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Short communication

High-performance liquid chromatographic quantitation of phloridzin in apple seed, leaf and callus

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Abstract

A quick and simple method was developed for the analysis of phloridzin in apple leaf, stem and callus. The method involves a simple extraction with ethanol–water (30:70) followed by quantitation on a reversed-phase column at 280 nm and offers several advantages over other methods described in the literature.

1. Introduction

Phloridzin has been claimed to be the major glycoside present in apple seed [1–3] and has been associated with seed dormancy [4,5]. In our apple breeding program, aimed at selecting varieties with a lower chilling requirement to break dormancy, a quick and accurate method to quantitate phloridzin in apple (seed, leaf and callus) will be required. Phloridzin in apple seed coats can be separated by thin-layer chromatography, solvent eluted and quantitated by a colorimetric method [5]. However, this method is very tedious and not very precise. More recently, phloridzin has been quantitated by HPLC [6]. I have previously reported a GC method for phloridzin quantitation in apple seed [7] using the trimethylsilyl derivative (TMS).

The HPLC procedure for phloridzin analysis [6] involves a tedious extraction with two separate solvents for several hours with large amounts of solvents in a Soxhlet extractor, followed by evaporation and further quantitation on a reversed-phase column using a very expensive and toxic solvent (acetonitrile). Our GC procedure involves a simple extraction in a test tube for 10 min, using an inexpensive and less toxic solvent system (ethanol–water, 30:70) but it is followed by the tedious formation of the TMS derivative and quantitation. Hence, both methods are not very suitable for routine screening of a large number of samples (as will be required in our breeding program).

In this communication, a simplified procedure is reported for phloridzin quantitation in apple (seed, leaf and callus), suitable for routine analysis of a large number of samples. The procedure combines the advantages of simple phloridzin extraction used in the previously described GC method [7] followed by HPLC quantitation [6]. Also, since the solvent (acetonitrile) used with the HPLC method described in the literature [6] is very expensive in Brazil, we have replaced it with ethanol which is considerably less expensive besides being less toxic. The low cost and less toxicity of the solvent are very important factors as we plan to analyze large number of samples over the next few years.

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2. Experimental

2.1. Instrumentation

An Instrumentos Cientificos high-performance liquid chromatograph (Model 480 C, São Paulo, Brazil) equipped with two pumps, a Rheodyne injector and a variable-UV detector (Model 435) was used.

2.2. Reagents and methods

Phloridzin was purchased from Sigma (St Louis, MO, USA) and recrystallized twice from ethylacetate. Its purity was checked from the melting point data (107–108°C), Infrared (IR) spectrum [8] and by its elution pattern on a reversed-phase HPLC (C_{18} column, 5 μm, 15 cm × 4.1 mm I.D.) with UV detection (280 nm). Under several gradient combinations of solvents (ethanol–water, 12:88 and ethanol), standard phloridzin eluted as a single peak.

2.3. Column

A 5 μm reversed-phase column (C_{18}, 15 cm × 4.1 mm I.D., Merck, Rio de Janeiro, Brazil) and a reversed-phase precolumn (2 cm × 4.1 mm I.D., Merck) were used. Both the column and the precolumn were thermostated in an oven at 30°C.

2.4. Chromatography conditions

For isocratic elution the solvent used was ethanol–water (20:80). For gradient elution, ethanol–water (12:88) (solution A) and ethanol (solution B) were used. The best resolution was obtained with the gradient presented in Table 1 at a flow-rate of 1 ml/min. Under these conditions, phloridzin eluted as a single peak and appeared to be ca. 99% pure as judged by area measurement, with a retention time (t_R) of ca. 8 min.

Table 1
Gradient system used for analysis of phloridzin in apple callus

Time (min)	Solvent B (%)
0	10
10	20
12	10

2.5. Extraction procedure

Seed coat

The methodology for phloridzin extraction has been previously described [7] and uses a quick extraction (about 10 min) with an inexpensive solvent system of ethanol–water (30:70).

Leaf and callus

The callus samples were obtained from plants regenerated "in vitro" and frozen until extraction. Leaf and callus tissues were allowed to attain room temperature, dried at 50°C in an oven (with forced air) for 24 h to a constant mass and ground (400 μm). Approximately 100 mg of the dried sample was weighed in a test tube and extracted three times with ethanol–water (30:70) as standard phloridzin was very soluble in this solvent. The extracts were pooled, diluted, filtered and injected onto the HPLC system. In the case of callus, initial tests showed that the concentration of phloridzin was very small and hence, the combined extracts after phloridzin extraction were evaporated to dryness and dissolved in 100 μl of ethanol–water (30:70) filtered and injected onto the HPLC system.

2.6. Efficiency of phloridzin extraction

The efficiency of the extraction procedure was checked by spiking two samples each of leaf, seed coat and callus (highest and lowest phloridzin contents) with a known quantity of standard phloridzin, followed by requantitation of the sample.

2.7. Reproducibility of the chromatographic system

HPLC system

The reproducibility was tested by injecting 1 μg of standard phloridzin ten times and determining the t_R and area under the peak. The standard deviations for t_R and area under the peak were determined.

GC system

The details have been previously described [7].

2.8. Preparation of the standard curve, identification and quantitation of phloridzin in samples

The standard curve was obtained by injecting known quantities of phloridzin, calculating the area under peak by multiplying the peak height with the peak width at half peak height (no integrator was available) and computer plotting the quantity of phloridzin injected against the area of the peak using linear regression. Phloridzin was identified in the samples by comparison of the t_R with the standard and its concentration was calculated from the peak area.

3. Results and discussion

3.1. Chromatography

We tried to develop an inexpensive solvent system for the analysis of phloridzin using an isocratic elution as we plan to analyze a large number of samples. The development of an isocratic system was possible for the analysis of phloridzin in apple seed and leaf and acceptable results were obtained with ethanol–water (20:80) as the eluting solvent. With this solvent system, the elution time for phloridzin was ca. 8 min with the peak shape being good. However, the use of an isocratic system was not possible in the case of callus samples due to the significantly lower phloridzin concentration (100 times lower). In this case we developed a gradient elution and the best results were obtained with the two-solvent system described in Table 1.

The reproducibility of the chromatographic system was good as judged by the standard deviation data on t_R and the area obtained under the peak being 2.5 and 3.3%, respectively, when 1 μg of standard was injected ten times over a 6-h period. The peak shape was good as was the sensitivity of the instrument (100 ng were easily detected).

3.2. Recovery

As can be observed from the data in Table 2, recovery of phloridzin (standard added to apple seed coat, leaf and callus samples was good, varying from 87 to 92%.

3.3. Quantitation

The equation (linear regression) for the calibration curve was $y = 0.9175x - 0.00847$. Typical chromatograms obtained for seed coat, leaf and callus samples are shown in Fig. 1a, b and c, respectively.

Phloridzin concentrations determined in the seed coat of eight apple varieties are presented in Table 3. As can be observed, a good variation in phloridzin concentration was found within the varieties. The chromatograms were quite clean and in all cases one major peak was recorded (Fig. 1a). As can be observed from Table 3, the concentrations determined by isocratic and gradient elution are essentially the same.

Also presented in Table 3 are the concentrations of phloridzin in eight apple seed coat

Table 2
Average recovery of standard phloridzin added to apple seed coat, leaf, and callus

	Recovery (%)	
	Gradient elution	Isocratic elution
Seed coat	92 ± 0.09	90 ± 0.10
Leaf	88 ± 0.14	92 ± 0.12
Callus	87 ± 0.4	87 ± 0.48

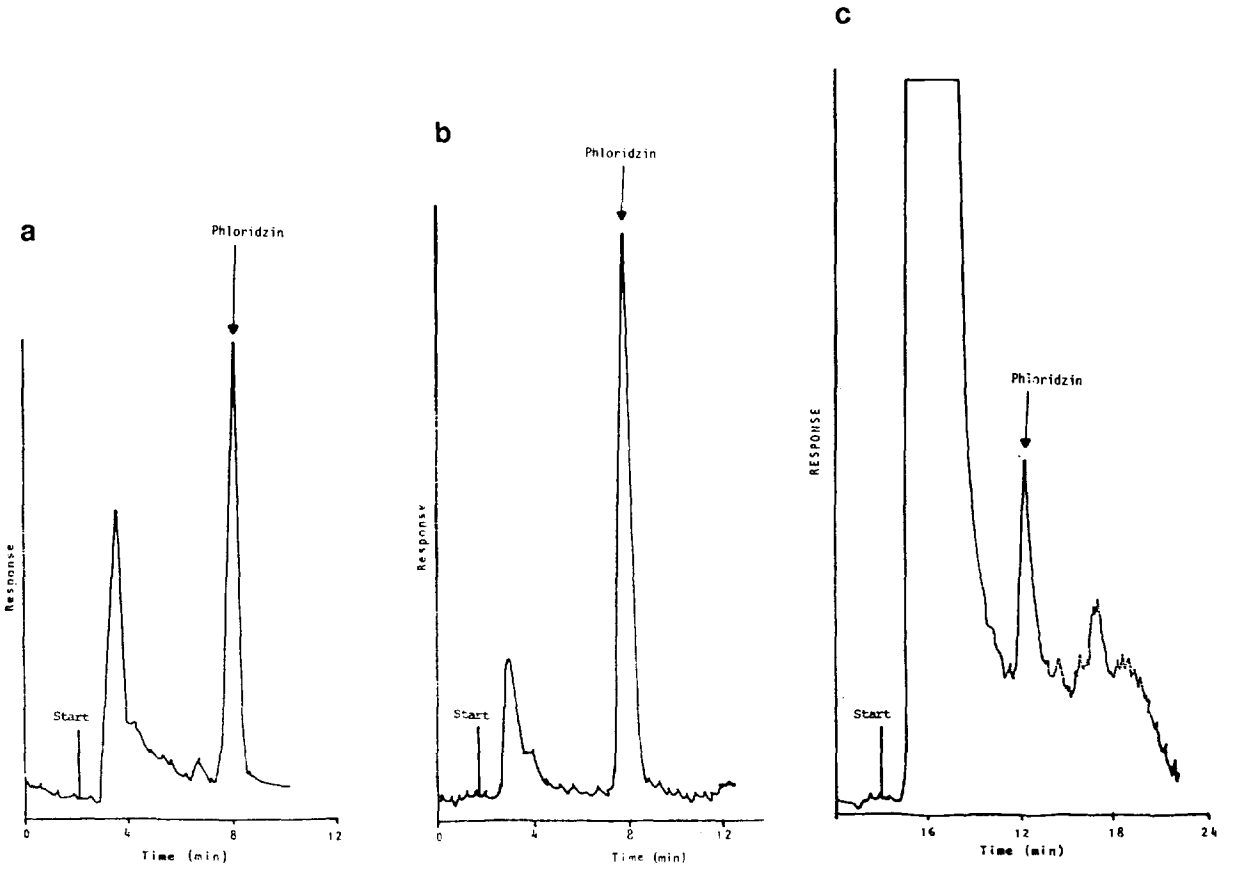

Fig. 1. Typical chromatograms obtained for (a) seed coat sample, (b) leaf sample, (c) callus sample.

Table 3
Concentration[a] (g/100 g of dry mass of seed) of phloridzin in the seed coat of 8 apple varieties determined by HPLC and GC [7]

Variety	GC	HPLC[b]	HPLC[c]
Ivete	6.21 ± 0.06	5.20 ± 0.40	5.14 ± 0.04
Ozark Gold	1.77 ± 0.01	1.22 ± 0.02	1.10 ± 0.02
Brasil	1.06 ± 0.01	0.73 ± 0.04	0.78 ± 0.05
Rainha catarina	0.92 ± 0.05	0.69 ± 0.02	0.70 ± 0.02
Winter banana	0.46 ± 0.02	0.32 ± 0.01	0.29 ± 0.01
Hawai	0.41 ± 0.01	0.30 ± 0.04	0.20 ± 0.05
Golden delicious	0.39 ± 0.02	0.20 ± 0.01	0.21 ± 0.01
Paulista	0.24 ± 0.00	0.20 ± 0.01	0.16 ± 0.01

[a] Average of two determinations.
[b] Gradient.
[c] Isocratic elution.

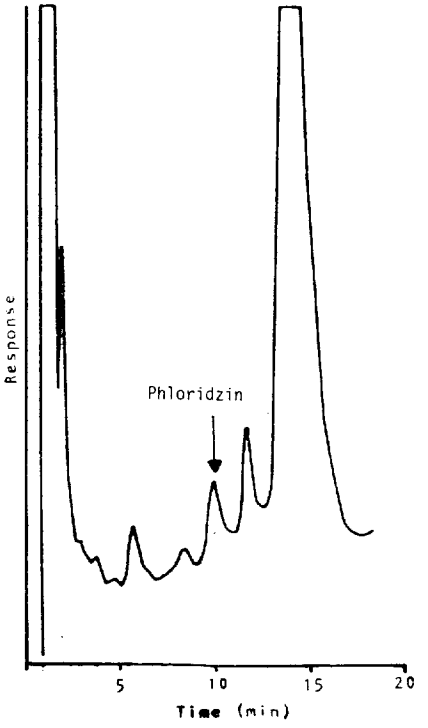

Fig. 2. Typical chromatogram obtained with TMS derivative of ethanolic extract of apple seed coat [7].

varieties, determined by GC using the TMS derivative [7]. In Fig. 2, a typical chromatogram obtained is presented. As can be seen from the chromatogram, several peaks were obtained and the peak corresponding to phloridzin is ca. 5% of the total peak area. Although the peak shape was reasonably good, there was a fair amount of tailing. However, the results obtained with GC were consistently higher compared with those obtained with HPLC (Table 1). The consistently higher results could be due to the fact that the peak corresponding to TMS-phloridzin is not homogeneous and may contain unresolved contaminating peaks. On the other hand, it appears that the HPLC peak in the natural sample corresponding to phloridzin appears to be pure as judged by its peak shape. This supposition is further supported by the fact that identical results were obtained for phloridzin analysis in the case of seed coat, with isocratic and gradient elution (which is known to possess a higher resolution capacity).

Table 4 shows the concentrations of phloridzin in leaf and callus of eight apple varieties. Typical chromatograms obtained with isocratic elution

Table 4
Concentration[a] (g/100 g of dry mass) of phloridzin in leaf and callus tissue in eight apple varieties determined by HPLC

Variety	Leaf[b]	Callus[c]		
		Sub cultures		
		1	2	3 to 5
Winter banana	2.49 ± 0.01	0.051 ± 0.002	0.035 ± 0.003	N.D.
Golden delicious	3.48 ± 0.11	0.044 ± 0.001	0.042 ± 0.003	N.D.
Ozark Gold	4.83 ± 0.09	0.012 ± 0.001	0.010 ± 0.006	N.D.
Brasil	5.82 ± 0.05	0.012 ± 0.003	0.002 ± 0.006	N.D.
Paulista	7.14 ± 0.02	N.D.	N.D.	N.D.
Ivete	8.38 ± 0.10	N.D.	N.D.	N.D.
Hawai	8.62 ± 0.04	N.D.	N.D.	N.D.
Rainha catarina	10.87 ± 0.07	N.D.	N.D.	N.D.

[a] Average of two determinations.
[b] Isocratic elution.
[c] Gradient elution.
N.D. = Not detected.

with leaf and callus samples are shown in Fig. 1b and c, respectively. As can be observed from Fig. 1b, a clean chromatogram was obtained with leaf sample.

The chromatograms obtained with callus samples were not very clean and a large peak eluted with the solvent in all samples. This peak interfered with the phloridzin peak and only with gradient elution could this peak be separated. Hence, in the case of callus sample, the use of gradient elution was indispensable.

The amount of phloridzin found in leaf was significantly higher than that found in the seed coat, which, in turn, was significantly higher than that found in the callus sample. Using our currently developed HPLC method for phloridzin analysis, we are carrying out further studies to confirm and establish correlations between seed dormancy and phloridzin concentrations in apple. For analysis of seed coat and leaf samples we are using isocratic elution while for callus tissue we are using gradient elution.

4. Conclusions

The HPLC method described in this paper is quite convenient and is being routinely used for the quantitation of phloridzin in apple seed, leaf (isocratic elution) and callus (gradient elution). Our method offers several advantages over the methods described in the literature since it utilizes an inexpensive solvent extraction and HPLC analysis.

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