different protons, and the NOESY experiment showed clearly that in both compounds glucose was linked to the hydroxyl in 2' position of phloretin (NOEs of anomeric protons of glucose with the H-3' of phloretin). NOE crosspeaks were detected among neighbour protons within each sugar ring. For xylose strong 1–3 and 1–$5_B$ NOEs were observed in accordance with a $\beta$-pyranose type of sugar (Homans, 1990) and a weak 1–2 NOE peak was also detected. For glucose, strong 1–5 and 1–3 NOEs were observed, indicating also a $\beta$-pyranose sugar type, and a weak 1–4 NOE as well. A strong inter-residue NOE peak was seen connecting the anomeric proton of xylose with protons $6_A$ and $6_B$ of glucose for compound **1**, which strongly supported a 1–6 type of linkage (Homans, 1990; Tomás-Lorente *et al.*, 1992), confirmed by an additional NOE (weak) between the same anomeric proton of xylose and proton 5 of glucose. This last was confirmed by the absence of the 6-hydroxyl signal of glucose in the disaccharide and for a low field shift of proton $6''_A$ (+0·25 ppm) and $6''_B$ (+0·12 ppm) of the disaccharide with respect to phloridzin. All these data indicate that the structure of compound **1** is 2',4',6',4-tetrahydroxydihydrochalcone-2'-*O*-(6''-$\beta$-D-xylopyranosyl)-$\beta$-D-glucopyranoside and that of compound **2** (phloridzin) is 2',4',6',4-tetrahydroxydihydrochalcone-2'-*O*-$\beta$-D-glucopyranoside.

**Table 2. $^1$H NMR complete assignment of dihydrochalcone protons by COSY and NOESY**

| Protons | Compound **1** | | Compound **2** | |
|---|---|---|---|---|
| | $\delta$ (ppm) | Multiplet | $\delta$ (ppm) | Multiplet |
| *Aglycone* | | | | |
| 2,6 | 7·03 | d | 7·03 | d |
| 3,5 | 6·64 | d | 6·63 | d |
| 3' | 6·19 | s | 6·12 | s |
| 5' | 5·92 | s | 5·92 | s |
| $\alpha$ | 3·25 | m | 3·31 | m |
| $\alpha'$ | 3·42 | m | 3·41 | m |
| $\beta,\beta'$ | 2·77 | t | 2·78 | t |
| *Glucose* | | | | |
| 1'' | 4·89 | d ($J$ = 8Hz) | 4·93 | d ($J$ = 8Hz) |
| 2'' | 3·30 | m | 3·27 | m |
| 3'' | 3·26 | m | 3·30 | m |
| 4'' | 3·21 | m | 3·18 | m |
| 5'' | 3·52 | m | 3·33 | m |
| $6''_A$ | 3·95 | d | 3·70 | m |
| $6''_B$ | 3·61 | m | 3·50 | m |
| OH-2'' | 5·34 | d | 5·32 | d |
| OH-3'' | 5·25 | d | 5·18 | d |
| OH-4'' | 5·18 | d | 5·08 | d |
| OH-6'' | — | — | 4·62 | d |
| *Xylose* | | | | |
| 1''' | 4·17 | d ($J$ = 8Hz) | | |
| 2''' | 2·99 | m | | |
| 3''' | 3·07 | m | | |
| 4''' | 3·27 | m | | |
| $5'''_A$ | 2·97 | m | | |
| $5'''_B$ | 2·99 | m | | |

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