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Original research paper

Dihydrochalcones in old apple varieties from Croatia

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Summary

Dihydrochalcones are the only subgroup of polyphenols that is specific to apples. They showed many beneficial bioactivities. In this work, the aim was to analyze dihydrochalcones in old apple varieties from Croatia to see if varieties differ amongst themselves according to dihydrochalcone percentage. Dihydrochalcones were extracted from the peel and flesh of five old apple varieties (Lještarka, Ljetna rebrača, Slavonska srčika, Zimnjara, Adamova zvijezda) and from wild apples (crabapples). Reverse-phase high performance liquid chromatography was used to identify dihydrochalcones (phloretin-2'-xyloglucoside and phloretin-2'-glucoside in the flesh; phloretin-2'-glucoside in the peel) and to express their percentage in relation to all other polyphenol subclasses. Dihydrochalcones occupied from 3 to 12.4 % in the flesh and from 0.8 to 10.8 % in the peel. Slavonska srčika and Adamova zvijezda showed higher dihydrochalcone percent in the flesh and peel. Principal component analysis also confirmed that Slavonska srčika and Adamova zvijezda differed from other varieties by higher dihydrochalcone percent. The pattern in the dihydrochalcone distribution was not shown. Although dihydrochalcones do not occupy a high portion in the polyphenol content, they are still an important feature of old varieties.

Keywords: phloretin, phloridzin, dihydrochalcones, old varieties, apple

Introduction

Dihydrochalcones are a subgroup of polyphenolic compounds. Structurally they differ from some usual polyphenols (flavonoids) because an open structure and a differently numbered carbon skeleton (Tomás-Barberán and Clifford, 2000). They can be found in apples in which they actually represent a specific feature. The main dihydrochalcones found in apple's flesh are phloretin-2'-xyloglucoside and phloretin-2'-glucoside while the peel is characterized by the phloretin-2'-glucoside (Tsao et al., 2003).
The interest in dihydrochalcones increased in recent couple of decades since they are known to have some beneficial bioactivities (Ehrenkranz et al., 2005). Mostly, their beneficial effects were connected to the effects on diabetes and glucose level lowering. It was shown that dihydrochalcones can reduce blood glucose levels in mice (Masumoto et al., 2009; Kobori et al., 2012) which can be

helpful in the prevention of hyperglycemia. A potential anti-diabetic drug on the basis of phloretin-2'-glucoside has been developed (Ikumi et al., 2008; Sakuma et al., 2009). Due to these beneficial activities, dihydrochalcones are a subject of many studies.
Old apple varieties have been grown in the past and now days they are actually neglected. According to our earlier studies, they can be a good source of dihydrochalcones (Jakobek et al., 2013, Jakobek and Barron, 2016). Namely, it was shown that some old varieties have the potential to have higher dihydrochalcone content in the flesh (Jakobek et al., 2013, Jakobek and Barron, 2016) in comparison to commercial varieties (Khanizadeh et al., 2008; Lamperi et al., 2008). Therefore, the dihydrochalcone content might be an important characteristic of these old varieties. By analyzing dihydrochalcones which showed many potential bioactivities, the important characteristic of apples is being analyzed, which can impact the recognition of these old varieties as an important source of polyphenols. Furthermore, apple varieties differ by the portion of certain polyphenolic subgroups, like phenolic acids or flavan-3-ols (Jakobek and Barron, 2016). Namely, varieties with a higher phenolic acid portion usually have a lower flavan-3-ol portion and vice versa. Mentioned differences are usually dependent on genetic variability. Since, dihydrochalcones are specific to apples, it would be interesting to know if the dihydrochalcone portion also follows a certain pattern. That would be another reason to recognize some old apple varieties as an important source of dihydrochalcones.
The aim of this study was to identify dihydrochalcones in six old apple varieties by using reversed-phase high performance liquid chromatography with photodiode array detection (RP-HPLC-PDA) and to express their percentage in relation to all other polyphenol subclasses. Furthermore, the aim was to see if there are some differences between varieties based on the dihydrochalcone percentage. That is why the data were analyzed with one-way ANOVA and post-hoc Tukey test. Principal component analysis was used to see possible clustering of varieties. Additionally, the dihydrochalcone percentage was compared to the percentage of other polyphenols to see if a pattern in a dihydrochalcone distribution could be seen.

Materials and Methods

Chemicals
Dihydrochalcone standard was purchased from Extrasynthese (Genay, France) (phloretin-2'-*O*-glucoside (phloridzin - 1046 ≥ 95 %)). Other chemicals were purchased from Fluka (Buchs, Switzerland) (*ortho*-phosphoric acid (85 %, HPLC grade)), J. T. Baker (Deventer, Netherlands) (HPLC grade methanol) and Kemika (Zagreb, Croatia) (hydrochloric acid (36.2 %)).

Samples and sample preparation
Old apple varieties (*Malus domestica*) (Lještarka, Ljetna rebrača, Slavonska srčika, Zimnjara, Adamova zvijezda, and a wild variety (crabapple)) were harvested in October/November 2015 in a family orchard (Mr. Veić, Mihaljevci, near Požega, Croatia). One kilogram of apples was peeled, the peel was pooled and homogenized for 15 seconds in small portions using a coffee blender. The flesh was then cut into smaller pieces, after removing the core and the seeds. Flesh was pooled and homogenized for 1 minute with a stick blender. Samples were immediately placed in a freezer at -18 °C.

Polyphenol extraction
0.1 % HCl in methanol was used to extract polyphenols from the peel, and 80 % methanol to extract polyphenols from the flesh (Jakobek et al., 2015). Shortly, samples of flesh and peel were weighed (0.2 g) in cuvettes, mixed with 1.5 ml of extraction solvent and vortexed (Grant Bio, Cambridgeshire, England). Cuvettes were placed in the ultrasonic bath (Bandelin Sonorex, RK 100, Berlin, Germany) for 15 minutes and then centrifuged (Minispin, Eppendorf AG, Germany). After removing extracts, residues were extracted one more time with 0.5 ml of extraction solvent. Extracts were combined and filtered through a 0.45 μm polytetrafluoroethylene (PTFE) syringe filter. Two parallel extracts were prepared for each peel or flesh sample.

Reversed phase high-performance liquid chromatography with photodiode array detection (RP-HPLC-PDA)
Varian HPLC system (Varian Inc., Palo Alto, USA) which consists of ProStar 230 solvent delivery module, ProStar 330 PDA detector, OmniSpher C18 column (250 x 4.6 mm, 5 μm) and guard column (ChromSep 1 cm x 3 mm) was used to analyze polyphenols according to our earlier developed method (Jakobek et al., 2015). Mobile phases were 0.1% phosphoric acid in water (A) and 100 % methanol (B). The gradient was: 5% B (0 min), 25 % B (5 min), 34 % B (14 min), 37% B (25 min), 40% B (30 min), 49% B (34 min), 50% B (35 min), 51% B (58 min), 55% B (60 min), 80% B (62 min), 80% B (65 min), 5% B (67 min) and 5% B (72 min). Other conditions were: the flow rate 0.8 ml min^{-1}, injection volume 20 μl, spectra recording from 190-600 nm. Standard phloretin-2'-glucoside (200 mg L^{-1}) was prepared in ethanol and analyzed with the same method. Chromatograms were scanned at 280, 320, 360 and 510 nm. Dihydrochalcones were detected at 280 nm. Phloretin-2'-glucoside in the flesh and peel was identified by comparison of retention times and spectral data with the once from standard. Phloretin-2'-xyloglucoside was tentatively identified in the flesh with the help of literature data (Tsao et al., 2003). Other polyphenolic subgroups were recognized by their characteristic spectra features (UV/Vis maximum of flavan-3-ols, phenolic acids, flavonols, anthocyanins at 271; 301 or 314; 350; and 510 nm, respectively). Peak areas of dihydrochalcones at 280 nm (phloretin-2'-glucoside and phloretin-2'-xyloglucoside), flavan-3-ols at 280 nm, phenolic acids at 310 nm, flavonols at 360 nm and anthocyanins at 510 nm were expressed as percentages in the total peak area.
Statistical analysis
Two extracts from each flesh and peel were prepared. Extracts were analyzed once with the RP-HPLC-PDA method. Dihydrochalcone percentage and percentages of other polyphenolic subclasses was based on n = 2. The data were analyzed with post-hoc Tukey pairwise comparison tests which are auxiliary to one-way ANOVA. Furthermore, dihydrochalcone percentages in flesh and peel were analyzed with principal component analysis in order to visualize possible clustering of varieties. All the statistical analyzes were carried out using the analytical software Statistica (Statsoft, Tulsa).

Results and Discussion

The description of old apple varieties is shown in Table 1. Three varieties had red or light red peel while the peel in others was yellowish. Five of these varieties were already analyzed before

(Jakobek et al., 2013; Jakobek and Barron, 2016) but Ljetna rebrača was analyzed for the first time, to the best of our knowledge. It should be mentioned that some of these apples could be present in other surrounding countries with similar or different names, but for this work apples originated from Croatia. Moreover, one variety Slavonska srčika is considered as indigenous for Croatia which gives this variety a special importance.

Table 1. Description of old apple varieties

Apple variety	Harvest maturity	Flesh color	Fruit skin color
Lještarka	Late	Yellowish	Red
Ljetna rebrača	Late	Yellowish	Red
Slavonska srčika	Late	Yellowish	Light red
Zimnjara	Late	Yellowish	Yellow green
Adamova zvijezda	Late	Yellowish	Yellowish
Wild apple	Late	Yellowish	Yellowish

Fig. 1 shows chromatograms of flesh and peel scanned at 280 nm. Dihydrochalcones (Fig. 2) were identified as phloretin-2'-xyloglucoside and phloretin-2'-glucoside in the flesh and phloretin-2'-glucoside in the peel. They showed a characteristic UV/Vis absorption at 276 nm. This agrees with the literature data (Tsao et al., 2003).

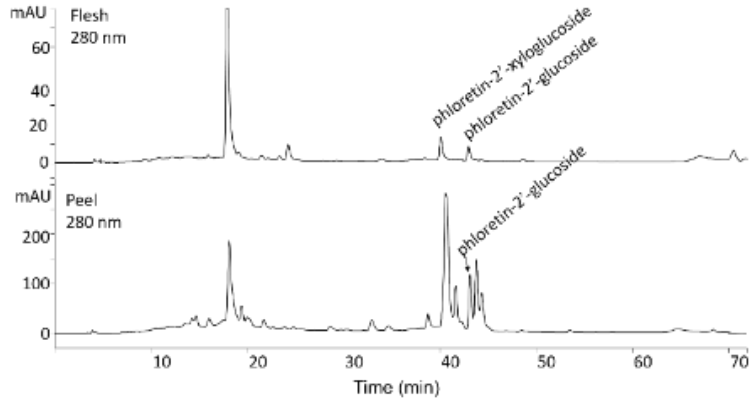

Fig 1. Chromatogram of apple flesh and peel scanned at 280 nm with dihydrochalcones identified in apples (Slavonska srčika variety)

The percentage of dihydrochalcones (Fig. 2) was calculated as a percentage of their peak areas in relation to percentage of peak areas of all other polyphenols at 280, 320, 360 and 510 nm. The results are shown in Fig. 3. In the flesh, the dihydrochalcone percentage varied from 3.7 to 12.4 %. Varieties that had high dihydrochalcone percentage in the flesh were Adamova zvijezda, Zimnjara, Slavonska srčika and Lještarka. Those varieties were also statistically similar. Varieties Ljetna rebrača and wild apples had lower portion of dihydrochalcones.

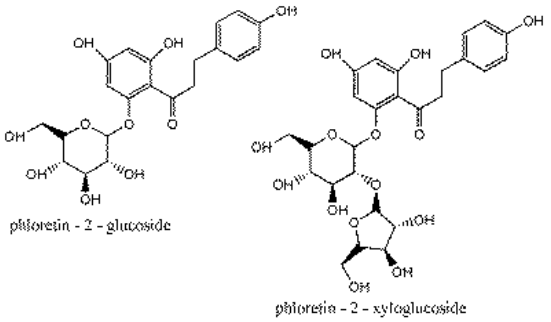

Fig. 2. Dihydrochalcones from apples: phloretin-2'-glucoside and phloretin-2'-xyloglucoside

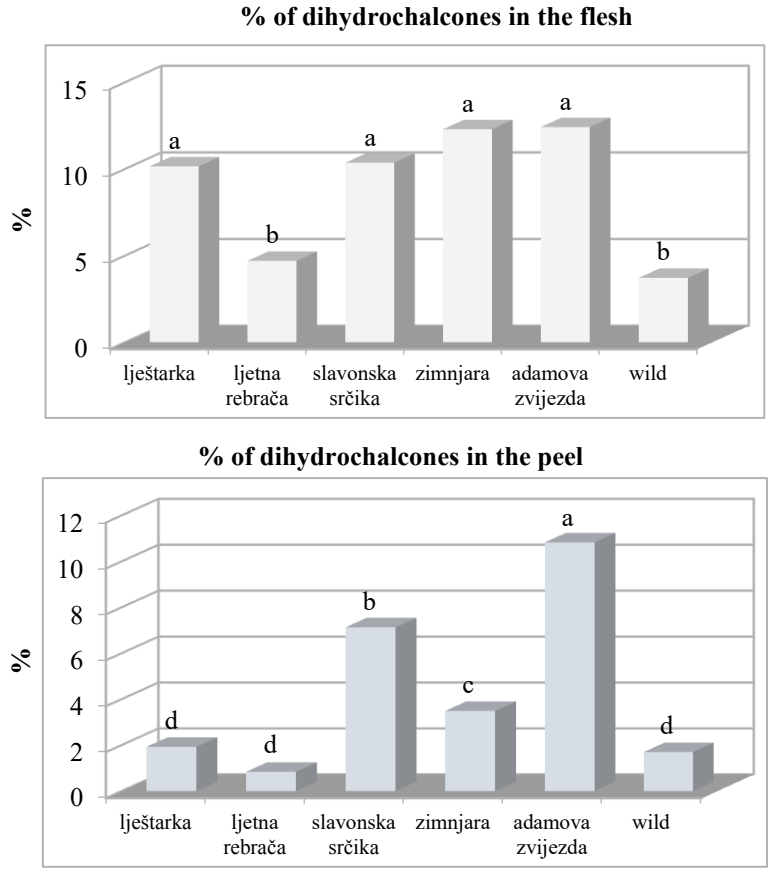

Fig. 3. Dihydrochalcone percentage in the flesh and peel of old apple varieties

In the peel (Fig. 3.), the dihydrochalcone percentage was between 0.8 to 10.8 %. Varieties with the highest dihydrochalcone percentage were Adamova zvijezda and Slavonska srčika. Zimnjara, Lještarka, wild apple and Ljetna rebrača were characterized with the lower dihydrochalcone percentage. In general, two varieties can be highlighted with somewhat higher dihydrochalcone percentage in both, flesh and peel: Adamova zvijezda and Slavonska srčika. The differences are statistically significant.

To see if varieties cluster according to the dihydrochalcone percentage, principal component analysis was conducted. This is a statistical tool that enables better visualization of data and possible clustering (Fig. 4.). All six varieties showed good separation, which points to their differences according to the dihydrochalcone percentage. Adamova zvijezda and Slavonska srčika positioned in the upper right corner of the diagram which could mean they are similar according to dihydrochalcones and it could be suggested that they create a separate cluster. Wild apple and Ljetna rebrača separated into the upper left corner, and Lještarka and Zimnjara positioned in the lower part of the diagram. In general, it is visible that varieties differ by the dihydrochalcone percentage and possible grouping into three clusters could be seen.

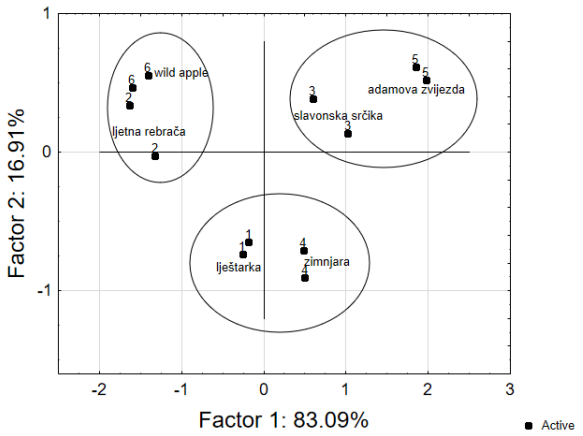

Fig. 4. Principal component analysis (PCA) of dihydrochalcone percentage in the flesh and peel of old apple varieties

Fig. 5 shows percentage distribution of all polyphenol subclasses. In the flesh, a certain pattern in the polyphenol distribution could be seen. Namely, varieties with the higher phenolic acid percentage had lower flavan-3-ol percentage and vice versa. This agrees with earlier studies (Ceymann et al., 2012; Jakobek and Barron, 2016). But there was no visible pattern connected to the dihydrochalcone distribution. In the peel, a pattern in the polyphenol distribution could not be seen. In earlier study (Voltz and McGhie, 2011) most of the variability in polyphenols was ascribed to genetic variability and exceptions were flavonols which were mostly affected by environment. The genetic variability could be the reason for the polyphenol variability in our study. Dihydrochalcones are studied more in the last few decades due to their potential beneficial effect like the effect on the

type 2 diabetes, weight loss and hyperglycemia (Ehrenkranz et al., 2005). Their possibility to reduce blood glucose levels was shown in animal studies on mice (Kobori et al., 2012; Masumoto et al., 2009). Furthermore, phloridzin conjugates showed the potential as an oral anti-diabetic drug (Ikumi et al., 2008; Sakuma et al., 2009). It was suggested that some products with high phloridzin content like pomace of unripe apples could be used for the reduction of postprandial glycemia (Makarova et al., 2015). The binding of phloridzin to human serum albumin, the most abundant protein in the human plasma which is considered a transporter of many drugs and chemical contaminants, was studied too (Yue et al., 2011). Phloridzin binds to the human serum albumin via hydrophobic force and hydrogen bonds (Yue et al., 2011). Since dihydrochalcones have shown many potential bioactivities, and they are studied as a possible drug, their content in natural food sources could be important.

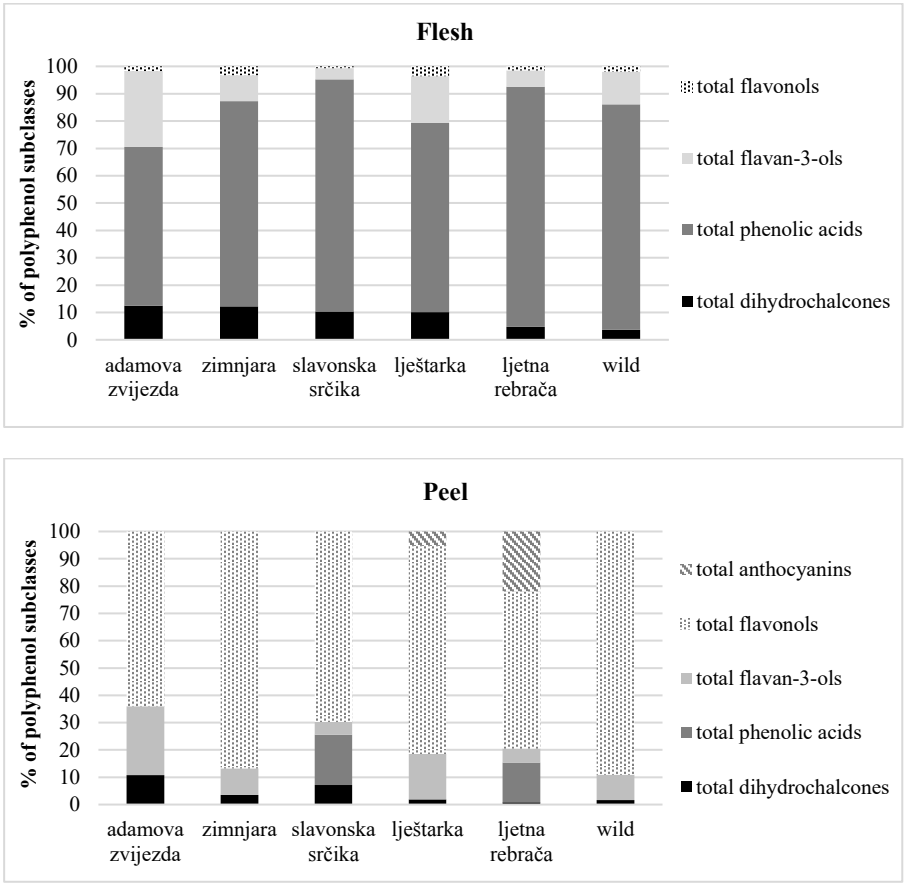

Fig. 5. Percentages of all polyphenol subclasses in flesh and peel of old apple varieties

Dihydrochalcones are specific to apples and their products. Their content can vary depending on the variety (Jakobek and Barron, 2016.; Khanizadeh et al., 2008; Veberic et al., 2005; Wojdyło et al., 2008) and they can be found in flesh and peel. Moreover, the content can be some 4 to 9 times higher in the peel than in the flesh (Khanizadeh et al., 2008; Tsao et al., 2003). When eating apple, many people discard the peel which means that some dihydrochalcones are actually discarded. In this case, the apple flesh as a source of dihydrochalcones can be very important. Some varieties that have higher content or portion of dihydrochalcones in the flesh can be important. In this work, varieties with the higher dihydrochalcone portion in the flesh were Adamova zvijezda, Zimnjara, Slavonska srčika. But in general, if the dihydrochalcone percentage in the flesh and peel together is taken into account, Slavonska srčika and Adamova zvijezda might be important. The dihydrochalcone portion in these old varieties might be considered as their specific feature.

Conclusions

In this work, dihydrochalcones from old apple varieties were analyzed. The usual dihydrochalcones were identified, phloretin-2'-xyloglucoside and phloretin-2'-glucoside in the flesh and phloretin-2'-glucoside in the peel. The dihydrochalcone percentage varied in the flesh from 3.7 to 12.4 %. In the peel, the dihydrochalcone percentage was between 0.8 to 10.8 %. Two varieties could be highlighted with somewhat higher dihydrochalcone percentage in both, flesh and peel: Adamova zvijezda and Slavonska srčika. Principal component analysis showed that apple varieties differ by the dihydrochalcone percentage. The pattern in the dihydrochalcone distribution was not shown.

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