Journal of Food Composition and Analysis 45 (2016) 9–15

Contents lists available at ScienceDirect

Journal of Food Composition and Analysis

journal homepage: www.elsevier.com/locate/jfca

Original Research Article

# Ancient apple varieties from Croatia as a source of bioactive polyphenolic compounds

Lidija Jakobek [a,*], Andrew R. Barron [b]

[a] J.J. Strossmayer University of Osijek, Faculty of Food Technology Osijek, Department of Applied Chemistry and Ecology, Franje Kuhača 20, HR 31000 Osijek, Croatia
[b] Yale University, Department of Statistics, 24 Hillhouse Ave., New Haven, CT 06511, USA

ARTICLE INFO

*Article history:*
Received 11 July 2015
Received in revised form 2 September 2015
Accepted 15 September 2015
Available online 24 September 2015

*Keywords:*
Food analysis
Apples
*Malus domestica*
Ancient varieties
Wild apple
Food composition
Polyphenols
RP-HPLC-PDA
Bioactive compounds

ABSTRACT

Several ancient apple varieties and a wild apple variety grown in Croatia were analysed for the polyphenol content and compared to two varieties grown in USA. In the flesh, flavanols, dihydrochalcones and phenolic acids (24 to 137, 23 to 109, 3 to 238 mg $kg^{-1}$ of fresh weight (FW), respectively) were found. Peel contained flavanols, dihydrochalcones, phenolic acids, flavonols and anthocyanins (65 to 690, 21 to 141, 0 to 107, 205 to 1223, 0 to 213 mg $kg^{-1}$ FW, respectively). The wild apple was characterized by much higher flavanol and phenolic acid content in the flesh (301 and 734 mg $kg^{-1}$ FW, respectively) while the peel was similar to other apples. The polyphenol profile was similar to apples from USA. The varieties Zimnjara, Lještarka and Adamova zvijezda could be highlighted as sources of polyphenols. Varieties are categorized by the content of dihydrochalcones and flavanols in the flesh (whether that content is high or low), and by the relative portion of phenolic acids and flavanols in the flesh (high phenolic acid proportion, lower flavanol proportion and vice versa). There was not observed to be as strong a pattern for categorizing differences in the peel.



## 1. Introduction

Apples have been grown for many centuries allowing the development of many different varieties, with different sensory and organoleptic properties. Though numerous varieties were developed over the time, only some of them are grown commercially today. Old varieties can still be found in individual orchards. Because of favourable differences in their characteristics that developed through many years, many of these old varieties should be preserved for the future. In Croatia, many of these varieties still exist (Jakobek et al., 2013) in individual orchards where they are well adapted to environmental and geographic features and can mature from early summer to the end of autumn. In our previous work, ancient summer varieties of apples from Croatia were analysed (Jakobek et al., 2013). Earlier study showed that old varieties have higher sensory and nutritional qualities when compared to commercial ones (Golden Delicious) (Donno et al., 2012). Iacopini et al. (2010) also showed that old local apple varieties, in particular Ruggine and Panaia-red, have higher level of phenolic compounds and antioxidant activity than commercial ones (Golden Delicious and Stark Delicious).

One of important quality features of apples is the amount of polyphenols. Five major polyphenolic groups are found in apples: flavanols, phenolic acids, flavonols, dihydrochalcones, and anthocyanins (Ceymann et al., 2012; Khanizadeh et al., 2008; Lamperi et al., 2008; Tsao et al., 2003; Vrhovšek et al., 2004; Wojdyło et al., 2008). The same polyphenols were identified in ancient apple cultivars from Poland (Wojdyło et al., 2008), Italy (Iacopini et al., 2010) and Croatia (Jakobek et al., 2013) and in commercial ones from Canada (Khanizadeh et al., 2008; Lamperi et al., 2008; Tsao et al., 2003; Vrhovšek et al., 2004), Italy (Ceymann et al., 2012; Lamperi et al., 2008; Vrhovšek et al., 2004), Poland (Wojdyło et al., 2008), Switzerland (Ceymann et al., 2012), and Austria and Slovenia (Veberic et al., 2005). Polyphenols are studied intensively due to many positive effects like their anticancerogenic properties (Veeriah et al., 2007; Yang and Liu, 2009), and effect on the prevention of cardiovascular diseases and cancer (Arts and Hollman, 2005; Hollman et al., 2011). Polyphenols from apples have shown antiviral properties (Suárez et al., 2010), the inhibition of Helicobacter pylory (Pastene et al., 2009) and Staphylococcal enterotoxin A (Rasooly et al., 2010), the effect on melanogenesis

* Corresponding author. Tel.: +385 31 224 325; fax: +385 31 207 115.
*E-mail addresses:* lidija.jakobek@ptfos.hr (L. Jakobek), andrew.barron@yale.edu (A.R. Barron).

http://dx.doi.org/10.1016/j.jfca.2015.09.007
0889-1575/

(Shoji et al., 2005) and the possibility to regulate plasma cholesterol metabolism (Tenore et al., 2013). Still, the bioactivities of polyphenols are not yet fully explained (Hollman et al., 2011). To have a positive influence on the human health, polyphenols should be released during digestion and then absorbed in efficient amount. Although many earlier studies described that they are absorbed in low quantities (Manach et al., 2005), the latest research points out their higher absorption (Czank et al., 2013). Also, polyphenols can interact with many other dietary constituents like carbohydrates, proteins, and lipids, which can affect their bioaccessibility, bioavailability and bioactivity (Jakobek, 2015). Due to many positive effects, polyphenols in general and apple polyphenols in particular are intensively studied.

The aim of this study was to analyse polyphenols in old, local autumn apple varieties and in a wild apple grown in Croatia (region Slavonia). These apples have not been studied in depth before (to the best of our knowledge) and could be a good source of bioactive polyphenolic compounds. In addition, the polyphenol profile of these apples was compared to two varieties grown in USA. Polyphenols were determined in the peel and in the flesh, and analysed with the use of reversed phase high-performance liquid chromatography coupled with a photodiode array detector (RP-HPLC-PDA). The reason for analysing bioactive polyphenolic compounds in old apple varieties was to enable the recognition of those varieties that could be a valuable source of polyphenols since the polyphenol amount represents one of important quality features of apples. Besides, the potential disappearance of these ancient varieties will be prevented, which can enable the preservation of apple biodiversity. The preservation of biodiversity will enable the selection of varieties with higher potential for positive bioactivities.

## 2. Materials and methods

### 2.1. Samples and sample preparation

Twelve old, local apple varieties (*Malus domestica*) (Lještarka, Božićnica, Kolerova, Srčika, Ivanlija, Duga, Zimnjara, Citronka, Zlatica, Paradija, Adamova zvijezda and Slavonska Srčika), and one wild variety (crab apple) were harvested in maturity in October 2013 in Croatia (family orchard (M. Veić), Mihaljevci, near Požega). Since these apples are not commercial types of apples, they are not grown in high amounts, so fruits (approximately 1 kg) could be harvested from only one tree and from one orchard. Apples were stored in the refrigerator no more than one week before sample preparation. Two apple cultivars grown in USA (Cortland and Russet, popular in North America and United Kingdom) were picked in maturity in October 2013 (Norton Brothers Fruit Farm and Hickory Hill Orchard, Cheshire, Connecticut), delivered to Croatia, Osijek, within one week and immediately subjected to sample preparation. Approximately, 1 kg of each apple variety was peeled with a consumer-grade peeler. Care was taken to peel apples as best as possible to get peel samples without flesh. The peel of the same variety was pooled and homogenized using a blender and stored at −18 °C for not more than 1 month. The core and the seeds were removed from the flesh, the flesh was cut into smaller pieces, and the flesh was pooled and homogenized with a stick blender. Homogenous flesh samples were also stored at −18 °C for not more than 1 month.

### 2.2. Chemicals

Chemicals were purchased from several firms: gallic acid monohydrate (398225, ≥98%), (+)-catechin hydrate (C1251, ≥98%), (−)-epicatechin (E1753, ≥90%), chlorogenic acid (C3878, ≥95%), *p*-coumaric acid (C9008, ≥98%), quercetin dihydrate (Q0125, ≥98%), and quercetin-3-β-D-glucoside (isoquercitrin – 17793, ≥90%) were purchased from Sigma–Aldrich (St. Louis, MO, USA); procyanidin B1 (0983, ≥80%), procyanidin B2 (0984, ≥90%), cyanidin-3-*O*-galactoside chloride (ideain chloride – 0923 S, ≥97%), cyanidin-3-*O*-glucoside chloride (kuromanin chloride – 0915 S, ≥ 96), quercetin-3-*O*-galactoside (hyperoside – 1027 S, ≥98%), quercetin-3-*O*-rhamnoside (quercitrin – 1236 S, ≥98.5%), phloretin-2′-*O*-glucoside (phloridzin – 1046, ≥99%), and phloretin (1044, ≥99%) from Extrasynthese (Genay, France); ortho-phosphoric acid (85%, HPLC grade) from Fluka (Buchs, Switzerland); HPLC grade methanol from J. T. Baker (Deventer, Netherlands); and hydrochloric acid (36.2%), sodium carbonate and Folin–Ciocalteu reagent from Kemika (Zagreb, Croatia).

### 2.3. Polyphenol extraction

Polyphenols were extracted separately from the peel (0.1% HCl in methanol) and from the flesh (80% methanol in water) according to our previously developed procedure (Jakobek et al., 2015). Samples of flesh or peel were weighed (0.2 or 0.75 g of flesh, 0.5 g of peel), mixed with extraction solvents (5 mL), vortexed (vortex, Grant Bio, Cambridgeshire, England) and then placed in an ultrasonic bath (Bandelin Sonorex, RK 100, Berlin, Germany) for 15 min. These extracts were filtered and the residue was extracted again in 2 mL of extraction solvent. The extracts from these two extraction steps were combined. One mL of prepared extract was filtered through a 0.45 μm polytetrafluoroethylene (PTFE) syringe filter and 20 μL was directly injected into the RP-HPLC-PDA system. From each peel or flesh, two extracts were made and each extract was analysed once with RP-HPLC-PDA, and two times with the Folin–Ciocalteu method.

### 2.4. Total polyphenol determination

Total polyphenols were determined by the Folin–Ciocalteu micro method (Waterhouse, 2015). Each extract (20 μL) was mixed with distilled water (1580 μL), Folin–Ciocalteu reagent (100 μL) and a sodium carbonate solution (200 g $L^{-1}$) (300 μL), and the mixture was incubated in a water bath at 40 °C (30 min). The absorbance was read at 765 nm on a UV-Vis spectrophotometer (JP Selecta S.A., UV 2005, Barcelona, Spain). Gallic acid solutions (0 to 500 mg $L^{-1}$) were measured with the same procedure. A calibration curve of gallic acid was created (absorbance vs concentration) and used for the total polyphenol content calculation (mg of gallic acid equivalents (GAE) per kg of fresh fruit weight (FW)).

### 2.5. Reversed phase high performance liquid chromatography with photodiode array detection (RP-HPLC-PDA)

Individual polyphenols were determined by using the RP-HPLC-PDA method validated in our previous study (Jakobek et al., 2015). The instrument was a Varian system (Varian Inc., Palo Alto, USA) (ProStar 230 solvent delivery module, ProStar 330 PDA detector, OmniSpher C18 column (250 × 4.6 mm, 5 μm), guard column (ChromSep 1 cm × 3 mm)). Phosphoric acid 0.1% in water was used as a mobile phase A, while mobile phase B was 100% HPLC grade methanol. The gradient was as follows: 0 min 5% B; 0 to 5 min from 5 to 25% B, 5 to 14 min from 25 to 34% B, 14 to 25 min from 34 to 37% B, 25 to 30 min from 37 to 40% B, 30 to 34 min from 40 to 49% B, 34 to 35 min from 49 to 50% B, 35 to 58 min from 50 to 51% B, 58 to 60 min from 51 to 55% B, 60 to 62 min from 55 to 80% B, 62 to 65 min 80% B, 65 to 67 min from 80 to 5% B, 67 to 72 min 5% B; with flow rate = 0.8 mL $min^{-1}$. Extracts and standards were injected in a volume of 20 μL. UV-Vis spectra were recorded in a wavelength range from 190 to 600 nm. The detection wavelength was 280 nm

for flavanols and dihydrochalcones, 320 nm for phenolic acids, 360 nm for flavonols and 510 nm for anthocyanins.

Calibration curves were made by preparing stock standards, diluting them and injecting into the RP-HPLC-PDA system as follows: (+)-catechin 2–250 mg $L^{-1}$, (−)-epicatechin 2–250 mg $L^{-1}$, chlorogenic acid 1–92 mg $L^{-1}$, *p*-coumaric acid 1–98 mg $L^{-1}$, quercetin 1–132 mg $L^{-1}$, quercetin-3-rhamnoside 5–180 mg $L^{-1}$, quercetin-3-galactoside 5–180 mg $L^{-1}$, quercetin-3-glucoside 5–180 mg $L^{-1}$ (prepared in methanol); phloretin 1–200 mg $L^{-1}$, phloridzin 1–200 mg $L^{-1}$ (prepared in ethanol); procyanidin B1 0.8–160 mg $L^{-1}$, procyanidin B2 1–225 mg $L^{-1}$ (prepared in water); cyanidin-3-galactoside 1–240 mg $L^{-1}$, cyanidin-3-glucoside 1–240 mg $L^{-1}$ (prepared in 0.1% HCl in methanol) according to our earlier work (Jakobek et al., 2015). The method was linear ($r^2$ 0.9702–0.9999), precision (coefficient of variation for the flesh 1–17% and peel 0.6–22%) and recovery (from 92 to 112%) were also determined. The limit of detection (LOD) in mg $kg^{-1}$ for the flesh and peel, respectively: (+)-catechin 0.7, 0.3; (−)-epicatechin 1.1, 0.5; chlorogenic acid 0.5, 0.2; *p*-coumaric acid 0.3, 0.1; quercetin 0.7, 0.3; quercetin-3-rhamnoside 1.1, 0.4; quercetin-3-galactoside 1.4, 0.6; quercetin-3-glucoside 0.3, 0.1; phloretin 0.5, 0.2; phloridzin 0.5, 0.2; procyanidin B1 1, 0.4; procyanidin B2 4.2, 1.7; cyanidin-3-galactoside and cyanidin-3-glucoside 4.6, 1.8.

Polyphenols were identified by the comparison of the retention times and spectral data with the ones from standards and by spiking extracts with standards. Some peaks were tentatively identified by the help of literature data (Jakobek et al., 2013; Tsao et al., 2003) (*p*-coumaroylquinic acid, quercetin-xyloside, phloretin-2′-xyloglucoside). Identified compounds were quantified using calibration curves of authentic standards. Tentatively identified compounds were quantified by using the following calibration curves: *p*-coumaroylquinic acid by using the *p*-coumaric acid calibration curve, quercetin derivatives and quercetin-xyloside by using the quercetin calibration curve, and phloretin-2-xyloglucoside by using the phloretin calibration curve.

### 2.6. Statistical analyses

Two extracts were prepared from the flesh and two from the peel of each apple variety and each extract was measured once on the RP-HPLC-PDA and twice by using the Folin–Ciocalteu method. The mean values and the coefficient of variation were based on $n = 2$ and $n = 4$ for RP-HPLC-PDA and Folin–Ciocalteu method, respectively, and they were reported in tables (Tables 1 and 2). Perspective three-dimensional plots were constructed to investigate the clustering of points from apple varieties according to the polyphenol content in three main polyphenol groups (flavanols, dihydralchalcones and phenolic acid for flesh; flavonols, dihydralchalcones and flavanols for peel).

## 3. Results and discussion

### 3.1. Identification of polyphenolic compounds

Polyphenols were analysed in apples through many studies, but usually the focus was oriented to commercial apples which are grown in high quantity and not to old varieties of apples. Old varieties are spread over many countries and usually share common history and sometimes names or there is a possibility that the same variety has different names. In neighbour countries like in Bosnia and Hercegovina, Serbia, Slovenia, Hungary and Austria, some old apple varieties have similar names, but it is often very difficult to compare these apples. Apples analyzed in this study are apples grown and picked in Croatia, region Slavonia, but it is possible that similar apples exist also in the broader region.

In old apple varieties, five classes of polyphenols were identified: flavanols, dihydrochalcones, phenolic acids, flavonols and anthocyanins in accordance with our earlier study (Jakobek et al., 2015). Some compounds were tentatively identified with the help of literature data (Jakobek et al., 2013; Tsao et al., 2003) like *p*-coumaroylquinic acid, phloretin-2′-xyloglucoside and quercetin-3-xyloside. Two quercetin derivatives were not identified. All compounds are listed in Tables 1 and 2. In earlier studies, the same compounds were identified in commercial (Ceymann et al., 2012; Escarpa and González, 1998; Khanizadeh et al., 2008; Tsao et al., 2003; Vrhovšek et al., 2004; Wojdyło et al., 2008) and in other ancient apple varieties (Iacopini et al., 2010; Jakobek et al., 2013; Wojdyło et al., 2008).

### 3.2. The polyphenol content

The flesh of apples grown in Croatia (Table 1) contained 23.7 to 137.1 mg $kg^{-1}$ of fresh weight (FW) of flavanols, 23.0 to 109.3 mg $kg^{-1}$ FW of dihydrochalcones, and 3.3 to 238.3 mg $kg^{-1}$ 1 FW of phenolic acids. The amount of polyphenols in apples we studied from USA is in a lower range of polyphenols of old apple varieties from Croatia. A wild apple (crab apple) showed much higher amount of flavanols (300.6 mg $kg^{-1}$ FW) and phenolic acids (733.8 mg $kg^{-1}$ FW) and a similar content of dihydrochalcones (93.7 mg $kg^{-1}$ FW). The amount is in accordance with the literature data in commercial (Carbone et al., 2011; Ceymann et al., 2012; Escarpa and González, 1998; Guyot et al., 2002; Khanizadeh et al., 2008; Lamperi et al., 2008; Le Bourvellec et al., 2011; Thompson-Witrick et al., 2014; Tsao et al., 2003; Veberic et al., 2005) and other ancient varieties (Iacopini et al., 2010; Jakobek et al., 2013). Slightly higher content of individual flavanols was reported in some studies (Ceymann et al., 2012; Escarpa and González, 1998; Le Bourvellec et al., 2011) and also in the Phenol explorer database for peeled cider and dessert apples (http://phenol-explorer.eu/). Phenol explorer also reported slightly lower content of individual dihydrochalcones in dessert apples and higher content in cider apples (http://phenol-explorer.eu/). In our previous work (Jakobek et al., 2013), old summer varieties of apples showed somewhat higher amount of (−)-epicatechin and chlorogenic acid, while other compounds were found in similar content. According to the results, some apple varieties are phenolic acid dominated (Zimnjara, Zlatica, Ivanlija), and some flavanol dominated (Citronka, Slavonska srčika, Duga, Srčika, Adamova zvijezda, Božićnica, Lještarka). The varieties that can be highlighted by their high content of polyphenols are Zimnjara and wild apple. The amount of dihydrochalcones, which are characteristic apple polyphenols, can be highlighted in variety Zimnjara, while the wild apple variety is rich in flavanols and phenolic acids. Overall, the varieties which could serve as a good source of polyphenols in the flesh are Zimnjara and wild apple.

The peel (Table 2) contained 65.1 to 690.2 mg $kg^{-1}$ FW of flavanols, 20.5 to 141.3 mg $kg^{-1}$ FW dihydrochalcones, 0 to 106.8 mg $kg^{-1}$ FW of phenolic acids, and 204.8 to 1222.7 mg $kg^{-1}$ 1 FW of flavonols. Anthocyanins were found in the peel of two varieties (Lještarka and Božićnica) (53.0 to 213.2 mg $kg^{-1}$ FW) and were not identified in other apples with slightly reddish colour. The polyphenol amount was similar to the amount of varieties from USA and wild apple. The polyphenol level in apples from USA is in the lower level of Croatian apples. The literature also reported similar content of polyphenols (Carbone et al., 2011; Escarpa and González, 1998; Guyot et al., 2002; Khanizadeh et al., 2008; Lamperi et al., 2008; Le Bourvellec et al., 2011; Thompson-Witrick et al., 2014; Tsao et al., 2003; Veberic et al., 2005) or slightly higher amounts of individual flavanols or chlorogenic acid (Escarpa and González, 1998; Lamperi et al., 2008; Le Bourvellec et al., 2011; Veberic et al., 2005). In our previous study, old summer type apple

**Table 1**
The content of polyphenols (mg $kg^{-1}$ fresh weight)* in the flesh of old apple varieties.

| Polyphenols | Croatia | | | | | | | | | | | | USA | | Croatia |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Red peel | | | | | Yellow, green peel | | | | | | | Red peel | Yellow green peel | Yellow green peel |
| | Lještarka | Božićnica | Kolerova | Srčika | Ivanlija | Duga | Zimnjara | Citronka | Zlatica | Paradija | Adamova. Zvijezda | Slavonska Srčika | Cortland | Russet | Wild |
| **Flavanols** | | | | | | | | | | | | | | | |
| Procyanidin B1 | 15.4 | 13.1 | 14.8 | 17.8 | 7.8 | 14.2 | 27.0 | 15.4 | 15.6 | 15.4 | 18.7 | 16.8 | 5.9 | 8.9 | 25.4 |
| (+)-Catechin | 3.8 | 5.5 | 5.4 | 7.9 | 4.8 | 17.3 | 9.6 | 9.5 | 12.5 | 3.0 | 10.3 | 8.4 | 4.4 | 5.3 | 102.5 |
| Procyanidin B2 | 70.4 | 66.8 | nd | 74.4 | 26.7 | 72.2 | 79.3 | 76.8 | 70.9 | 22.8 | 68.0 | 73.3 | nd | 27.0 | |
| (−)-Epicatechin | 7.0 | 18.8 | 3.6 | 11.1 | 5.2 | 17.2 | 18.7 | 35.4 | 14.4 | 3.8 | 10.8 | 30.0 | 11.4 | 6.8 | 172.7 |
| **Total** | 96.6 | 104.3 | 23.7 | 111.2 | 44.6 | 121.0 | 134.5 | 137.1 | 113.3 | 45.0 | 107.8 | 128.5 | 21.8 | 48.0 | 300.6 |
| **Dihydrochalcones** | | | | | | | | | | | | | | | |
| Phloretin 2′-xyloglucoside[a] | 47.6 | 47.4 | 45.9 | 49.0 | 15.3 | 43.8 | 58.2 | 51.4 | 48.4 | 13.8 | 44.4 | 47.1 | 16.4 | 16.0 | 50.8 |
| Phloridzin | 30.2 | 34.2 | 29.5 | 31.1 | 10.5 | 27.0 | 51.2 | 36.1 | 31.0 | 9.2 | 26.4 | 34.6 | nd | 15.0 | 42.9 |
| **Total** | 77.8 | 81.6 | 75.5 | 80.2 | 25.7 | 70.8 | 109.3 | 87.5 | 79.4 | 23.0 | 70.9 | 81.7 | 16.4 | 31.0 | 93.7 |
| **Phenolic acids** | | | | | | | | | | | | | | | |
| Chlorogenic acid | 4.9 | 46.0 | 20.6 | 26.5 | 82.6 | 1.6 | 222.0 | 74.0 | 151.9 | 39.1 | 11.1 | 84.4 | 3.6 | 24.2 | 733.8 |
| *p*-Coumaroylquinic acid[a] | 2.7 | 5.8 | 1.3 | 5.7 | 2.6 | 1.7 | 16.2 | 3.6 | 6.2 | 2.9 | 1.0 | 7.9 | 2.9 | 8.6 | |
| **Total** | 7.6 | 51.8 | 21.9 | 32.3 | 85.1 | 3.3 | 238.3 | 77.6 | 158.1 | 42.0 | 12.2 | 92.2 | 6.5 | 32.8 | 733.8 |
| **TOTAL (HPLC)** | 182.0 | 237.8 | 121.1 | 223.6 | 155.5 | 195.0 | 482.1 | 302.2 | 350.8 | 110.0 | 190.8 | 302.4 | 44.7 | 111.8 | 1128.1 |
| TOTAL (FC)[b] | 265.1 | 884.2 | 459.0 | 448.3 | 250.0 | 661.0 | 1375.7 | 581.4 | 946.8 | 432.2 | 676.1 | 596.4 | 181.8 | 250.8 | 4992.9 |

LOD in mg $kg^{-1}$: procyanidin B1 1.0, (+)-catechin 0.7; procyanidin B2 4.2; (−)-epicatechin 1.1; phloretin 0.5; phloridzin 0.5; chlorogenic acid 0.5, *p*-coumaric acid 0.3.

[a] Tentatively identified.

[b] Total polyphenols obtained by Folin Ciocalteu (FC) method based on two extracts each measured twice ($n=4$), expressed in mg gallic acid equivalent per kg of fresh flesh weight.

* Data based on two extracts each measured once ($n=2$), coefficient of variation from 1 to 17%. Nd, not detected.

**Table 2**
The content of polyphenols (mg $kg^{-1}$ fresh weight)* in the peel of old apple varieties.

| Polyphenols | Croatia | | | | | | | | | | | | USA | | Croatia |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Red peel | | | | | Yellow, green peel | | | | | | | Red peel | Yellow green peel | Yellow green peel |
| | Lještarka | Božićnica | Kolerova | Srčika | Ivanlija | Duga | Zimnjara | Citronka | Zlatica | Paradija | Adamova Zvijezda | Slavonska srcika | Cortland | Russet | Wild |
| **Flavanols** | | | | | | | | | | | | | | | |
| Procyanidin B1 | 98.2 | 30.6 | 27.8 | 28.1 | 8.6 | 28.3 | 25.2 | | 13.4 | 7.8 | 23.9 | 17.9 | 12.7 | 20.7 | |
| (+)-Catechin | 4.2 | 65.1 | 137.9 | 29.6 | 14.8 | 123.5 | 72.8 | 35.4 | 94.0 | 8.0 | 244.9 | 99.9 | 30.6 | 8.8 | 7.4 |
| Procyanidin B2 | 68.5 | 85.6 | 167.9 | 59.2 | 47.4 | 74.0 | 36.6 | 56.6 | 62.5 | 34.7 | 61.5 | 90.4 | 49.0 | 39.6 | 51.2 |
| (−)-Epicatechin | 58.8 | 253.0 | 244.5 | 141.9 | 67.7 | 292.1 | 61.2 | 36.9 | 152.6 | 14.5 | 360.0 | 164.2 | 76.4 | 3.3 | 80.4 |
| **Total** | 229.7 | 434.3 | 578.2 | 258.8 | 138.4 | 517.9 | 195.8 | 128.9 | 322.6 | 65.1 | 690.2 | 372.4 | 168.8 | 72.40 | 139.0 |
| **Dihydrochalcones** | | | | | | | | | | | | | | | |
| Phloridzin | 47.5 | 111.1 | 52.1 | 67.9 | 25.6 | 103.0 | 104.3 | 31.6 | 52.3 | 20.5 | 74.2 | 141.3 | 30.8 | 25.4 | 18.5 |
| **Total** | 47.5 | 111.1 | 52.1 | 67.9 | 25.6 | 103.0 | 104.3 | 31.6 | 52.3 | 20.5 | 74.2 | 141.3 | 30.8 | 25.4 | 18.5 |
| **Phenolic acids** | | | | | | | | | | | | | | | |
| Chlorogenic acid | 6.9 | 22.9 | 18.7 | nd | 5.1 | nd | 86.9 | 32.3 | 53.7 | 2.4 | 106.8 | 63.1 | 11.0 | nd | 7.7 |
| **Total** | 6.9 | 22.9 | 18.7 | | 5.1 | | 86.9 | 32.3 | 53.7 | 2.4 | 106.8 | 63.1 | 11.0 | | 7.7 |
| **Flavonols** | | | | | | | | | | | | | | | |
| Quercetin-3-galactoside | 500.8 | 292.1 | 255.8 | 232.5 | 193.0 | 143.9 | 113.6 | 111.2 | 195.0 | 109.7 | 113.8 | 194.3 | 121.2 | 100.7 | 225.0 |
| Quercetin-3-glucoside | 392.1 | 161.9 | 60.0 | 210.4 | 163.0 | 36.6 | 72.4 | 54.1 | 104.5 | 30.7 | 77.8 | 146.8 | 77.4 | nd | 59.4 |
| Quercetin derivative 1 | 55.1 | 9.1 | 16.3 | 6.7 | 22.2 | 10.5 | 10.8 | 7.5 | 17.6 | 9.6 | 22.5 | 18.6 | 15.4 | 7.7 | 19.3 |
| Quercetin derivative 2 | 15.2 | 7.5 | 7.4 | 11.1 | 4.1 | 4.4 | 1.7 | | 5.0 | 3.1 | 5.4 | 1.4 | 3.3 | nd | 4.4 |
| Quercetin-3-xyloside[a] | 34.9 | 10.4 | 39.1 | 16.8 | 19.7 | 10.6 | 15.9 | 8.9 | 10.7 | 10.6 | 30.5 | 44.4 | 17.9 | 8.8 | 23.2 |
| Quercetin-3-rhamnoside | 120.7 | 38.3 | 74.4 | 90.7 | 62.6 | 35.8 | 22.9 | 19.2 | 34.3 | 30.2 | 15.7 | 64.5 | 31.6 | 30.7 | 64.8 |
| Quercetin | 104.0 | 16.3 | 31.8 | 29.2 | 15.5 | 12.9 | 5.4 | 7.0 | 17.9 | 11.1 | 7.8 | 16.2 | 17.6 | 4.1 | 13.0 |
| **Total** | 1222.7 | 535.7 | 484.7 | 597.3 | 480.2 | 254.7 | 242.7 | 207.9 | 384.9 | 204.8 | 273.5 | 486.2 | 284.4 | 152.0 | 408.9 |
| **Anthocyanins** | | | | | | | | | | | | | | | |
| Cyanidin-3-galactoside | 213.2 | 53.0 | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | 25.8 | nd | nd |
| **Total** | 213.2 | 53.0 | | | | | | | | | | | 25.8 | | |
| **TOTAL (HPLC)** | 1720.1 | 1156.8 | 1133.7 | 924.1 | 649.3 | 875.6 | 629.7 | 400.6 | 813.5 | 292.9 | 1144.7 | 1063.0 | 520.8 | 249.8 | 574.2 |
| TOTAL (FC)[b] | 3879.9 | 3378.2 | 4168.1 | 3334.6 | 1975.6 | 3807.5 | 2702.3 | 1672.6 | 3043.3 | 917.5 | 3768.9 | 3518.8 | 1839.6 | 831.2 | 2254.4 |

LOD in mg $kg^{-1}$: procyanidin B1 0.4, (+)-catechin 0.3; procyanidin B2 1.7; (−)-epicatechin 0.5; phloridzin 0.2; chlorogenic acid 0.2, quercetin-3-galactoside 0.6; quercetin-3-glucoside 0.1; quercetin 0.3; quercetin-3-rhamnoside 0.4; cyanidin-3-galactoside 1.8.

[a] Tentetively identified.

[b] Total polyphenols obtained by Folin Ciocalteu (FC) method based on two extracts each measured twice ($n=4$), expressed in mg gallic acid equivalent per kg of fresh peel weight.

* Data based on two extracts each measured once ($n=2$), coefficient of variation from 0.6 to 22%. Nd, not detected.

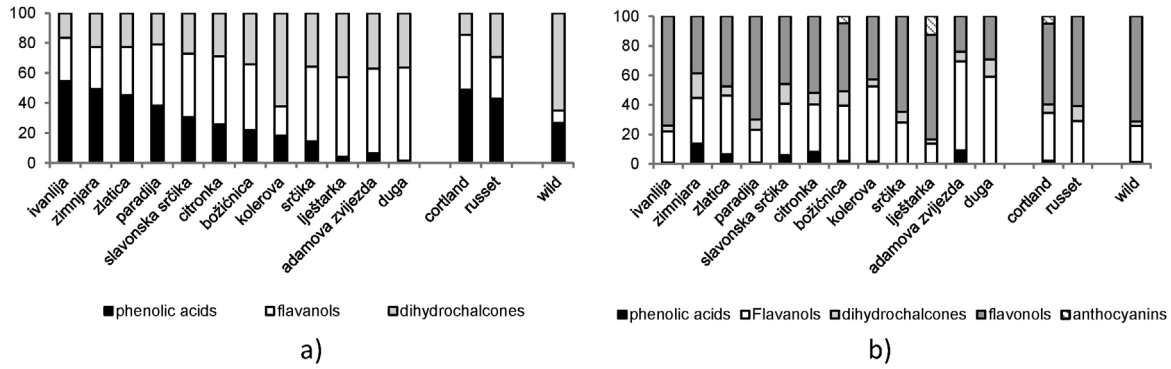


**Fig. 1.** The percentage distribution of polyphenol groups in the flesh (a) and in the peel (b).

varieties had higher amount of (−)-epicatechin and chlorogenic acid, while the amount of other compounds was similar (Jakobek et al., 2013). According to the results, the varieties that can be highlighted due to the high polyphenol content in the peel are Lještarka, Božićnica, Kolerova, Adamova zvijezda and Slavonska srčika. It could be mentioned that variety Lještarka has much higher amount of quercetin derivatives, even two to six time higher than other varieties. The highest amount of flavanols and phenolic acids was found in Adamova Zvijezda. Overall, these two varieties, Lještarka and Adamova zvijezda can be highlighted among other varieties due to the high amount of mentioned polyphenols in the peel. It could be mentioned that during the usual preparation of peel samples by apple peeling, very small amounts of flesh could remain in the peel sample. But care was taken in the preparation so that the flesh remains are minimal which should not influence the presented results.

The content of total polyphenols determined by the Folin–Ciocalteu method (Tables 1 and 2) was higher than the content determined by HPLC, which is the usual trend (Ceymann et al., 2012; Iacopini et al., 2010; Lamperi et al., 2008). But the same varieties that gave the highest total polyphenol content by the HPLC method generally show the highest total polyphenol content with the Folin–Ciocalteu method. The correlation coefficient between the results obtained by these two analytical techniques were $R^2$ = 0.94 and 0.78 for the flesh and peel, respectively. When evaluating apples as a source of polyphenols, it should be mentioned that the flesh contributes more to the intake of polyphenols. Namely, the peel represents only about 10% of the whole fruit and it is not always consumed. That is why some of the varieties which have more polyphenols in the flesh could be better sources of polyphenols. While the peel doesn't contribute to the polyphenol intake as much as flesh does, it does contain important polyphenol group such as quercetin derivatives, and it has a high accumulation of polyphenols. That is why some of the varieties are highlighted as varieties rich in polyphenols in the peel. Polymeric procyanidins were not determined in this study and since they are present in old apple varieties (the degree of polymerization up to 10) (Hellström and Matilla, 2008; Jakobek et al., 2013) their determination will be the focus of future study.

### 3.3. Classification of apples

Polyphenol groups from all varieties from Croatia and USA were projected in a three-dimensional diagram (Supplementary material, Fig. S1). In the flesh, the projection included the amount of phenolic acids, flavanols and dihydrochalcones (Supplementary material Fig. S1a). It appears that the varieties can be categorized by the amount of flavanols and dihydrochalcones in the flesh: a group with lower (Paradija, Ivanlija, Kolerova, Cortland and Russet) and a group with higher content of flavanols and dihydrochalcones.

For the peel (Supplementary material Fig. S1b), the three-dimensions are the amounts of flavonols, flavanols and dihydrochalcones. Compared to the flesh, the pattern in the peel does not show as strong a clustering according to which varieties can be categorized.

Fig. 1 shows the distribution of polyphenols. In the flesh (Fig. 1a), dihydrochalcones (15–65%), flavanols (8–62%) and phenolic acids (2–55%) can be present in high portion. It could be also seen that some varieties are richer in phenolic acids and some in flavanols. Namely, varieties, in which phenolic acids occupy higher portion, have also lower flavanol portion, and vice versa. This could mean that varieties can be classified into the ones with higher flavanol or higher phenolic acid portion in the flesh which is in accordance with earlier study (Ceymann et al., 2012). The mayor polyphenols in the peel (Fig. 1b) are flavonols (up to 74%) and flavanols (up to 60%), while the portion of other polyphenols is lower (phenolic acids up to 14%, dihydrochalcones up to 17%, anthocyanins up to 12%). There are not strong patterns for distinguishing different groups of apples according to the peel.

The differences in the polyphenol content among varieties could be the result of genetic variability as it was reported by Volz and McGhie (2011). Namely, differences among genotypes accounted for 46 to 97% of the total variation in the concentration of total polyphenols and polyphenol groups in the flesh and in the peel, except for the flavonols (quercetin derivatives) (Volz and McGhie, 2011). Flavonols in the peel are more susceptible to changes in the environment since they are sensitive to the light and temperature changes (Volz and McGhie, 2011). They protect the plant from UV radiation, and higher temperature and exposure to light seem to promote their metabolism (Volz and McGhie, 2011). In the studied apples, their high flavonol amount (quercetin derivatives) in the peel could be the result of environmental conditions and it is very likely that it could change from year to year. On the other hand, the content of other polyphenols, especially in the flesh, could be the results of the genetic variability. Environmental influence could be the reason for the lack of pattern for the categorization of apple varieties according to the peel polyphenols.

## 4. Conclusion

The polyphenol profile of old apple varieties is similar to other old and commercial apple varieties and varieties from USA. The result point to some old varieties of autumn apples that could be a good source of polyphenols, like Zimnjara because of the high total polyphenol and total dihydrochalcone content in the flesh. The peel of varieties Lještarka and Adamova zvijezda could be

highlighted due to high quercetin derivative and flavanol content, respectively. The wild apple or crab apple variety is characterized by much higher total polyphenol content, and higher phenolic acid and flavanol content in the flesh. This distinguishes the flesh of the wild apple variety among all others, while the peel is similar to all other varieties. When all of these results are summed up, the varieties richest in polyphenols are Zimnjara, Lještarka, Adamova zvijezda and wild apple. Furthermore, apple varieties could be classified according to the dihydrochalcone and flavanol content, considering their low or high content in the flesh. Another important characteristic of apple flesh is the portion of phenolic acids and flavanols. Apple varieties with high phenolic acid proportion, usually have lower flavanol proportion, and vice versa. Clustering of variety differences according to the apple peel is not as pronounced.

## Acknowledgements

We thank Mr. M. Veić for his donation of apple samples for this research. This study was funded by the J.J. Strossmayer University of Osijek project: Characterization of polyphenols in old apple cultivars.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jfca.2015.09.007.

## References

Arts, I.C.W., Hollman, P.C.H., 2005. Polyphenols and disease risk in epidemiologic studies. Am. J. Clin. Nutr. 81, 317S–325S.

Carbone, K., Giannini, B., Picchi, V., Scalzo, R.L., Cecchini, F., 2011. Phenolic composition and free radical scavenging activity of different apple varieties in relation to the cultivar, tissue type and storage. Food Chem. 127, 493–500.

Ceymann, M., Arrigoni, E., Schärer, H., Nising, A.B., Hurrell, R.F., 2012. Identification of apples rich in health-promoting flavan-3-ols and phenolic acids by measuring the polyphenol profile. J. Food Compos. Anal. 26, 128–135.

Czank, C., Cassidy, A., Zhang, Q., Morrison, D.J., Preston, T., Kroon, P.A., Botting, N.P., Kay, C.D., 2013. Human metabolism and elimination of the anthocyanin, cyanidin-3-glucoside: a $^{13}$C-tracer study$^{1-3}$. Am. J. Clin. Nutr. 97, 995–1003.

Database on polyphenol content in foods. Retrieved August 18, 2015 from: http://phenol-explorer.eu/.

Donno, D., Beccaro, G.L., Mellano, M.G., Torello Marinoni, D., Cerutti, A.K., Canterino, S., Bounous, G., 2012. Application of sensory, nutraceutical and genetic techniques to create a quality profile of ancient apple cultivars. J. Food Qual. 35, 169–181.

Escarpa, A., González, M.C., 1998. High performance liquid chromatography with diode-array detection for the determination of phenolic compounds in peel and pulp from different apple varieties. J. Chromatogr. A 823, 331–337.

Guyot, S., Le Bourvellec, C., Marnet, N., Drilleau, J.F., 2002. Porcyanidins are the most abundant polyphenols in dessert apples at maturity. Lebensmittel-Wissenschaft und Technologie 35, 289–291.

Hellström, J.K., Matilla, P.H., 2008. HPLC determination of extractable and unextractable proanthocyanidins in plant material. J. Agric. Food Chem. 56, 7617–7624.

Hollman, P.C.H., Cassidy, A., Comte, B., Heinonen, M., Richelle, M., Richling, E., Serafini, M., Scalbert, A., Sies, H., Vidry, S., 2011. The biological relevance of direct antioxidant effects of polyphenols for cardiovascular health in humans is not established. J. Nutr. 141, 989S–1009S.

Iacopini, P., Camangi, F., Stefani, A., Sebastiani, L., 2010. Antiradical potential of ancient apple varieties of *Malus x domestica* Borkh. In a peroxynitrite-induced oxidative process. J. Food Compos. Anal. 23, 518–524.

Jakobek, L., García-Villalba, R., Tomás-Barberán, F.A., 2013. Polyphenolic characterisation of old apple varieties from Southeastern European region. J. Food Compos. Anal. 31, 199–211.

Jakobek, L., 2015. Interactions of polyphenols with carbohydrates, lipids and proteins. Food Chem. 175, 556–567.

Jakobek, L., Boc, M., Barron, R.A., 2015. Optimization of ultrasonic-assisted extraction of phenolic compounds from apples. Food Anal. Methods, http://dx.doi.org/10.1007/s12161-015-0161-3.

Khanizadeh, S., Tsao, R., Rekika, D., Yang, R., Charles, M.T., Rupasinghe, H.P.V., 2008. Polyphenol composition and total antioxidant capacity of selected apple genotypes for processing. J. Food Compos. Anal. 21, 396–401.

Lamperi, L., Chiuminatto, U., Cincinelli, A., Galvan, P., Giordani, E., Lepri, L., Del Bubba, M., 2008. Polyphenol levels and free radical scavenging activities of four apple cultivars from integrated and organic farming in different Italian areas. J. Agric. Food Chem. 56, 6536–6546.

Le Bourvellec, C., Bouzerzour, K., Ginies, C., Regis, S., Plé, Y., Renard, C.M.G.C., 2011. Phenolic and polysaccharidic composition of applesauce is close to that of apple flesh. J. Food Compos. Anal. 24, 537–547.

Manach, C., Williamson, G., Morand, C., Scalbert, A., Rémésy, C., 2005. Bioavailability and bioefficacy of polyphenols in humans, I. Review of 97 bioavailability studies. Am. J. Clin. Nutr. 81, 230S–242S.

Pastene, E., Troncoso, M., Figueroa, G., Alarcón, J., Speisky, H., 2009. Association between polymerization degree of apple peel polyphenols and inhibition of *Helicobacter pylori* Urease. J. Agric. Food Chem. 57, 416–424.

Rasooly, R., Do, P.M., Friedman, M., 2010. Inhibition of biological activity of Staphylococcal enterotoxin A (SEA) by apple juice and apple polyphenols. J. Agric. Food Chem. 58, 5421–5426.

Shoji, T., Masumoto, S., Moriichi, N., Kobori, M., Kanda, T., Shinmoto, H., Tsushida, T., 2005. Procyanidin trimers to pentamers fractionated from apple inhibit melanogenesis in B16 mouse melanoma cells. J. Agric. Food Chem. 53, 6105–6111.

Suárez, B., Álvarez, A.L., Garcia, Y.D., del Barrio, G., Lobo, A.P., Parra, F., 2010. Phenolic profiles, antioxidant activity and in vitro antiviral properties of apple pomace. Food Chem. 120, 339–342.

Tenore, G.C., Campiglia, P., Ritieni, A., Novellino, E., 2013. *In vitro* bioaccessibility, bioavailability and plasma protein interaction of polyphenols from Annurca apple (*M. pumila Miller cv Annurca*). Food Chem. 141, 3519–3524.

Thompson-Witrick, K.A., Goodrich, K.M., Neilson, A.P., Hurley, G.M., Stewart, A.C., 2014. Characterization of the polyphenol composition of twenty cultivars of cider, processing, and dessert apples (Malus x domestica Borkh.) grown in Virginia. J. Agric. Food Chem. 62, 10181–10191.

Tsao, R., Yang, R., Young, J.C., Zhu, H., 2003. Polyphenolic profiles in eight apple cultivars using high-performance liquid chromatography (HPLC). J. Agric. Food Chem. 51, 6347–6353.

Veberic, R., Trobec, M., Herbinger, K., Hofer, M., Grill, D., Stampar, F., 2005. Phenolic compounds in some apple (*Malus domestica* Borkh) cultivars of organic and integrated production. J. Sci. Food Agric. 85, 1687–1694.

Veeriah, S., Hofmann, T., Glei, M., Dietrich, H., Will, F., Schreier, P., Knaup, B., Pool-Zobel, B.L., 2007. Apple polyphenols and products formed in the gut differently inhibit survival of human cell lines derived from colon adenoma (LT97) and carcinoma (HT29). J. Agric. Food Chem. 55, 2892–2900.

Volz, R.K., McGhie, T.K., 2011. Genetic variability in apple fruit polyphenol composition in *Malus x domestica* and *Malus sieversii* germplasm grown in New Zealand. J. Agric. Food Chem. 59, 11509–11521.

Vrhovšek, U., Rigo, A., Tonon, D., Mattivi, F., 2004. Quantitation of polyphenols in different apple varieties. J. Agric. Food Chem. 52, 6532–6538.

Waterhouse A, Folin-Ciocalteau micro-method for total phenol in wine. http://waterhouse.ucdavis.edu/faqs/folin-ciocalteau-micro-method-for-total-phenol-in-wine (Accessed 1.05.15).

Wojdyło, A., Oszmiański, J., Laskowski, P., 2008. Polyphenolic compounds and antioxidant activity of new and old apple varieties. J. Agric. Food Chem. 56, 6520–6530.

Yang, J., Liu, R.H., 2009. Synergistic effect of apple extracts and quercetin 3-β-*D*-glucoside combination on antiproliferative activity in MCF-7 human breast cancer cells in vitro. J. Agric. Food Chem. 57, 8581–8586.