Detection of Phloridzin in Strawberries (*Fragaria x ananassa* Duch.) by HPLC–PDA–MS/MS and NMR Spectroscopy

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The phenolic profile of strawberry fruits (*Fragaria x ananassa* Duch., Rosaceae) was investigated by high-performance liquid chromatography with photodiode array detection. A peak displaying retention time and UV spectral data identical to those of phloridzin (phloretin 2′-*O*-β-D-glucoside), a dihydrochalcone glucoside so far considered characteristic of apples, was monitored. For further characterization, crude extracts of strawberries were purified on polyamide, and the target compound was isolated by preparative and analytical HPLC. Structure elucidation was performed on the basis of APCI- and ESI-MS in the negative ion mode as well as by 1D and 2D NMR spectroscopy using authentic phloridzin for comparison. The D-configuration of the sugar moiety was established by HPLC analysis of the corresponding acyclic 1-deoxy-1-(*N*-acetyl-α-methylbenzylamino)alditol acetate. Apart from its chemotaxonomic relevance, this first report on the occurrence of phloridzin in strawberries is of particular interest for the authenticity control of strawberry products such as juices, jams, and fruit preparations since phloridzin has so far been used for the detection of fraudulent admixtures.

KEYWORDS: Strawberry fruits; *Fragaria x ananassa* Duch.; phloridzin; authenticity control; HPLC; mass spectrometry; NMR spectroscopy

INTRODUCTION

Strawberries are one of the most important fruits and are either consumed as fresh fruits or used for industrial food production. Due to their high antioxidant capacity, which is mainly attributed to their flavonoid content (*1*), strawberries have also attracted nutritional interest. Gallic, ellagic, and 4-hydroxybenzoic acids (*2−4*) as well as hydroxycinnamates such as feruloylglucose, *p*-coumaric and caffeic acids (*3*), and glycosides of quercetin and kaempferol (*5−7*) have so far been detected in strawberries. The anthocyanin fraction of strawberries is mainly composed of pelargonidin and, to a lesser extent, cyanidin glycosides (*8*).

Apart from their nutritional relevance, phenolics play an important role in food analysis since they have been widely recognized as indicators of adulterations of fruit juices, (*9, 10*) jellies (*11*), jams (*12, 13*), and purees (*14*). The dihydrochalcone glycosides phloridzin (phloretin 2′-*O*-β-D-glucoside) and phloretin 2′-*O*-(6″-β-D-xylosyl)-β-D-glucoside have so far been regarded as characteristic constituents of apples (*15−19*) and have therefore been used as markers of fraudulent admixtures. Phloridzin has recently been identified in rose hip (*Rosa canina* L.) (*20*). However, the identification was only based on mass

spectrometry, which does not allow unambiguous determination of the sugar moiety. Furthermore, chemotaxonomic relevance of these findings and implications for authenticity control have not been realized.

Recently, a new process for the production of canned cherries and strawberries with improved textural properties was developed (*21*). The formation of calcium chelates was shown to be only partly responsible for fruit firming. Since cross-linkage of plant cell wall polymers via hydroxycinnamates, in particular ferulic acid, is known to largely affect textural attributes of plants and foods (*22*), involvement of peroxidase and phenolics was suggested (*21*). Therefore, the phenolic profile of strawberry fruits was investigated using HPLC with diode array and mass spectrometric detection. In the present study, the isolation and structure elucidation of phloridzin in strawberries is reported for the first time.

MATERIAL AND METHODS

Standards. Standards used for identification purposes by HPLC−PDA and MS were obtained from Roth (Karlsruhe, Germany). Phloridzin was purchased from Fluka (Buchs, Switzerland).

Sample Preparation for Analytical HPLC. Individually quick-frozen (IQF) strawberry fruits of the cultivars Honeoye, Elsanta, and Senga Sengana were extracted as described previously (*19*). Fully ripe fruits (100 g) were homogenized using a blender and extracted by stirring with 100 mL of acetone for 1 h at ambient temperature. After

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Detection of Phloridzin in Strawberries

J. Agric. Food Chem., Vol. 51, No. 10, 2003 **2897**

centrifugation, acetone was evaporated in vacuo at 30 °C. The aqueous solution was adjusted to pH 2 and extracted three times with 50 mL of ethyl acetate. After evaporation to dryness, the residues were dissolved in 2 mL of methanol, membrane filtered (0.2 μm), and used for HPLC analysis.

Achenes of the cultivar Senga Sengana could easily be separated during juice production with a decanter since they were sticking to the wall of the bucket used for collecting the pomace. They were immediately frozen using liquid nitrogen. After the achenes were crushed in a mortar, the plant material was extracted as described for IQF fruits.

HPLC Analyses. The separation of phenolic compounds was carried out as described earlier (*19*). An HP HPLC series 1100 (Hewlett-Packard, Waldbronn, Germany) was used equipped with ChemStation software, a degasser, model G1322A, a binary gradient pump, model G1312A, a thermoautosampler, model G1329/1330A, a column oven, model G1316A, and a diode array detector, model G1315A. The column used was an Aqua 5 μm, 125 Å particle size C18 (250 mm × 4.6 mm i.d.) from Phenomenex (Torrance, CA) and a security guard C18 ODS (4.0 mm × 3.0 mm i.d.). The column was operated at a temperature of 25 °C. The mobile phase consisted of 2% (v/v) acetic acid in water (eluent A) and 0.5% acetic acid in water and acetonitrile (50:50, v/v; eluent B). The gradient program was as follows: 10% B to 55% B (50 min), 55% B to 100% B (10 min), 100% B to 10% B (5 min). The injection volume for all samples was 10 μL. Simultaneous monitoring was performed at 280 nm (dihydrochalcones, catechins, proanthocyanidins, benzoic acids), 320 nm (hydroxycinnamic acids), and 370 nm (flavonols) at a flow rate of 1 mL/min. Spectra were recorded from 200 to 600 nm (peak width 0.2 min, data rate 1.25 s^{-1}).

HPLC−MS Analyses. LC−MS analyses were performed using the same stationary phase and eluents as described above. The system consisted of an HPLC pump, Pro Star (Varian, Darmstadt, Germany), and a UV detector, model ABI 785 A (Applied Biosystems, Weiterstadt, Germany). The UV detector and mass spectrometer were connected in series. Negative ion mass spectra of the column eluate (*m/z* 10−800) were recorded using a TSQ 700 mass spectrometer (Finnigan MAT, San Jose, CA) fitted with an APCI source. The temperature of the capillary was set to 200 °C, and that of the vaporizer was kept at 450 °C. Nitrogen was used as the sheath gas at a pressure of 70 psi. Collision-induced dissociation spectra were obtained at 22 eV using argon as the collision gas (2.5 mTorr).

MS Analyses. The substance isolated for NMR analysis was also investigated by negative ion ESI mass spectrometry. A syringe pump (Harvard Apparatus 22, Southnatick, MA) operated at a flow rate of 5 μL/min was connected to the mass spectrometer mentioned above. Spectra were recorded from *m/z* 50 to *m/z* 650. The spray voltage was 3.5 kV. Collision-induced dissociation spectra were obtained at 21 eV using argon as collision gas (2.5 mTorr).

Preparative Isolation of Phloridzin. *Extraction.* Ripe IQF strawberries (2.8 kg) of the cultivar Senga Sengana (Poland) harvested in 2001 were thawed using a microwave oven and homogenized in a blender. The puree was extracted by stirring with 3 L of acetone at ambient temperature for 1.5 h. After filtration, acetone was removed in vacuo, and the aqueous solution was extracted with ethyl acetate as described above. The organic solvent was evaporated in vacuo, and the residue was dissolved in water.

A glass column (volume 110 mL) was filled with polyamide MN SC6 (particle size 0.05−0.16 mm; Macherey Nagel, Dueren, Germany) and successively conditioned with 100 mL of TFA (0.01%), 150 mL of water, 80 mL of methanol (40%), 160 mL of methanol (100%), and 100 mL of TFA (0.01%). After the aqueous strawberry extract was applied, the column was washed with 130 mL of water and 80 mL of methanol (40%) and dried under gentle vacuum. Phenolic compounds were eluted with 160 mL of methanol (100%) and collected. The eluate was evaporated to dryness, and the flask flushed with nitrogen and stored at −80 °C.

Preparative HPLC. For preparative isolation of phloridzin, an HPLC system (Bischoff, Leonberg, Germany) consisting of an LC-CaDI 22-14 control unit, two HPLC compact pumps, a LAMBDA 1000 UV detector, and a dynamic mixing chamber (Knauer, Berlin, Germany) was used. Chromatographic runs were recorded with Bischoff McDAcq
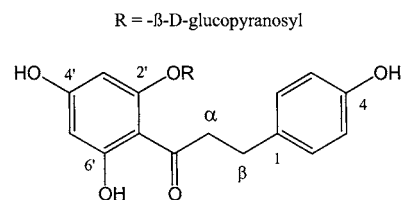
R = -ß-D-glucopyranosyl

Figure 1. Structure of the dihydrochalcone glycoside phloridzin.

32 software. The column used was a Phenomenex (Torrance, CA) C18 Aqua (250 × 21.2 mm i.d., particle size 5 μm, pore size 125 Å) operated at room temperature. The mobile phase was the same as used for analytical HPLC. The gradient program was as follows: 55% B to 65% B (10 min), 65% B to 100% B (5 min), at a flow rate of 10 mL/min. Monitoring was performed at 280 nm. Aliquots of 250 μL were applied per injection, and the effluent was collected from 14.5 to 15.5 min. The solution was stored at − 80 °C in a nitrogen atmosphere. During solvent evaporation in vacuo, small portions of water were added occasionally to avoid acid hydrolysis of the analyte. Finally, the samples were lyophilized for storage. The residue was dissolved in 1.5 mL of methanol, and aliquots of 20 μL were taken for further purification by analytical HPLC as described above. The effluent was collected from 45 to 45.5 min. The solvent was evaporated, and the samples were stored at −80 °C in a nitrogen atmosphere.

NMR Analysis. NMR spectra were recorded on a Varian Unity Inova 500 spectrometer. The ^{1}H and ^{13}C chemical shifts were referenced to solvent signals at $\delta_{H/C}$ 8.71/149.8 (pyridine-d_5), relative to that of TMS. All 1D (^{1}H, DPFGNOE) and 2D (DQFCOSY, GHSQC, GHMQC, G = "gradient enhanced") NMR measurements were performed using standard Varian pulse sequences. ^{1}H−^{13}C correlation spectra were recorded by GHSQC (J_{C-H} = 140 Hz) for the determination of proton-bearing carbons and GHMQC ($^nJ_{C-H}$ optimized for 5 Hz) for multibond correlations (HMBC).

Determination of the Configuration of the Sugar Moiety. The configuration of the glucopyranosyl moiety of phloridzin was determined following the method of Oshima and Kumanotani (*23*) by preparation of the 1-(N-acetyl-α-methylbenzylamino)-1-desoxyalditol pentaacetate derivative of the sugar moiety and consecutive HPLC analysis. The corresponding derivatives of L- and D-glucose were used for comparison.

The HP HPLC system described above was used for separation. The column was a RP 18 Spherisorb, 2 μm (125 × 4.6 mm), operated at ambient temperature at a flow rate of 1 mL/min. The mobile phase consisted of acetonitrile and water (38:62, v/v). Monitoring was performed at 220 nm.

RESULTS

Phenolic compounds extracted from strawberry fruits cv. Senga Sengana were separated by HPLC. Apart from a number of compounds, the characterization of which is a matter of current investigations, an unknown peak, **X** (t_R = 44 min), coeluting with authentic phloridzin (**Figure 1**) was detected. UV spectral data of compound **X** and the standard were identical, showing maxima of 285 and 230 nm, respectively. Negative ion ESI-MS of the isolated compound provided a quasi molecular ion peak [M − H]$^-$ at *m/z* 435 which was assigned to phloridzin, and a fragment of *m/z* 273 which was identified as the phloretin aglycon. The negative ion APCI mass spectrum displayed signals at *m/z* 495 and 273 which were assigned to an adduct of phloridzin and acetic acid [M + CH$_3$COOH − H]$^-$ and the phloretin aglycon [M − 162 − H], respectively. Mass spectrometric analysis of the phloridzin reference also showed the formation of acetic acid adducts as well as identical fragmentation.

Further mass spectral analysis of the sample and phloridzin reference showed identical fragmentation up to MS^2 of the aglycon (**Table 1**).

Table 1. Characteristic Ions of Compound **X** and Phloridzin Reference Substance

	$[M - H]^-$ m/z	HPLC–ESI-MS/MS experiment, m/z (rel intens)
reference substance	435	MS^2 [435]: 435 (2), 273 (100), 167 (6)
		MS^3 [435 → 273]: 273 (27), 179 (11), 167 (100) 125 (6), 123 (17), 119 (7)
substance isolated from peak **X**	435	MS^2 [435]: 435 (4), 273 (100), 167 (7)
		MS^3 [435 → 273]: 273 (30), 179 (11), 167 (100), 125 (5), 123 (17), 119 (6)

Comparison of the ^{1}H NMR spectra of the commercial standard and the isolated phloridzin in different solvents such as MeOH-d_4 and D$_2$O containing pyridine-d_5 confirmed the presence of phloridzin. Moreover, in contrast to spectra recorded in MeOH-d_4 (overlap of six protons between δ 3.37 and 3.52 ppm; data not shown), the use of pyridine-d_5 as a solvent (solvent effect) allowed the complete assignment of all protons and carbons by 2D NMR spectroscopy including DQFCOSY, GHSQC, and GHMQC. Data for phloridzin: ^{1}H NMR [500 MHz, pyridine-d_5/D$_2$O (34:1, v/v), ppm] δ_H 7.35 (2H, d, J = 8.0 Hz, H-2, H-6), 7.16 (2H, d, J = 8.0 Hz, H-3, H-5), 6.98 (1H, d, J = 2.2 Hz, H-3′), 6.64 (1H, d, J = 2.2 Hz, H-5′), 3.95 (1H, dt, J = 17.1, 7.2 Hz, H-α), 3.84 (1H, dt, J = 17.1, 7.2 Hz, H-α), 3.12 (2H, m, 2 H-β), 5.69 (1H, d, J = 7.6 Hz, Glu H-1), 4.50 (1H, dd, J = 2.0, 12.0 Hz, Glu H-6), 4.37 (2H, overlapped, Glu H-2,3), 4.32 (1H, dd, J = 5.7, 12.0 Hz, Glu H-6), 4.27 (1H, t$_{br}$, J = 9.6 Hz, Glu H-4), 4.11 (1H, ddd, J = 2.0, 5.7, 9.5 Hz, Glu H-5);

^{13}C NMR [^{13}C shifts were derived from GHSQC and GHMQC at 500 MHz, pyridine-d_5/D$_2$O (34:1, v/v), ppm] δ_C 133.0 (C-1), 129.8 (C-2), 115.9 (C-3), 157 (C-4), 106.6 (C-1′), 162.3 (C-2′), 95.3 (C-3′), 166.4 (C-4′)*, 97.8 (C-5′), 167.2 (C-6′)*, 206.1 (C=O), 45.7 (C-α), 29.4 (C-β), 102.2 (C-Glu-1), 74.5 (C-Glu-2), 78.3 (C-Glu-3), 70.9 (C-Glu-4), 78.3 (C-Glu-5), 62.0 (C-Glu-6). The asterisks indicate that the assignments may be interchanged.

The determination of the absolute configuration of the sugar moiety of phloridzin was performed according to a modified method described by Oshima and Kumanotani (*23*). HPLC analysis using internal diastereomeric standards of the L-MBA-alditol acetates of D- and L-glucose revealed a D-configuration of the glucose of the isolated phloridzin. Retention times of diastereomeric reference samples were 12.5 min for the D-MBA derivative and 11.4 min for the L-MBA derivative.

Quantification of phloridzin was performed for freeze-dried fruits of the three cultivars Honeoye, Senga Sengana, and Elsanta, which contained 4.9 ± 0.2, 4.6 ± 0.2, and 1.9 ± 0.1 mg/100 g, respectively, on a dry matter basis. However, phloridzin could not be detected in strawberry achenes.

DISCUSSION

Dihydrochalcone glycosides have so far been considered to be restricted to the genus *Malus* L. and have therefore been used as a marker for the detection of fraudulent admixtures (*10*, *15*, *16*, *18*, *24*). It was not until 1993 that the structures of phloretin glucoside (phloridzin) and phloretin xylosylglucoside were unambiguously established by NMR spectroscopy (*17*). Phloretin xylosylgalactoside has only been tentatively identified in Golden Delicious apples (*25*). 3-Hydroxyphloridzin has been detected in freeze-dried apple pomace (*26*). Using mass spectrometry and NMR spectroscopy, phloridzin has been identified in 3 of 18 populations examined of *Lithophragma affine* A. Gray (Saxifragaceae), which marked the first report of phloridzin outside the Rosaceae family (*27*). Very recently, the phenolic profile of rose hip (*R. canina* L., Rosaceae) was investigated by LC−ESI-MS/MS (*20*). Among 21 phenolic

compounds characterized, 4 with a pseudo molecular ion [M − H]$^-$ of m/z 435 were detected. Two of them were tentatively assigned to taxifolin pentosides, on the basis of the fragmentation of the aglycon. Product ion spectra of the remaining compounds showed identical fragment ions at m/z 273. Whereas the identity of the first remained to be determined, the fragmentation pattern of the aglycons allowed the assignment of the second to phloretin.

While rose hip has only limited relevance in the food market, the detection of phloridzin in strawberries is of considerable interest not only from a chemotaxonomic point of view but especially with respect to authenticity control of strawberry products. According to ref. *28*, adulteration mainly arises since it can be profitable and since adulterants can be easily mixed into a puree or juice and are difficult to detect. As described by others, adulteration of fruit products practiced by the admixture of fruits of less commercial value is a common practice (*11*). Apple puree has been reported to be a likely adulterant of raspberry puree (*29*). The observation that the presence of phloridzin in commercial pear juice is indicative of undeclared addition of apple juice (*9*) has been confirmed by investigations of authentic pear samples in our laboratory.

While in apple fruits phloridzin is mainly localized in the seeds and skin (*30*), strawberry achenes were found to be devoid of phloridzin in the present study. The contents of phloridzin as determined in the receptacles of three strawberry cultivars ranged from 1.9 to 4.9 mg/100 g of dry matter, which roughly corresponds to the same amount per kilogram of fresh fruit (*3*). Compared to the contents of strawberry phenolics other than phloridzin (*3*), it becomes evident that phloridzin may be classified as a minor constituent. Direct comparison with the results reported by other authors was not possible since quantification of flavonoids has been based on their aglycons (*2*, *31*, *32*).

Although strawberry phenolics have been the subject of various investigations, surprisingly phloridzin has not previously been detected. Therefore, only improved chromatographic performance, combined with selective and sensitive mass spectrometric techniques, allowed the detection even of minor phenolic constituents (*19*, *33*). Since Rosaceae plants have been shown to contain phenolic compounds displaying MS fragmentation similar to that of phloridzin (*20*), further investigations by NMR spectroscopy were a prerequisite for unambiguous identification. Therefore, a combination of tandem mass spectrometry and NMR spectroscopy was used in our study. Contamination of samples, glassware, and the HPLC system by the authentic standard material was ruled out by analyzing blanks which were shown to be devoid of phloridzin.

Apples are known to contain both phloretin glucoside and phloretin xylosylglucoside in comparatively large amounts. Since in our study the xyloglucoside was not detectable in strawberries, the lack of this compound may be used for authenticity control of strawberry products. Whereas simultaneous detection of phloridzin and phloretin xylosylglucoside, especially at higher levels, is an indicator of fraudulent

Detection of Phloridzin in Strawberries

J. Agric. Food Chem., Vol. 51, No. 10, 2003 **2899**

admixtures, phloridzin solely detected at low amounts points to strawberry origin.

Our findings and the results of previous studies (*27, 20*) support the assumption of a more widespread occurrence of phloridzin. It is expected that further studies of the phenolic profile of other Rosales will corroborate our findings. Therefore, the almost dogmatic view that phloridzin is exclusively found within the genus *Malus* L. should be revised.

ABBREVIATIONS USED

MBA, L-α-methylbenzylamine; TFA, trifluoroacetic acid.

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