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Polyphenol content, profile, and distribution in old, traditional apple varieties

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ABSTRACT

Apples are an important source of polyphenols in the human diet. They have also shown many potentially beneficial effects on human health. Old, traditional apple varieties grown in the past could also be valuable varieties but little is known about their polyphenolic compounds and characteristics in general. The aim of this study was to collect 25 old, traditional apple varieties, to determine their polyphenolic profile and the amounts of total polyphenols in the peel and flesh, and to compare them with a commercial variety. To the best of our knowledge, some of those varieties have never been studied before ('Mašanka', 'Bobovac', 'Batulenka', 'Krastavka'). Total polyphenols were determined by using the Folin-Ciocalteu method and individual polyphenol identification was done by using an RP-HPLC. The flesh contained 170 to 941 mg kg⁻¹ fresh weight (FW) of total polyphenols, and the peel contained 931 to 3791 mg kg⁻¹ FW. In comparison to the commercial variety, the peel of all old varieties had higher polyphenol content, while the flesh of only some old varieties was richer in polyphenols. Principal component analysis showed possible clustering. Eighteen individual polyphenols were distributed in apple peel and flesh. The dominant polyphenol subgroups in the peel were flavonols (18 to 80 %) and flavan-3-ols (6 to 66 %), and in the flesh those subgroups were phenolic acids (41 to 85 %) and flavan-3-ols (3-49 %).

Introduction

Many different varieties of apples have been developed over the centuries (Musacchi and Serra, 2018). Most of them are nowadays neglected and grown only in some orchards. Only commercial varieties are grown in high quantities. Some countries preserve those old varieties in germplasm banks (Lo Piccolo et al., 2019) while in some countries that process is just beginning.

Many studies have shown potentially beneficial effects of apples on human health in general, like on cardiovascular health (Bondonno et al., 2017) or diabetes (Shoji et al., 2017; Sun et al., 2016). But, in recent times, some studies are pointing more specifically to the significance of old varieties of apples in comparison to new varieties (Kschonsek et

al., 2019; Vegro et al., 2016). Old varieties have been shown to be much better for people with intolerance to apples (Kschonsek et al., 2019). Namely, it was found that people with birch-pollen allergy often develop an intolerance to apples. Those individuals tolerate old apple varieties better than new ones. This was explained by the higher amount of polyphenols in old varieties (Kschonsek et al., 2019). More studies are necessary to confirm this property. Another study has also shown that less allergenic genotypes of apples occur in old varieties, especially those that came before the "green revolution". This is a period during which the genetic improvement of plants was greatly accelerated (Vegro et al., 2016). Due to the emerging significance of old varieties, it is necessary to preserve them.

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Apples contain various polyphenols belonging to phenolic acid, flavonols, flavan-3-ols, anthocyanins, and dihydrochalcone subgroups (Zhao et al., 2019; Woydyło et al., 2008). Polyphenols have been studied well in commercial varieties but not in old varieties of apples.

The aim of this study was to collect 25 different old varieties of apples in Croatia, to extract polyphenolic compounds, and to determine their total phenolic content. The other aim was to identify individual polyphenols in these varieties. The results were compared to the commercial variety 'Idared'. To the best of our knowledge, several of the varieties in this study have never been studied before.

Materials and methods

Apple samples

Twenty-five varieties of apples were collected in 2018, in three orchards in Croatia (orchard in Mihaljevci ((45°22'47.6"N 17°40'34.3"E) owned by Mirko Veić; orchard in Gornji Tkalec (45°58'24.0"N 16°27'12.0"E); orchard in Rude (45°46'35.6"N 15°40'35.8"E)). One variety, Božićnica, was collected from two orchards and it is called Božićnica 1 and Božićnica 2 here. Approximately one kg of apples was peeled, the peel was pooled together and homogenized with a coffee grinder. The flesh was cut in quarters, the core was removed, the flesh was pooled together and it was homogenized with a stick blender. All samples were immediately frozen overnight. Extracts were prepared the next day.

Extraction procedure

Polyphenols were extracted with a procedure already described in the literature (Jakobek et al., 2015). Shortly, 0.2 g of flesh or peel was weighed, 1.5 ml of 80% methanol in water was added in a plastic cuvette. The reaction mixture was put in an ultrasonic bath for 15 minutes and then centrifuged for 10 min at 10 000 rpm. The extract was separated from the residue by pipetting it into another plastic cuvette. Extracts were filtrated through a 0.45 µm PTFE filters. Two parallel extracts were prepared from each variety.

Total polyphenol determination

Total polyphenols were determined by using the Folin-Ciocalteu method. 20 µl of an extract, 1580 µl of distilled water, 100 µl of the Folin-Ciocalteu reagent, and 300 µl of the Na₂CO₃ solution (200 g/l) were added in a glass cuvette, homogenized in a vortex, incubated at 40 °C for 30 min (IN 30, Memmert, Schwabach, Germany), and

the absorbance was measured at 765 nm (UV-Vis spectrophotometer, UV-1280, Shimadzu, Kyoto, Japan). A blank solution contained distilled water instead of the polyphenol extract. Total polyphenols were expressed in mg/l of gallic acid equivalent and then recalculated into mg/kg fresh apple weight (FW).

High performance liquid chromatography identification of polyphenols

The identification of polyphenols in extracts was conducted using an Agilent HPLC system 1260 Infinity II (Agilent technology, Santa Clara, CA, USA) consisting of a quaternary pump, PDA detector, a vialsampler, a poroshell 120 EC C-18 column (4.6 x 100 mm, 2.7 µm), and a poroshell 120 EC-C18 4.6 µm guard-column. Mobile phases were 0.1% H₃PO₄ (mobile phase A) and 100% methanol (mobile phase B). The gradient was established for the separation of polyphenols: 0 min 5% B, 5 min 25% B, 14 min 34% B, 25 min 37% B, 30 min 40% B, 34 min 49% B, 35 min 50% B, 58 min 51% B, 60 min 55% B, 62 min 80% B, 65 min 80% B, 67 min 5% B, 72 min 5% B. The flow was 0.8 ml/min with an injection volume of 10 µl. Standards of polyphenols were injected in different concentration ranges to construct calibration curves. All calibration curves were linear (r² 0.9927 to 1.0000). The identification of polyphenols in extracts was done by comparing the UV/Vis spectrum and retention times of peaks in extracts with those of authentic standards. Additional identification was done by adding standards in extracts (spiking).

Statistical analysis

All extracts were prepared in two parallels, each measured once with Folin-Ciocalteu methods (n=2) and once with HPLC (n=2). Means ± standard deviations were presented. Total polyphenols were analysed with the post-hoc Tukey test to see any possible grouping amongst varieties. Principal component analysis (PCA) was used to analyse and visualize the grouping of apple varieties.

Results and discussion

Total polyphenols in old apple varieties are shown in Fig. 1. The flesh of old varieties had 170 to 941 mg/kg FW of total polyphenols, while the peel contained much higher quantities, from 931 to 3791 mg/kg FW. These values are in accordance with amounts already reported in the literature (Donno et al., 2012; Drogoudi and Pantelidis, 2011). For example, it was reported that the flesh contains 312 – 1290 mg/kg FW and that the peel contains 2857 – 6300 mg/kg FW of total polyphenols (Drogoudi

and Pantelidis, 2011). Looking at the peel polyphenols, all old varieties had higher measured amounts of total polyphenols than the commercial variety 'Idared'. Apple varieties with statistically significantly higher amounts of polyphenols in the peel in comparison to the commercial variety are 'Kolerova srčika', 'Pisanika', 'Božićnica 2', 'Lještarka', and 'Mašanka'. In the flesh, some old varieties had higher measured amounts of polyphenols and some had lower in comparison to the commercial variety 'Idared'. But, statistically, the flesh of the commercial variety does not differ significantly from the old varieties. Nevertheless, the old varieties that can be highlighted as having more polyphenols in the flesh are 'Kolerova srčika', 'Krastavka', 'Gravenstein', and 'Wild Apples'. This agrees with some earlier studies where it was shown that old varieties had higher amounts of polyphenols than new varieties (Donno et al., 2012).

To see any possible clustering of varieties, principal component analysis was conducted on the total polyphenols from both the flesh and the peel (Fig. 2). Some possible clustering of varieties can be seen. Old varieties clustered separately from the commercial variety 'Idared'. Among the old varieties, a possible clustering divides the varieties into three possible clusters. In one group, 'Kolerova srčika', 'Božićnica 2', 'Lještarka' and 'Wild Apples' are grouped together. A variety 'Pisanika' has some differences that separate it from other varieties. A second cluster can be seen for the varieties 'Zlatica', 'Batulenka', 'Citronka', and 'Zimnjara'. Other varieties belong to the third cluster. In general, principal component analysis which takes into account more factors (total polyphenols in the flesh and peel) suggests possible differences between old and commercial varieties.

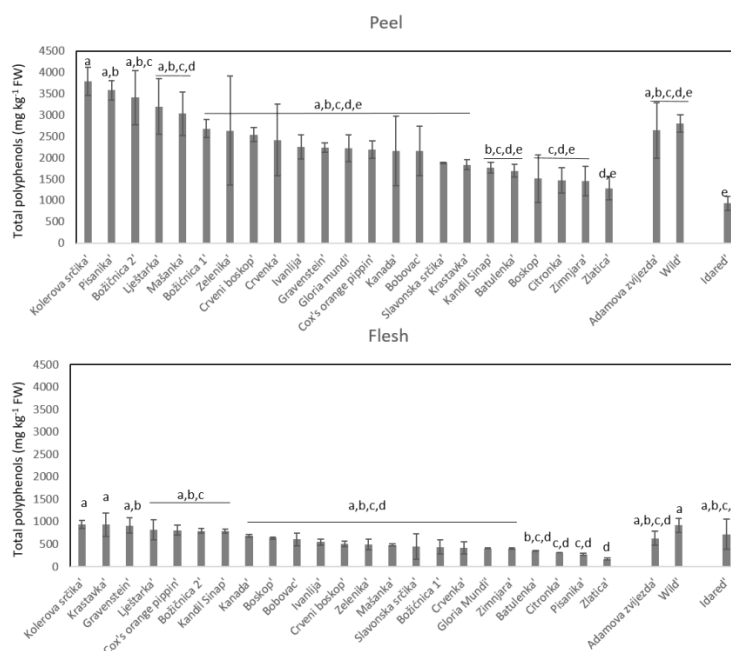


Fig. 1. Total polyphenol content in the peel and the flesh of old apple varieties (mg/kg FW) and in the commercial variety 'Idared'

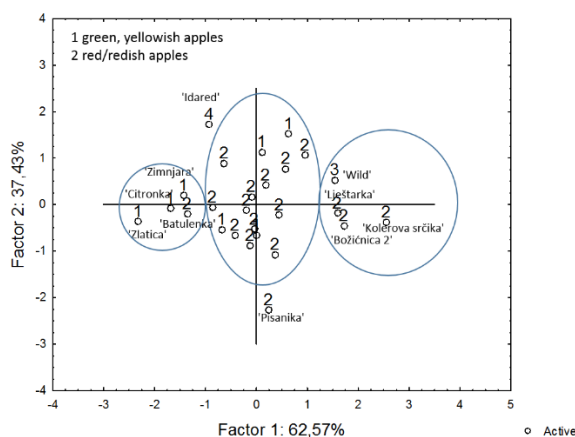


Fig. 2. Principal component analysis of the total polyphenols of the peel and flesh of old apple varieties and the commercial variety 'Idared' (1 – green, yellowish apples, 2 – red/reddish apples, 3 – wild apples, 4 – 'Idared')

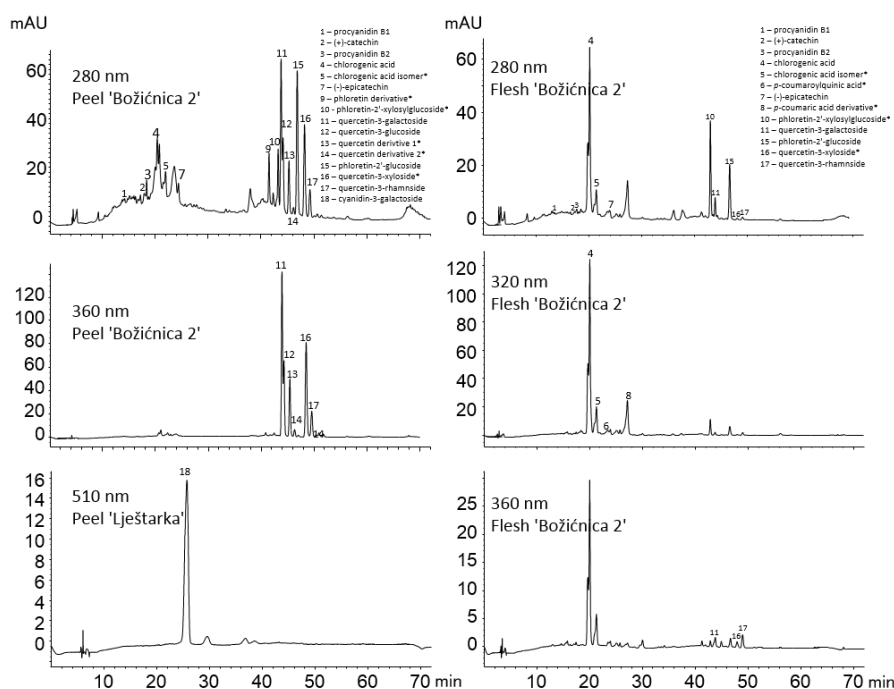


Fig. 3. Polyphenolic compounds identified in old apple varieties. Peaks: 1 – procyanidin B1, 2 – (+)-catechin, 3 – procyanidin B2, 4 – chlorogenic acid, 5 – chlorogenic acid isomer*, 6 – *p*-coumaroylquinic acid*, 7 – (-)-epicatechin, 8 – *p*-coumaric acid derivative*, 9 – phloretin derivative*, 10 – phloretin-2'-xyloxyglucoside*, 11 – quercetin-3-galactoside, 12 – quercetin-3-glucoside, 13 – quercetin derivative 1*, 14 – quercetin derivative 2*, 15 – phloretin-2'-glucoside, 16 – quercetin-3-xyloside*, 17 – quercetin-3-rhamnoside, 18 – cyanidin-3-galactoside. * - tentatively identified

In order to determine the profile of polyphenols in the peel and flesh, the extracts were analysed using a RP-HPLC method. In the old varieties of apples, a total of 18 polyphenols were identified/tentatively identified according to their UV/Vis spectrum and retention times (Fig. 3). The identified compounds belong to flavan-3-ol (peaks 1-3 and 7), dihydrochalcone (peaks 9, 10, and 15), phenolic acid (peaks 4-6, and 8), flavonol (peaks 11-14, 16-17), and anthocyanin (peak 18) subgroups, which agrees with the literature (Kschonsek et al., 2019; Li et al., 2019; Lo Piccolo et al., 2019). Amongst the identified compounds, 16 compounds have been identified in the peel and 13 in the flesh (Fig. 3).

Figs. 4 and 5 show the UV/Vis spectrum of each of those polyphenols from the peel and the flesh, overlaid with the corresponding spectrum of an authentic standard, if available. Four *flavan-3-ols* have been identified (procyanidin B1, (+)-catechin, procyanidin B2, (-)-epicatechin). Each of these showed that the maximum in the UV/Vis spectrum occurs at 280 nm, which agrees with the spectral maximum of authentic standards as well as with their retention times. Similar spectral maxima were reported in the literature (Giomaro et al., 2014; Wojdyło et al., 2008). In the *dihydrochalcone* group, the compound which showed a maximum at 285 nm, typical for dihydrochalcones, was identified as phloretin-2'-glucoside (peak 15). The spectrum and retention time agree with that of the

standard. The other two peaks in the dihydrochalcone group, peak 9 and 10, could be phloretin derivatives. Peak 9 had the spectral characteristics typical of phloretin derivatives. Peak 10 had a typical dihydrochalcone spectrum with the maximum at 285 nm, and was tentatively identified according to the literature (Giomaro et al., 2014; Kschonsek et al., 2019; Wojdyło et al., 2008) as phloretin-2'-xyloxyglucoside. *Phenolic acids* found in apples were chlorogenic acid (peak 4), chlorogenic acid isomer (peak 5), *p*-coumaroylquinic acid (peak 6), and *p*-coumaric acid derivative (peak 8). The UV/Vis spectrum of chlorogenic acid (max at 330 nm with shoulder at 300 nm) agrees with that of its standard, as does its retention time, and it agrees with literature data (Giomaro et al., 2014; Wojdyło et al., 2008). According to the UV/Vis spectrum of peak 5 (max at 330 nm with shoulder at 300 nm), it is similar to chlorogenic acid, so, that compound could be a chlorogenic acid isomer. Peak 6 had an UV/Vis max at 310 nm, which is similar to the spectrum of *p*-coumaroylquinic acid. According to typical UV/Vis spectra and literature data (Giomaro et al., 2014; Wojdyło et al., 2008), this peak was tentatively identified as *p*-coumaroylquinic acid. Peak 8 had a similar spectrum as *p*-coumaric acid (UV/Vis max at 310 nm) and could be another *p*-coumaric acid derivative. Six *flavonols* were found in apples, mostly in the peel. Three of them (peak 11, 12, and 17) were identified as quercetin-3-

galactoside (UV/Vis max 255 and 360 nm), quercetin-3-glucoside (UV/Vis max 255 and 355 nm), and quercetin-3-rhamnoside (UV/Vis max 255 and 350 nm), according to the agreement of each spectrum with the spectrum of standards and with their retention times. Earlier studies also reported similar maxima in UV/Vis spectra (Giomaro et al., 2014; Wojdyło et al., 2008). The other two peaks, peak 13 and 14, showed spectra typical for flavonols (UV/Vis maximum at 255 and 355), and according to these spectral characteristics and available literature (Giomaro et al., 2014), they could be derivatives of quercetin. The tentative identification of peak 16 is quercetin-3-xyloside, according to its spectral characteristics (max at 255 and 355) and available literature data (Wojdyło et al., 2008). Only one *anthocyanin* was identified in the apple peel: cyanidin-3-galactoside. Its UV/Vis spectrum (UV/Vis maximum at 275 and 520 nm) agrees with that of an authentic standard, with its retention time and with UV/Vis spectra

reported in the literature (Giomaro et al., 2014; Wojdyło et al., 2008).

Figs. 6 and 7 show the percentage distribution of identified polyphenols in the peel and flesh. In the peel (Figure 6), flavonols are present in the highest percentage (18 to 80 %), followed by flavan-3-ols (6 to 66 %). phenolic acids (1 to 27 %), dihydrochalcones (2 to 30 %), and anthocyanins (0 to 7 %) are present in the lowest percentage. Earlier studies also reported that the peel contains more flavonols and flavan-3-ols (Giomaro et al., 2014), similar to our study. In the flesh (Fig. 7), phenolic acids are the dominant polyphenol subgroup (41 to 85 %), followed by flavan-3-ols (3-49 %), while dihydrochalcones (2-8 %) and flavonols (2 to 16 %) are present in lower percentages. In earlier studies, phenolic acids (Giomaro et al., 2014; Lo Piccolo et al., 2019) and flavan-3-ols (Lo Piccolo et al., 2019) are reported to be the main polyphenolic constituents in apple pulp.

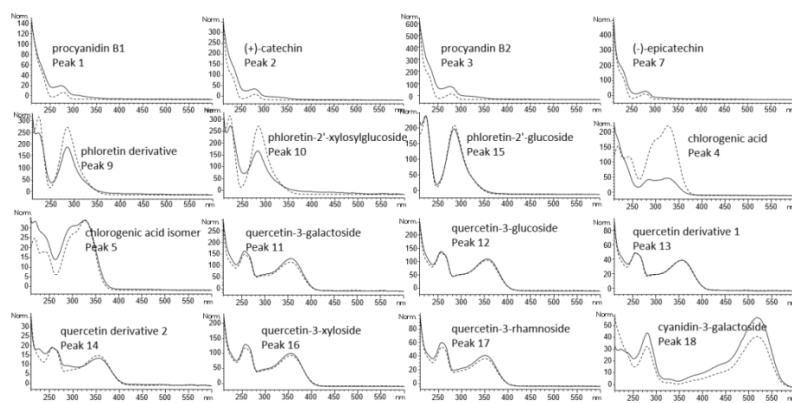


Fig. 4. UV/Vis spectrum of polyphenols in the peel of old apple varieties overlaid with the UV/Vis spectrum of an authentic standard (phloretin derivative, phloretin-2'-xyloglucoside overlaid with the spectrum of phloretin-2'-glucoside; chlorogenic acid isomer overlaid with the spectrum of chlorogenic acid, quercetin derivatives 1 and 2, and quercetin-3-xyloside overlaid with the quercetin-3-glucoside spectrum)

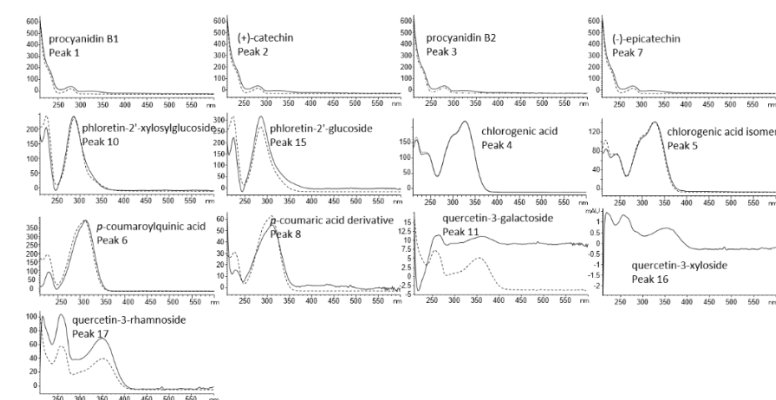


Fig. 5. UV/Vis spectrum of polyphenols in the flesh of old apple varieties overlaid with UV/Vis spectrum of authentic standard (phloretin-2'-xylosylglucoside overlaid with the spectrum of phloretin-2'-glucoside; chlorogenic acid isomer overlaid with the spectrum of chlorogenic acid, *p*-coumaric acid derivative and *p*-coumaroylquinic acid overlaid with the *p*-coumaric acid spectrum)

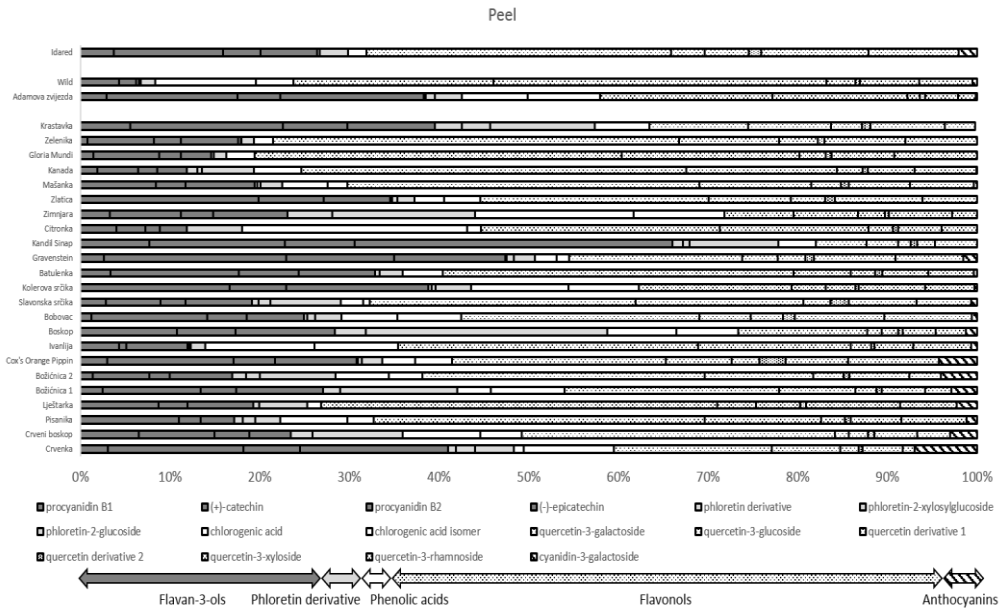


Fig. 6. Polyphenol distribution in apple peel

Polyphenolic compounds have shown potentially positive effects on human health through numerous studies (Hua et al., 2018; Serra et al., 2019, Silva et al., 2019). In order to determine their true role in the body, the bioaccessibility and bioavailability of polyphenols, and the influence of their metabolites and catabolites in the human body are being investigated (MacDonald and Wagner, 2012; Williamson and Clifford, 2017). Apples are a fruit commonly consumed throughout the year and are, therefore, a significant source of polyphenols. However, new research suggests that old apple cultivars are actually richer in polyphenols (Donno et al., 2012)

and have positive health effects (Kschonsek et al., 2019; Vegro et al., 2016). These studies certainly need to be confirmed. For these reasons of higher polyphenol content and potential health benefits, it is necessary to preserve old cultivars and create apple databases. The old apple cultivars grown in Croatia (Jakobek et al., 2013; Jakobek and Barron, 2016) have also been shown to be rich in polyphenolic compounds, and this is especially true of apple peel. Due to potential beneficial effects, further studies of bioaccessibility of old varieties will be conducted.

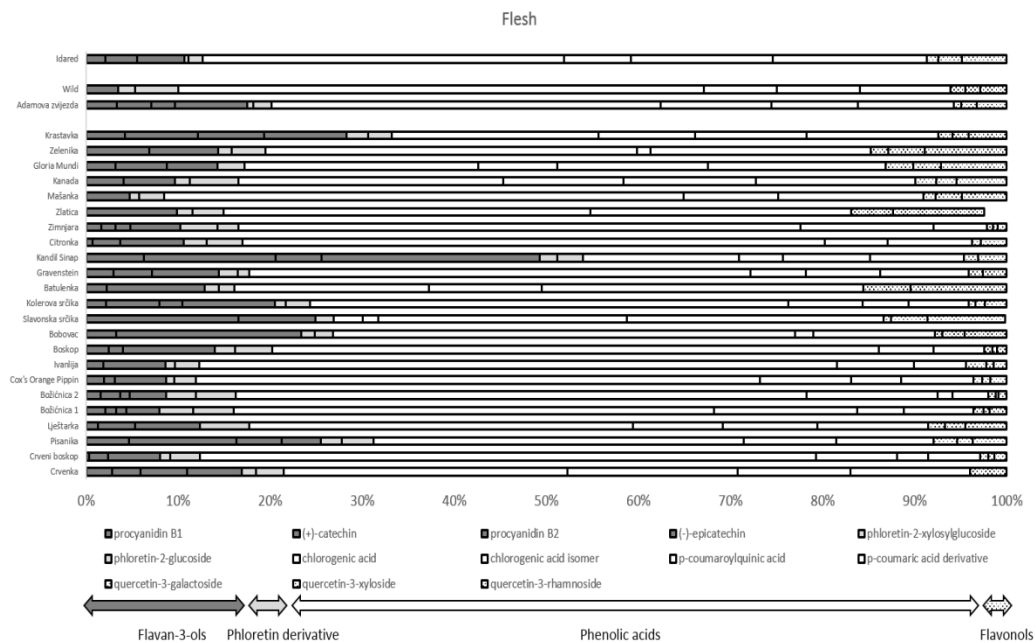


Fig. 7. Polyphenol distribution in apple flesh

Conclusion

In comparison to a commercial variety, some old, traditional varieties contain more polyphenols in the flesh and some contain less. But the peel of all old varieties had higher amounts of total polyphenols than the commercial variety. Eighteen different polyphenolic compounds were identified/tentatively identified in the old varieties, belonging to phenolic acid, flavonol, flavan-3-ol, dihydrochalcone, and anthocyanin subgroups. They all showed similar characteristics of their UV/Vis spectra to authentic standards. The distribution of these individual polyphenols was different in the peel and in the flesh. Flavonols and flavan-3-ols dominated in the peel, while phenolic acids and flavan-3-ols were the main polyphenols in the flesh. Further studies on traditional apple varieties are necessary to show their beneficial effects and to preserve them for future generations.

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