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Phloridzin as a marker for evaluation of fruit product's authenticity

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Abstract: Phloridzin (phloretin-2'-O-glucoside) is a phenolic compound characteristic of the genus Malus. This study aimed to evaluate phloridzin as a marker of undeclared addition of apples in fruit products. To test this proposal, the heat and oxidation stability of phloridzin was firstly confirmed. Then the distribution and variability of phloridzin in apples were studied, showing no difference between the tested apple varieties (Golden Delicious, Granny Smith, Rubin and Champion) but a significant difference in phloridzin content in seeds (2 380 ± 755 mg kg^{-1}) compared to peel, flesh and core, which contained less than 70 mg kg^{-1}. The effects of different stages of apple purée production at an industrial scale were also investigated. The kinetics of phloridzin diffusion from seeds to apple homogenate played an important role in the final phloridzin content in 16 analysed apple purées (26–39 mg kg^{-1}). Finally, the survey of phloridzin content in 31 fruit products in the market was carried out. Phloridzin was also measured in eight jams and fillings which did not declare the presence of apples on their labels; findings from 2 to 6 mg kg^{-1} indicate the addition of apples from 5% to 20%. It was confirmed that phloridzin appears to be a suitable marker for detecting the undeclared presence of apples, which are a cheap substitute for the declared fruit types.

Keywords: phloridzin; HPLC; apples; stability; technology; adulteration

Phenolic compounds are nutritionally and organoleptically important substances found in fruit. They influence bitterness and aroma of foods and contribute to the turbidity of apple juices (Khanizadeh et al. 2008). The largest and most thoroughly studied group of polyphenols are flavonoids, in which several thousand compounds, e.g. flavanones, catechins, anthocyanidins, isoflavones, chalcones and dihydroflavonols, are included. The object of the present study, phloridzin, belongs to the chalcones (Cook and Samman 1996; Nowakowska 2006; Kun et al. 2017; Zhou et al. 2017). Biosynthesis of phloridzin was completely described by Gosch et al. (2009, 2010). Phenolic compounds, such as phloridzin, are considered to be effective chemotaxonomic markers and an adequate tool for the evaluation of authenticity of food e.g. fruit juices, jams etc. (Vandercook 1977; Gomis et al. 2001; Dragovic-Uzelac et al. 2005; Tanriöven and Ekşi 2005; Spinelli et al. 2016; Xu et al. 2016).

Phloretin 2'-glucoside (phloridzin) and phloretin 2'-xylosilglucoside are used as markers of apple raw materials (Dragovic-Uzelac et al. 2005). The distribution of phloridzin in apples depends strongly on the apple variety (Khanizadeh et al. 2008; Valavanidis et al. 2009). Great differences are also found in various apple tissues (skin, flesh and core). The highest concentra-

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tion of phloridzin was found in apple seeds (Khanizadeh et al. 2008). Published data on the concentration of phloridzin in apples and some other plants are shown in Table 1. No significant differences in terms of phloridzin concentrations were observed between organic and conventionally grown apples (Valavanidis et al. 2009). The degradation of phloridzin by fungi was described. Phloridzin is transformed by fungi to phloretin, phloroglucinol and phloretic acid. Phloretic acid is transformed into p-hydroxybenzoic acid in the next step (Jayasankar et al. 1969). Small decreases of phloridzin content occurred during storage of apples in a regular and ultra-low oxygen environment (Awad et al. 2000). A significant decrease of phloretin glycosides was observed in juices during storage at a temperature of 25 °C for 9 months (Spanos et al. 1990). Furthermore, the low influence of a modified atmosphere and calcium ascorbate solution on phloridzin content was observed in apple slices (Aguayo et al. 2010). The assumption that phloridzin occurred only in apples persisted for a considerable period of time. Subsequent research revealed that phloridzin was also found in some other plants, see Table 1.

However, the content of phloridzin in other kinds of technologically important fruits is not at such high concentrations as in apples (Williams 1964; Iwashina 2000). In the literature, phloridzin is mentioned as a qualitative marker typical of apples, and its use for the detection of counterfeit products was discussed in these studies: Versari et al. (1997); Fügel et al. (2005); Górnaś et al. (2015) and Kamiloglu (2019).

This study aimed to evaluate phloridzin as a suitable characteristic marker for assessing the presence of undeclared apple raw material in food products despite the variety of used technologies.

MATERIAL AND METHODS

Samples

Apple cultivars (Golden Delicious, Granny Smith, Rubin, Champion and Idared; class I, Czech Republic) purchased in the Czech market were used. Samples (apples, crushed apples and apple purées – from the cultivar Idared) for the evaluation of the effect of technology on the content of phloridzin were obtained from the production line of the Hamé s.r.o. Company. Samples of apple purées (10–12 °Brix) were produced at the Hamé Company in Kunovice, the Czech Republic. The samples were taken directly from the production line (Hamé s.r.o). The production line used for the production of apple purée in the Hamé factory is described in Figure 1. The whole apples were boiled in steam and subsequently pulped in a pulper. Three parallel samples were taken from selected locations along the production line. The descriptions of analysed apple-based commercial products (fruit and/or apple content) are given in Tables 2 and 3. All commercial samples were purchased in the Czech market.

Determination of phloridzin content

Chemicals. A standard of phloridzin (phloridzin dihydrate, 99%) was purchased from Sigma-Aldrich (Prague, Czech Republic). Phosphoric acid was purchased from Lachema (Brno, Czech Republic). Acetonitrile and methanol were purchased from Merck (Prague, Czech Republic). Demineralised water (Milli Q quality) was used for the preparation of buffer, mobile phase, standard solutions and extraction of samples.

Instrumentation. HPLC analyses were performed on a Dionex HPLC instrument (Amedis, Prague, Czech Republic) consisting of a P680 pump, an Ulti-

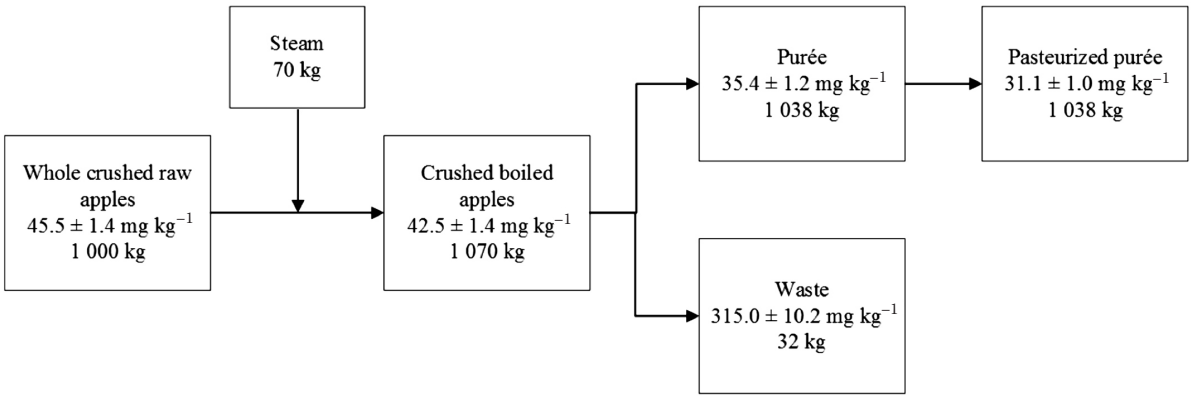

Figure 1. A common production line used for the production of apple purée (phloridzin content ± standard deviation and mass balance of raw material, product, semi-products and waste)

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Table 1. The content of phloridzin in apples and some other plants

Fruit	Phloridzin (mg kg^{-1})			References
	part of apple	min–max	average	
Apple	peel	100–150	–	Burda et al. 1990
	flesh	–	10	
Apple	seed coat	1.6–62.1	–	Jham 1996
Apple	peel	12–418	–	Escarpa and González 1998
	flesh	4–20	–	
Apple	peel	460–710	540	Awad et al. 2000
	flesh	–	80	
	seed	–	7 410	
	core	–	1 940	
Apple	peel	750–1 100	–	Awad and Jager 2001
Apple	pitted fruits	14.4–28	–	Lee et al. 2003
Apple	peel	37.6–172	–	Tsao et al. 2003
	flesh	8–24.6	–	
Apple	flesh and peel	6.4–91.1	–	Vrhovsek et al. 2004
Apple	peel	29.152–153.61	–	Petkovsek et al. 2007
	flesh	5.12–13.327	–	
Apple	peel	25.5–87.2	–	Khanizadeh et al. 2008
	flesh	6.5–16.1	–	
Apple	peel	710–2420	1 550	Łata et al. 2009
	flesh and peel	110–430	270	
Apple	peel	18–167	–	Valavanidis et al. 2009
	whole apple	6–96	–	
	peel	21–142	–	
	whole apple	8–84	–	
Apple	flesh + peel	–	9.7	Aguayo et al. 2010
Apple	flesh + peel	–	19.3	Feliciano et al. 2010
Apple	–	0.7–3.3		Bílková et al. 2020
Strawberries (dry matter)	–	–	0.38	Hilt et al. 2003
Cranberries	–	–	1.2	Turner et al. 2005
Mexican oregano (*Lippia graveolens*)	–	–	13.6	Lin et al. 2007
Rosa hip (*Rosa canina*)	–	–	0.037	Cunja et al. 2015

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Table 2. The content of phloridzin in selected products in the Czech market (declared apple content is given in round brackets; declared fruit content is given in square brackets; only for samples where it was attached)

Sample	Phloridzin (mg kg^{-1})	Estimated apple content (%) *
Plum jam with apples[a]	19.7 ± 0.5	60.6–68.6
Plum jam[b, c]	4.8 ± 0.2	14.8–16.7
Apple bakery filling (90%)[d]	6.1 ± 0.1	18.8–21.3
Plum bakery filling (220%)	34.7 ± 0.4	106.8–120.9
Apricot bakery filling (70%)[e]	1.5 ± 0.1	4.6–5.2
Strawberry bakery filling (70%)[e, i]	1.8 ± 0.2	5.5–6.3
Blueberry bakery filling (70%)[g, h, i]	3.0 ± 0.3	9.2–10.5
Sour cherry bakery filling (70%)[b, g]	4.2 ± 0.1	12.9–14.6
Peach jam[e, h]	1.8 ± 0.1	5.5–6.3
Apple baby food	25.0 ± 0.2	76.9–87.1
Strawberry baby food[a]	20.1 ± 0.2	61.8–70
Apple purée (95%)	31.4 ± 0.2	96.6–109.4
Apple purée (92%)	29.7 ± 0.5	91.4–103.5
Lubawa black currant jam[e]	1.7 ± 0.1	5.2–5.9
Apple-blueberry jam (35%)	13.9 ± 0.1	42.8–48.4
Apple-strawberry jam (35%)	15.6 ± 0.4	48–54.4
Apple-apricot jam (35%)[f]	8.2 ± 0.2	25.2–28.6
Apple-currant-strawberry jam (50%)	10.8 ± 0.5	33.2–37.6
Apple-apricot jam (35%)	12.6 ± 0.1	38.8–43.9
Apple-strawberry jam (35%)[f]	8.1 ± 0.2	24.9–28.2
Blueberry jam[c, d]	5.7 ± 0.1	17.5–19.9

*calculated at a confidence interval of 32.5–28.7 mg kg^{-1} phloridzin (confidence level: 0.95) for a pure apple purée; each value is expressed as the mean ± SD; there is no statistical difference between the samples denoted by the same letter ($P > 0.01$)

mate 3 000 photodiode array detector (Thermo Fisher Scientific, Bannockburn, USA) and an ASI 100 autosampler controlled by the Chromeleon 6.80 software package.

HPLC assay. Chromatographic separation of phloridzin was carried out on an Eclipse XBD C8 column (150 mm × 4.6 mm, 5 mm, Agilent). The column was purchased from Chromservis (Prague, Czech Republic). The column temperature was kept at 30 °C. The mobile phase consisted of 0.01 mol L^{-1} of phosphoric acid in water (solvent A) and 0.01 mol L^{-1} of phosphoric acid in acetonitrile (solvent B). The elution conditions were: 0–5 min, 5% B isocratic; 5–15 min, linear gradient 5–20% B, 15–20 min, linear gradient 20–40% B, 20–28 min, linear gradient 40–80% B, 28.1–35 min, 5% B isocratic. Flow rate was 1 mL min^{-1}. The quantification was at 190–400 nm wavelengths. For detec-

Table 3. The content of phloridzin in 100% apple juices from the Czech market

Sample	Phloridzin (mg kg^{-1})
Sample 1[d]	18.7 ± 0.7
Sample 2[a]	5.6 ± 0.1
Sample 3[a]	5.7 ± 0.2
Sample 4[a]	4.7 ± 0.1
Sample 5[a, b]	10.1 ± 0.3
Sample 6[b, c]	8.4 ± 0.6
Sample 7[a, c]	6.6 ± 0.2
Sample 8 (unfiltered)[d]	18.8 ± 0.5

Each value is expressed as the mean ± SD; there is no statistical difference between the apple juices denoted by the same letter ($P > 0.01$)

https://doi.org/10.17221/239/2020-CJFS

tion, the 280-nm wavelength was chosen as the most suitable wavelength. One analysis took 35 minutes. The quantitative analysis was based on an external standard method.

The phloridzin content was determined by an HPLC assay and the validation parameters of the HPLC assay were set as follows: linearity: 0–100 mg L^{-1}, repeatability: 3.5% (n = 7, concentration level 50 mg L^{-1}), recovery: 97% (concentration level 50 mg L^{-1}), recovery: 96% (concentration level 8 mg L^{-1}), limit of detection: 0.3 mg kg^{-1} and limit of quantification was 1 mg kg^{-1}.

Measurement of refraction. Measurement of refraction (expressed in °Brix) was done according to ISO 2173:2003 (Fruit and vegetable products — Determination of soluble solids — Refractometric method).

Optimisation of phloridzin extraction. The extraction time of phloridzin from a sample was optimised. The set of samples (raw apples and fruit baby foods) was extracted for two hours in methanol (80 : 20 methanol/water, methanol for HPLC, ≥ 99.9% Sigma-Aldrich; Germany) under ultrasound (Kraintek, Czech Republic). The ratio of the sample to methanol was 1 : 4. The samples for the optimisation of phloridzin extraction were taken at regular intervals (30 min).

Determination of thermal and oxidation stability of phloridzin

The apple purée sealed in a jar was heated in the water bath (100 °C) for 2 hours. The samples for the evaluation of thermal stability were taken at regular intervals (30 minutes).

For the evaluation of phloridzin oxidation stability, pulped apples were exposed to the air for one hour. The purée was aerated [air: mixture of N_2 (nitrogen) and O_2 (oxygen); SIAD Czech spol. s r.o., flow: 50 ml min^{-1}, 30 min] from a gas cylinder using a needle. The samples were taken at the beginning and end of the experiment. The phloridzin content was determined by the procedure mentioned in the chapter "Determination of phloridzin content".

To determine the influence of technological processing, the apples were crushed with a laboratory homogeniser and boiled in a sealed jar under nitrogen atmosphere for 60 minutes. The boiled apples were pushed through a fine mesh screen and the phloridzin content was analysed.

Sample treatment. The homogenised sample (5 g) was weighed in a 50-ml volumetric flask, and 45 ml of methanol-water mixture (20:80 v/v) was added. The sample was extracted in the ultrasonic bath at 25 °C for twenty minutes. Then the content was cooled down to 20 °C and made up to volume with methanol-water mixture (20:80 v/v). The filtrate (PTFE Filter, 25 mm, 0.45 μm) was analysed for phloridzin content.

Statistical analysis

The analyses were triplicated for each sample and mean ± SD values are given. All statistical analyses were performed using one-way ANOVA with post-hoc Tukey HSD Test (Excel 2016, Microsoft Corporation and Statistica 12.7).

RESULTS AND DISCUSSION

Phloridzin content in selected apple parts. The stability of phloridzin was determined. During phloridzin extraction, the maximum value of phloridzin concentration was reached after fifteen minutes. Then it remained constant. Based on these findings, it can be assumed that fifteen minutes is a sufficient time for phloridzin extraction.

As another parameter, the influence of oxygen action on the degradation of phloridzin content was addressed. The results showed that the phloridzin loss was insignificant during heating, and the degradation of phloridzin content was statistically insignificant during the oxygen action.

Table 4. The content of phloridzin in selected apple cultivars

	Granny Smith[a]	Golden Delicious[a]	Rubin[a]	Champion[a]
	phloridzin (mg kg^{-1})	phloridzin (mg kg^{-1})	phloridzin (mg kg^{-1})	phloridzin (mg kg^{-1})
Peel[b]	10.0 ± 0.4	34.3 ± 21.3	20.0 ± 4.9	18.8 ± 8.4
Flesh[b]	2.0 ± 0.8	3.4 ± 2.0	1.8 ± 0.5	6.9 ± 1.8
Core (without seeds)[b]	16.8 ± 11.8	18.9 ± 6.6	13.6 ± 7.3	66.7 ± 22.9
Seeds	2307 ± 337	2566 ± 1529	2911 ± 163	1734 ± 173

Each value is expressed as the mean ± SD; there is no statistical difference between the results denoted by the same letter (P > 0.01)

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Phloridzin content was measured in four apple cultivars. These apple cultivars commonly used for technological treatment were chosen: Granny Smith, Golden Delicious, Rubin and Champion. Four apples from each cultivar were precisely divided into four parts (peel, flesh, core and seed). The phloridzin content in selected apple parts is shown in Table 4. No statistically significant difference between apple cultivars was confirmed (Table 4). Statistically significant differences were observed between seeds and the other parts of apples (Table 4). The highest concentration of phloridzin was obtained from apple seeds. Higher concentrations were also in cores and peels. The results of our study are consistent with previous studies to a different extent. On the one hand, they corresponded to a considerable extent with the data published by Aguayo et al. (2010) (Table 1). Valavanidis et al. (2009) and Khanizadeh et al. (2008) also published similar values for the amount of phloridzin found in the apples. Furthermore, the concentration of phloridzin in peels closely matched the findings of Escarpa and González (1998), Tsao et al. 2003 and Valavanidis et al. (2009). However, the concentration of phloridzin in seeds in our study showed less similarity to the results from Awad et al. (2000). In the case of values published by Łata et al. (2009) and Awad and Jager (2001), the difference was more significant because they reported ten to a hundred times higher concentrations of phloridzin in the apples than in our results. Conversely, lower values than those measured were detected by Bílková et al. (2020). The differences in the published data could be due to different methods of detection, apple cultivars or the harvest period.

Influence of technology. The influence of technology on phloridzin content in the final products was studied. First, the simplified laboratory experiment using two apple cultivars (Idared and Granny Smith) was conducted. The concentration of phloridzin in the apple flesh was measured. The content of phloridzin was 5.0 ± 0.2 mg kg^{-1} and 2.6 ± 0.1 mg kg^{-1}, respectively. Phloridzin content in the purée made from steamed whole Idared apples was 58.4 ± 1.9 mg kg^{-1} and 10.3 ± 0.3 mg kg^{-1} in Granny Smith. From these results, it is evident that significant differences ($P < 0.01$) in phloridzin content were observed between apple purée prepared from whole apples and apple purée made from apple flesh (without seeds, peels). A higher concentration of phloridzin was observed in the boiled pulp, probably due to the movement of phloridzin from the phloridzin-rich parts of apples (seeds, cores, and peels). The results of the laboratory experiment were verified by using the technology for the production of apple purée (a mixture of different cultivars) (Figure 1).

The mass balance was estimated from dry matter (Figure 1). The highest concentration of phloridzin was found in the waste because the waste from the pulper contained a large quantity of seeds, cores and peels. Hardly any effect of heat treatment on phloridzin content was observed during pasteurisation. Only a slight decrease was noted. It was probably due to the inhomogeneity of industrial samples since the samples were processed in large volumes. The test on the production line showed that the final purée contained more phloridzin than apple flesh. The final content of phloridzin in the purée was 31.1 ± 1.0 mg kg^{-1}. Two commercially produced apple purées were also measured (declared apple content equalling 95 and 92%), and the content of phloridzin was 31.4 ± 0.2 mg kg^{-1} and 29.7 ± 0.5 mg kg^{-1}, respectively. After recalculation to 100% apple purée, the concentration of phloridzin was 33.1 ± 0.2 mg kg^{-1} and 32.3 ± 0.5 mg kg^{-1}, respectively. These measured values of phloridzin concentration in apple products were roughly consistent with the data published by Oszmiański et al. (2008) and measured values of commercially produced apple purées.

Phloridzin as a tool for assessment of food adulteration. Since our research and that of others have established the high concentration of phloridzin in apples, its concentration can be used to estimate apple content and/or detect food adulteration, especially from undeclared apple-product additives. The final content of phloridzin in apple juices is probably influenced by the technology used. Apple juice is produced by pressing raw apples, so there is no release of phloridzin from the cell walls and no diffusion from parts of the apple with a higher concentration into the apple pulp. If they had not been filtered and clarified like "sample 7 (unfiltered)", they would contain higher amounts of phloridzin, as various clarification methods can significantly reduce the content of phloridzin. Other technological processes that could affect the phloridzin content also include the processing of apple pulp by the addition of enzymes, various types of presses or pulp wash. Questions about the quantification of apple content are more difficult due to the natural variability of phloridzin in apples and the influence of the technology used on phloridzin in the apple purée. The phloridzin content range from 16 pure apple purées was 26–38.8 mg kg^{-1} (confidence interval 32.5–28.7 mg kg^{-1} phloridzin; confidence level: 0.95).

https://doi.org/10.17221/239/2020-CJFS

Because the concentration of phloridzin in the samples of apple purée was from 28.7 to 32.5 mg kg^{-1}, the estimation of apple content was calculated from this range. The results of measured samples are given in Tables 1 and 2. The estimated content of apples correlated well with its declared content, which is especially obvious from the samples of apple purées mentioned above. It follows that the content of phloridzin at the level of about 1 mg kg^{-1} (i.e. the presence of about 30 g of apples/kg products) significantly revealed the addition of apples to selected fruit products. The detectable addition of apples in fruit products corresponded very well with the data published by Dragovic-Uzelac et al. (2005). The concentration of phloridzin shown in Table 3 roughly corresponded with published data for commercially produced juices where the phloridzin content ranged from 2.3 to 38.0 mg L^{-1} (Kermasha et al. 1995; Versari et al. 1997; Schieber et al. 2001; Suárez et al. 2005; Karaman et al. 2010). Thus, the use of phloridzin as a marker of the authenticity of fruit products is promising.

CONCLUSION

The goal of this article was to confirm phloridzin as a marker suitable for apple content measurement. The concentration of phloridzin in selected apple varieties and different parts of apples as well as the stability of phloridzin during the production of apple purée was ascertained. It was confirmed that the highest concentration of phloridzin is in apple seeds followed by the core and peels. The kinetics of phloridzin diffusion from seeds to apple homogenate played an important role in final phloridzin content in 16 analysed apple purées (26–38.8 mg kg^{-1}). It was found that the content of phloridzin at the level of 1 mg kg^{-1} (i.e. the presence of approximately 30 g of apples/kg products) proves the addition of apples into fruit products. This was subsequently verified on selected products from the Czech market. Phloridzin was quantified also in eight jams and fillings that did not declare the presence of apples on their labels; findings from 2 to 6 mg kg^{-1} indicate the addition of apples from 5% to 20%. The content of phloridzin in apple juices was found at the level of 4.7–18.8 mg kg^{-1}. It was found that the influence of heat treatment or oxygen presence on the stability of phloridzin is not significant. Phloridzin is a suitable marker for detecting the presence of apples in the fruit-based product, and for an approximation of apple content it is necessary to use other markers such as sorbitol, malic acid, etc.

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