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Optimization of Ultrasonic-Assisted Extraction of Phenolic Compounds from Apples

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Abstract In order to optimize conditions for the extraction of polyphenols from apples, peel and flesh of apples were subjected to an extraction process with different solvents consisting of various ratios of methanol and water (40, 60, 80 %), 100 % methanol, or with methanol acidified with hydrochloric acid (0.1 %). Extractions were performed using an ultrasonic bath with time periods from 5 to 15 min. Total polyphenols and total anthocyanins were analyzed using the Folin-Ciocalteau or the pH differential method, respectively. Individual polyphenols were analyzed with reversed-phase high-performance liquid chromatography with photodiode array detection (RP-HPLC-PDA). The differences in polyphenol content were statistically analyzed using *t* tests, associated with a regression model. The results showed that an efficient extraction from the peel could be performed with 80 % methanol to extract flavonols, anthocyanins, dihydrochalcones, and flavan-3-ols. Acidified methanol could also be useful for the extraction of anthocyanins and flavonols from the peel. For the flesh, 80 % methanol could be a solvent of choice for flavan-3-ols, dihydrochalcones, and hydroxycinnamic acids.

Keywords Ultrasonic extraction · RP-HPLC · Apples · Old varieties · Phenolic compounds

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Introduction

Apples are a type of fruit used commonly in the human diet due to their availability during the whole year, pleasant taste, and presence of many nutrients like vitamins and other bioactive compounds. Many studies have associated apples and their nutrients with the positive influence on the human health. Bioactive polyphenols from apples have shown antiviral properties (Suárez et al. 2010), the inhibition of *Helicobacter pylory* (Pastene et al. 2009), and anticarcinogenic properties (Veeriah et al. 2007). Due to many positive effects, apples are studied intensively.

Polyphenolic compounds are found in apples in high amounts (Escarpa and González 1998; Tsao et al. 2003; Veberic et al. 2005; Khanizadeh et al. 2008; Wojdylo et al. 2008). The major polyphenolic groups are polimeric proanthocyanidins composed of several flavan-3-ol molecules, monomeric flavan-3-ols (flavanols), flavonols, anthocyanins, dihydrochalcones, and hydroxycinnamic acids (Escarpa and González 1998; Tsao et al. 2003; Veberic et al. 2005; Khanizadeh et al. 2008; Wojdylo et al. 2008). Flavonols and anthocyanins are usually found in the peel, although some red fleshed apples can have anthocyanins in the flesh as well (Jakobek et al. 2013). Proanthocyanidins/flavan-3-ols, dihydrochalcones, and hydroxycinnamic acids are the major polyphenol groups found in the apple flesh (Tsao et al. 2003; Khanizadeh et al. 2008; Lamperi et al. 2008; Balázs et al. 2012; Jakobek et al. 2013).

Polyphenols are usually extracted from homogenized apple samples by liquid–solid extraction with different solvents. Solvents usually used are methanol (van der Sluis et al. 2001; Napolitano et al. 2004), various ratios of water and methanol (Arts and Hollman 1998; Escarpa and González 1998; Tsao et al. 2003; Veberic et al. 2005; Khanizadeh et al. 2008; Lamperi et al. 2008; Iacopini et al. 2010; Suárez

et al. 2010), various ratios of water and acetone (Vanzani et al. 2005; Suárez et al. 2010), various ratios of water, acetone, and methanol (Hellström and Mattila 2008), or ratios of water, methanol acidified with hydrochloric acid (Wojdylo et al. 2008), acetic acid (Alonso-Salces et al. 2004, 2005), or formic acid (Łata and Tomala 2007). The extraction is sometimes performed by homogenizing solvent and apple material in different periods of time (Tsao et al. 2003; Łata and Tomala 2007; Khanizadeh et al. 2008; Lamperi et al. 2008; Iacopini et al. 2010; Suárez et al. 2010) after which extract are separated from apple material by filtration. The homogenization of apple material and solvents can be performed even with various shakers (Arts and Hollman 1998). Some studies used ultrasonic extraction (Escarpa and González 1998; van der Sluis et al. 2001; Napolitano et al. 2004; Alonso-Salces et al. 2005; Veberic et al. 2005; Wojdylo et al. 2008) which is more efficient and the extraction lasts shorter period of time. Usually, polyphenol extraction is performed separately from the apple peel and flesh (Escarpa and González 1998; Tsao et al. 2003; Alonso-Salces et al. 2005; Veberic et al. 2005; Khanizadeh et al. 2008; Lamperi et al. 2008) due to different polyphenol group found in peel and flesh, or it can be done in the whole apples (van der Sluis et al. 2001; Vanzani et al. 2005; Wojdylo et al. 2008).

Since extraction process is the most important process for the right determination of the polyphenol content, it is important to adjust extraction parameters which will perform the best isolation of polyphenols. The improper extraction time, solvent, and plant material/solvent ratio could lead to insufficient extraction of polyphenols. That is why for the proper determination and quantification of polyphenols, the proper extraction parameters should be chosen and optimized.

In this work, the extraction of polyphenolic compounds from apples was studied. The polyphenols were extracted by using different ratios of methanol and water and acidified methanol, in different time periods. Extractions were performed by the help of ultrasonic bath. The polyphenolic compound content was determined spectrophotometrically (Folin-Ciocalteau method for total polyphenols and pH differential method for total anthocyanins) and with reversed-phase high-performance liquid chromatography with photodiode array detection (RP-HPLC-PDA).

Materials and Methods

Samples and Sample Preparation

Two varieties of apples were used in this study: Lještarka and Idared. Old local apple (*Malus domestica*) variety "Lještarka" was harvested in Croatia, region Slavonia, in a family orchard (M. Veić) in Mihaljevci, near Požega. Apple had a dark red color. Apple Idared was purchased in a local supermarket. Samples of both varieties were prepared in the same way. Approximately, 1 kg of apples was peeled. The peeled fruits were cut into quarters, seeds and core were removed, and flesh was cut into smaller pieces. Flesh and peel were separately homogenized by using a stick blender. Samples were kept in a freezer at −18 °C no more than 1 month.

Chemicals

Chemicals used in this study were purchased from several firms: gallic acid monohydrate (398225), (+)-catechin hydrate (C1251), (−)-epicatechin (E1753), chlorogenic acid (C3878), quercetin dihydrate (Q0125), and quercetin-3-β-D-glucoside (isoquercitrin—17793) were purchased from Sigma-Aldrich (St. Louis, MO, USA); procyanidin B1 (epicatechin(4β-8)catechin—0983), procyanidin B2 (epicatechin(4β-8)epicatechin—0984), cyanidin-3-*O*-galactoside chloride (ideain chloride—0923 S), cyanidin-3-*O*-glucoside chloride (kuromanin chloride—0915 S), quercetin-3-*O*-galactoside (hyperoside—1027 S), quercetin-3-*O*-rhamnoside (quercitrin—1236 S), phloretin-2′-*O*-glucoside (phloridzin—1046), and phloretin (1044) from Extrasynthese (Genay, France); orto-phosphoric acid (85 %) from Fluka (Buchs, Switzerland); HPLC grade methanol from J.T. Baker (Netherlands); hydrochloric acid (36.2 %), potassium chloride, sodium acetate trihydrate, sodium carbonate, and Folin-Ciocalteu reagent from Kemika (Zagreb, Croatia).

Extraction of Phenolic Compounds

The aim was to study the influence of extraction solvent, extraction time, and fruit weight/solvent ratio on the efficiency of the total polyphenol and total anthocyanin extraction, separately from flesh and peel. This part of the experiment was conducted on the apple Idared. The extraction was performed in different time intervals, from 5 to 15 min, with the help of various extraction solvents which included different ratios of methanol and water (40 % methanol, 60 % methanol, 80 % methanol), 100 % methanol, and acidified methanol (0.1 % HCl in methanol)). Shortly, samples of flesh (0.2 g) or peel (0.5 g) were weight, mixed with 5 ml of different extraction solvents, vortexed, and placed in the ultrasonic bath for 5, 10, or 15 min. Extracts were filtered and used for the analysis of total polyphenols and total anthocyanins. The ratio of the flesh or peel weight to the solvent volume that efficiently extract the majority of polyphenols was examined too. Flesh and peel of different weight (0.1, 0.2, 0.5, and 0.75 g) in 5 ml of 80 % methanol were extracted 15 min by ultrasonic bath, and total polyphenols and total anthocyanins were analyzed in extracts. Additionally, to see how many polyphenols are left in the residue, residues that gave the highest amount of polyphenols

(0.1 and 0.2 g of flesh and peel) were subjected to a second (2 ml 80 % methanol) and third extraction (1 ml 80 % methanol).

The effect of extraction solvents on the individual polyphenols was studied in two varieties: Lještarka and Idared. Flesh (0.5 g) and peel (0.5 g) were extracted in all extraction solvents for 15 min in an ultrasonic bath. Extracts were additionally filtered through a 0.45-μm PTFE syringe filter, and 20 μl was directly injected into the RP-HPLC-PDA system.

Total Polyphenol and Total Anthocyanin Determination

Total polyphenols were determined by Folin-Ciocalteau micro method (Waterhouse 2014). An aliquot (20 μl) of extract was mixed with 1580 μl of distilled water and 100 μl of Folin-Ciocalteau reagent. Three hundred microliter of sodium carbonate solution (200 g l^{-1}) was added to the mixture. The mixture was incubated at 40 °C for 30 min in the water bath. The absorbance was read at 765 nm on a UV–vis spectrophotometer (JP Selecta UV 2005, Barcelona, Spain). Solutions of gallic acid from 0 to 500 mg l^{-1} were measured with the same procedure, for the creation of the calibration curve. Total polyphenolics were expressed as milligram of gallic acid equivalents (GAE) per kilogram of fresh fruit weight (FW).

Total anthocyanins were estimated by a pH differential method (Giusti and Wrolstad 2001). Each extract was diluted with two buffers: one was potassium chloride buffer (pH 1.0) (1.86 g KCl in 1 l of distilled water, pH value adjusted to 1.0 with concentrated HCl) and the other sodium acetate buffer (pH 4.5) (54.43 g $CH_3CO_2Na \cdot 3H_2O$ in 1 l of distilled water, pH value adjusted to 4.5 with concentrated HCl). The dilution factor (DF) was 20. Extracts were incubated in the dark for 15 min at room temperature. Absorbance of both dilutions was measured at 510 and 700 nm on a UV–vis spectrophotometer (JP Selecta UV 2005, Barcelona, Spain). The absorbance of the extract was calculated as $A_{extract}=(A_{510}-A_{700})_{\mathrm{pH\ 1,0}}-(A_{510}-A_{700})_{\mathrm{pH\ 4,5.}}$ The content of total anthocyanins was calculated according to the following equation:

$$\text{Total anthocyanins}\left(\text{mg } l^{-1}\right) = (A_{extract} \times MW \times DF \times 1000)/(\varepsilon \times l)$$

where (ε) is the molar extinction coefficient of cyanidin-3-glucoside (26,900 l mol^{-1} cm^{-1}), MW is the molar weight cyanidin-3-glucoside (449.2 g mol^{-1}), DF is the dilution factor, and l is the cuvette path length (1 cm). The results were expressed in milligram of cyanidin-3-glucoside equivalents (CGEs) per kilogram of fresh fruit.

High-Performance Liquid Chromatography Measurement

Individual polyphenols in extracts were determined by using RP-HPLC-PDA. Analytical system consisted of a Varian system (USA) equipped with a ProStar 230 solvent delivery module and a ProStar 330 PDA Detector. Compounds were separated on an OmniSpher C18 column (250×4.6 mm inner diameter, 5 μm, Varian, USA) protected with guard column (ChromSep 1 cm×3 mm, Varian, USA). Mobile phase A was 0.1 % phosphoric acid in water and mobile phase B 100 % HPLC grade methanol. 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}. Injection volume for samples and standards was 20 μl; compounds were separated at room temperature. A 10-min re-equilibration period was used between individual runs. UV–vis spectra were recorded in wavelength range from 190 to 600 nm. The detection wavelength was 280 nm for procyanidins, monomeric flavan-3-ols, and dihydrochalcones, 320 nm for phenolic acids, 360 nm for flavonols, and 510 nm for anthocyanins.

Identification was based on the comparison of retention times and spectral data with those of authentic standards. Furthermore, extracts were spiked with polyphenol standards which gave additional information on polyphenol identification. Calibration curves of the standards were made by preparing stock standards in 100 % methanol ((+)-catechin, (−)-epicatechin, chlorogenic acid, quercetin, quercetin-3-rhamnoside, quercetin-3-galactoside, quercetin-3-glucoside), in ethanol (phloretin, phloridzin), in water (procyanidin B1, procyanidin B2), or in acidified methanol (0.1 % HCl in methanol—cyanidin-3-galactoside, cyanidin-3-glucoside). Then, stock solutions were diluted and injected into RP-HPLC-PDA (0.8–160 mg l^{-1} (procyanidin B1); 1–225 mg l^{-1} (procyanidin B2); 2–250 mg l^{-1} ((+)-catechin, (−)-epicatechin); 1–200 mg l^{-1} (phloretin, phloridzin); 1–132 mg l^{-1} (quercetin); 5–180 mg l^{-1} (quercetin-3-rhamnoside, quercetin-3-glucoside, quercetin-3-galactoside); 1–240 mg l^{-1} (cyanidin-3-galactoside, cyanidin-3-glucoside); and 1–92 mg l^{-1} (chlorogenic acid). Identified compounds were quantified using calibration curves of authentic standards. All phenolic compounds showed a linear response within range studied (r^2=0.9702–0.9999). Precision of the method was evaluated by determining within-day variation of the HPLC analysis (within-day precision). Coefficients of variation (CV) of peak areas varied between 0.92 and 10.3 %. To determine recoveries, known amounts of standards (10–30 mg l^{-1}) were added to extracts prior to HPLC analysis.

The recoveries were in all cases higher than 92 %. In the calculation of final results, no correction for recovery was applied to data.

Statistical Analysis

All extracts were made in duplicate and analyzed two times for total polyphenols and total anthocyanins or once with RP-HPLC-PDA. Data presented are mean±standard deviation (SD). The results for total polyphenols and total anthocyanins after 15 min of extraction and the RP-HPLC-PDA results for individual polyphenols were analyzed using a regression model using contrasts for comparing different sets of solvent type, with phenolic compound indicators included for statistical control. Significance of differences of sets of solvent type in the regression model is determined by the *T* value in certain rows of the regression table and its associated *p* value at the 0.05 and 0.01 levels for significance and strong significance.

Results and Discussions

The aim of the study was to find extraction conditions for the analysis of polyphenols in apples. Since the extraction procedures can be time-consuming, with many steps like liophilization, extraction, evaporation, cleaning procedures, the aim of this study was to find conditions which will avoid too many steps and still give good results. To avoid liophilization, the extraction was studied in fresh apple material. The extractions were obtained separately in the peel and flesh of apples due to different compounds found in these materials. To reach a good extractability of compounds, fruit weight to solvent ratio, appropriate solvent, and extraction time were determined.

The Influence of the Extraction Solvent

Various solvents were examined for their extractability of polyphenols (40 % methanol, 60 % methanol, 80 % methanol, 100 % methanol, and acidified methanol (0.1 % HCl in methanol)), in different time periods, from 5 to 15 min in the ultrasonic bath (Tables 1 and 2). Total polyphenols were measured with the Folin-Ciocalteau method and total anthocyanins with the pH differential method. With respect to the time of the extraction, generally it could be seen that the amount of total polyphenols and total anthocyanins from both peel and flesh increased as the extraction time increased, for all solvents used. Extraction lasting for 30 min was also examined, and the results were almost equal to the results obtained after 15 min (data not shown). According to these results, 15 min in ultrasonic bath should be enough to efficiently extract polyphenols from flesh and peel.

For the peel (Table 1), when methanolic solvents without acid were compared, higher amount of total polyphenols were extracted by using 80 % methanol (2356 mg kg^{-1} FW) and higher amount of total anthocyanins with 80 to 100 % methanol (35.7 and 35.4 mg kg^{-1} FW, respectively). Methanol (40 %) was also shown to be a good extraction solvent, but higher percentages of methanol like 80 or 100 % methanol were shown to be better than solvents with higher ratio of water like 40 % methanol, due to the reduction of polyphenol oxidase activity by methanol. On the other hand, acidified methanol extracted even higher total polyphenols (2669 mg kg^{-1} FW) and total anthocyanins (37.9 mg kg^{-1} FW). In a slightly acidic environment, the higher content of polyphenols could be the result of the hydrolysis of polymeric molecules. According to the results, the polyphenols from the peel could be efficiently extracted with 80 % methanol or acidified methanol. Additionally, anthocyanins are more stable in acidic environment. Namely, anthocyanins change their form according to the pH value. Lower pH value is suitable for anthocyanins to be in the flavilium cation form which gives the red color to the extract and is suitable for the determination of anthocyanins according to their UV/Vis characteristics (Giusti and Wrolstad 2001), and this might cause slightly higher amounts. In the case of higher anthocyanin concentration in apple peel, acidified methanol could be a good choice.

For the flesh (Table 2), the highest amounts of total polyphenols were obtained by using 60 or 80 % of methanol (599 and 604 mg kg^{-1} FW, respectively). According to these results, more total polyphenols could be extracted from the flesh using 60 or 80 % methanol. Similar results were obtained in earlier studies. Namely, adequate extraction of catechins from apples was achieved with 60 to 100 % methanol (Arts and Hollman 1998).

Fruit Weight to Solvent Ratio

The fruit weight to solvent ratio is important because the solvent affect the amount of polyphenolics to be extracted. Namely, too much solvent could isolate a majority of polyphenolic compounds, but on the other hand, the extract could be diluted which requires additional concentration step and additional time. Not enough solvent can bring to an insufficient extraction. Different amounts of peel and flesh (0.1, 0.2, 0.5, and 0.75 g) were extracted in 5 ml of 80 % methanol, 15 min with ultrasonic bath (Fig. 1). Total polyphenols in extracts were measured by Folin-Ciocalteau method and total anthocyanins by pH differential method. The results showed that total polyphenols and total anthocyanins decreased when peel/solvent ratio increased. It is usual to expect lower amount of extracted compounds with higher amount of apple material per milliliter of solvent, due to a smaller contact area between extracted material and solvent. Moreover, after the extraction,

Table 1 The amount of polyphenolic compounds in the extracts of peel of apple Idared, obtained by using different extraction solvents and ultrasonic bath

Extraction solvent	Time (min)	Total polyphenols[a] (mg GAE kg^{-1} FW)	Total anthocyanins[b] (mg CGE kg^{-1} FW)
40 % methanol	5	1001.7±6.5	25.0±7.1
	10	1184.0±211.7	20.2±0.1
	15	2292.4±151.7	28.4±7.8
60 % methanol	5	1546.2±127.4	13.7±4.9
	10	1480.9±124.3	15.1±2.4
	15	1878.7±52.2	26.5±2.7
80 % methanol	5	1205.9±57.3	13.0±0.0
	10	924.4±18.7	17.5±2.3
	15	2356.4±167.1	35.7±2.7
100 % methanol	5	1179.5±103.0	18.1±2.3
	10	1093.0±51.3	16.4±4.6
	15	1949.2±13.2	35.4±1.8
Acidified methanol[c]	5	1371.7±53.0	10.2±4.8
	10	1622.2±26.3	21.9±2.4
	15	2669.2±394.2	37.9±3.6

[a] Total polyphenols determined by using Folin-Ciocalteau method, expressed as milligram of gallic acid equivalent (GAE) per kilogram of fresh weight (FW)

[b] Total anthocyaninis determined by using pH differential method, expressed as milligram of cyanidin-3-glucoside equivalent (CGE) per kilogram of fresh weight (FW)

[c] Methanol acidified with 0.1 % hydrochloric acid

residues of two systems that gave the highest amount of polyphenols (0.1 and 0.2 g in 5 ml of solvent) were subjected to additional second (2 ml of solvent) and third (1 ml of solvent) extractions. First and second extraction extracted most of polyphenols (93 to 96 % peel, 90 to 93 % flesh). The third extraction extracted additional smaller portion of polyphenols (4–7 % peel, 7–10 % flesh). To extract all polyphenols, it is necessary to conduct at least two extraction steps. Furthermore, since the weight of the fresh material, that gave the highest amount of polyphenols, is small (0.1 and 0.2 g), it could be suggested to choose higher weight (0.2 g) over smaller (0.1 g) and conduct the extraction three times. The number of extraction steps also depends on the apple variety and the amount of polyphenols.

Table 2 The amount of total polyphenolic compounds in the extract of flesh of apple Idared obtained by using different extraction solvents and ultrasonic bath

Extraction solvent	Time (min)	Total polyphenols[a] (mg kg^{-1} FW)
40 % methanol	5	384.9±8.9
	10	368.0±28.4
	15	456.2±42.3
60 % methanol	5	385.7±12.9
	10	425.4±30.6
	15	598.8±30.0
80 % methanol	5	529.5±84.3
	10	433.9±84.1
	15	604.0±31.6
100 % methanol	5	477.5±30.7
	10	452.0±60.0
	15	484.0±23.1
Acidified methanol[b]	5	416.6±15.1
	10	430.1±36.8
	15	493.0±62.3

[a] Total polyphenols determined by using Folin-Ciocalteau method, expressed as milligram of gallic acid equivalent (GAE) per kilogram of fresh weight (FW)

[b] Methanol acidified with 0.1 % hydrochloric acid

Finally, the optimized conditions for the extraction of polyphenols from apple material would be to extract polyphenols 15 min in the ultrasonic bath. Good extraction solvents to be used are 60 to 80 % methanol for the flesh and 80 % methanol or acidified methanol for the peel. After the first extraction, the residue should be extracted again for 15 min, in the ultrasonic bath to complete the polyphenol extraction. Extracts should be combined and used for the analysis of polyphenols. Depending on the apple variety, a third extraction of the residue could be suggested. The literature data supports the use of methanol/water mixtures as extraction solvents. In earlier studies, different ratios of methanol with water were shown to be the solvent of choice (Arts and Hollman 1998; Escarpa and González 1998; Tsao et al. 2003; Veberic et al. 2005; Khanizadeh et al. 2008; Lamperi et al. 2008; Iacopini et al. 2010; Suárez

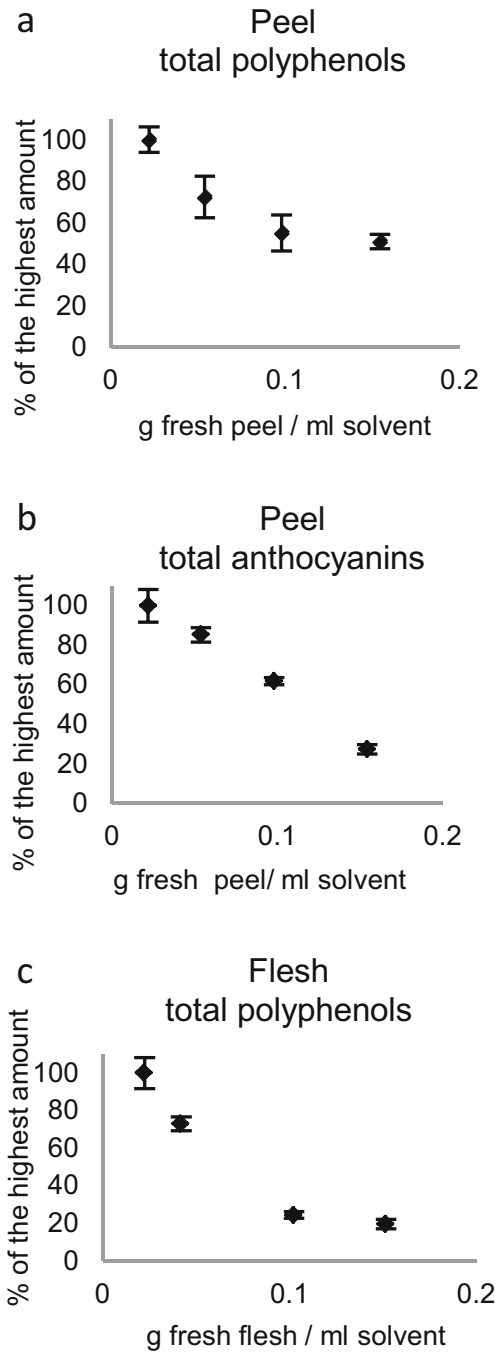

Fig. 1 The influence of fruit weight/solvent volume on the extractability of **a** total polyphenols from peel, **b** total anthocyanins from peel, **c** total polyphenols from flesh (Idared). Total polyphenols and total anthocyanins determined by using Folin-Ciocalteau and pH differential methods, respectively

et al. 2010). Furthermore, methanol can reduce the polyphenol oxidase activity, which can catalyze the oxidation and polymerization of polyphenols to brown pigments (Arts and Hollman 1998). By reducing polyphenol oxidase activity, methanol can protect polyphenols and allow better extraction. Acidified methanol was also used as a solvent (Ceymann et al. 2012), and it could be a good solution in the case of apple peel that contain anthocyanins. On the other hand, acetone was shown to be suitable for the extraction of polymeric proanthocyanidins (Hellström and Mattila 2008), but these compounds were not the aim of this study. The extraction of polymeric procyanidins from apples with different solvents will be the aim of the future study.

Analysis of Extracts with RP-HPLC-PDA

The RP-HPLC-PDA method was developed and validated for the determination of individual polyphenols. Compounds were identified by using spectral data and retention times of authentic standards. Additionally, extracts were spiked with known standards to confirm the identification. Some of them were tentatively identified by using literature data, like phloretin-2′-xyloglucoside (Tsao et al. 2003; Alonso-Salces et al. 2004; Pastene et al. 2009; Balázs et al. 2012; Jakobek et al. 2013), quercetin-3-xyloside (Jakobek et al. 2013), and *p*-coumaroylquinic acid (Tsao et al. 2003; Jakobek et al. 2013). Identified compounds are shown in Fig. 2. In the apple peel (Fig. 2), the most dominant compounds were quercetin derivatives. Several peaks could belong to kaempferol or (iso)rhamnetin derivatives, but they were not identified. Besides flavonols (quercetin derivatives), peel contained chlorogenic acid from the hydroxycinnamic acid group, cyanidin-3-galactoside from the anthocyanin group, some flavan-3-ols like (+)-catechin, (−)-epicatechin, procyanidin B2, and phloridzin from the dihydrochalcone group. All of these compounds were found in apple peel in earlier studies (Escarpa and González 1998; Tsao et al. 2003; Khanizadeh et al. 2008; Lamperi et al. 2008; Balázs et al. 2012; Jakobek et al. 2013). Favonols and anthocyanins are characteristic compounds of the peel. Earlier studies reported galactoside, glucoside, xyloside, arabonoside, and rhamnoside derivatives of quercetin (van der Sluis et al. 2001; Tsao et al. 2003; Khanizadeh et al. 2008; Lamperi et al. 2008) and various (iso)rhamnetin and kaempferol derivatives (Alonso-Salces et al. 2004; Jakobek et al. 2013). Anthocyanins reported in the peel are galactoside of cyanidin (van der Sluis et al. 2001; Tsao et al. 2003; Alonso-Salces et al. 2004; Khanizadeh et al. 2008), other cyanidin derivatives (Lamperi et al. 2008; Balázs et al. 2012; Jakobek et al. 2013), and even malvidin derivatives (Balázs et al. 2012).

In the flesh (Fig. 2), the most dominant compounds were phloridzin and phloretin-2′-xyloglucoside that belong to the group of dihydrochalchones, chlorogenic acid, and *p*-coumaroylquinic acid from hydrocycinnamic acid group, (+)-catechin, (−)-epicatechin from monomeric flavan-3-ols, and procyanidin B1 and procyanidin B2 from the dimeric procyanidins. The same compounds were identified in earlier studies (Escarpa and González 1998; Tsao et al. 2003; Napolitano et al. 2004; Khanizadeh et al. 2008; Lamperi et al. 2008; Ceymann et al.

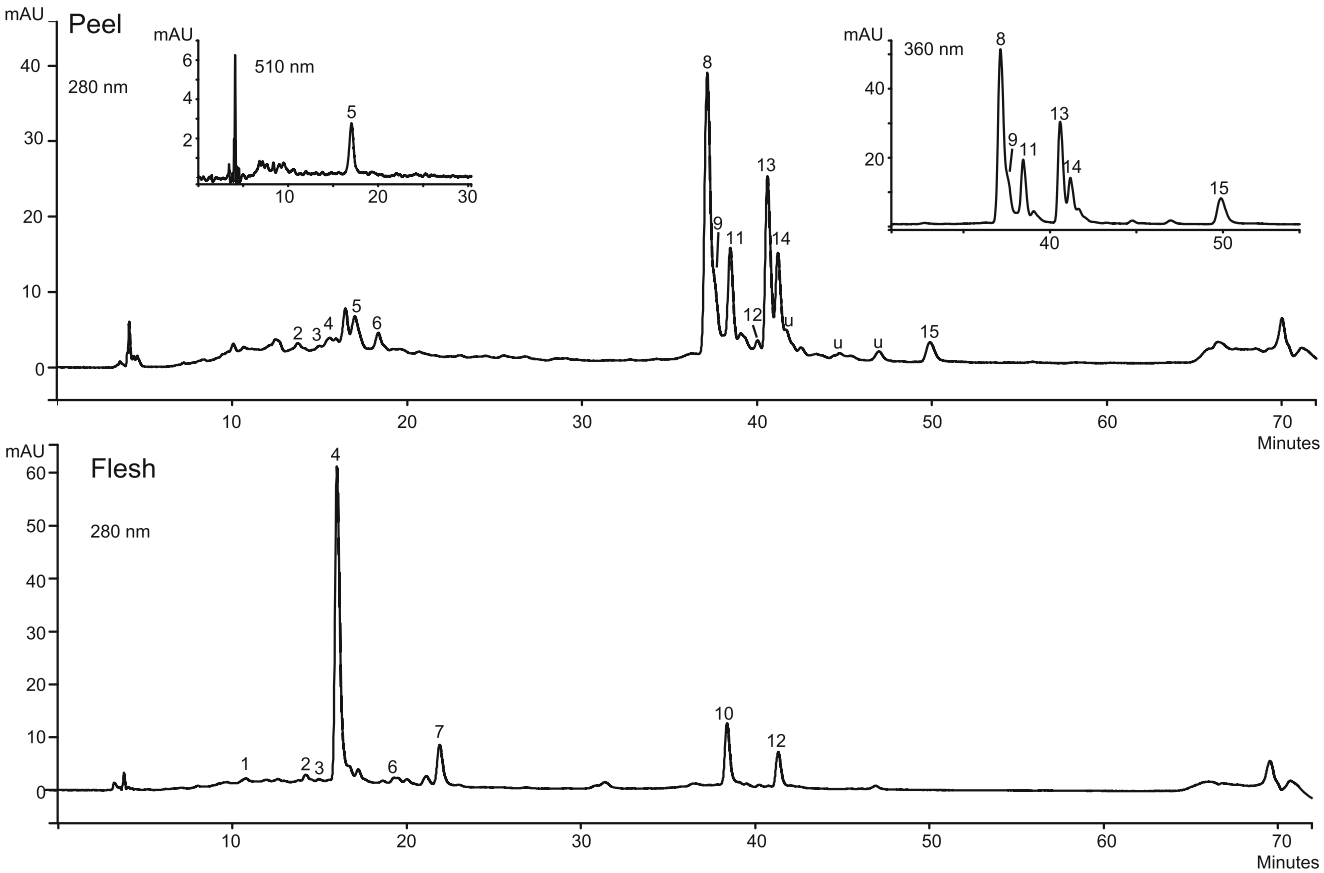

Fig. 2 Chromatogram of apple peel (0.1 % HCl in methanol) and flesh (80 % methanol). Peak identification: *1* procyanidin B1, *2* (+)-catechin, *3* procyanidin B2, *4* chlorogenic acid, *5* cyanidin-3-galactoside, *6* (−)-epicatechin, *7** *p*-coumaroylquinic acid, *8* quercetin-3-galactoside, *9* quercetin-3-glucoside, *10** phloretin 2′-xyloglucoside, *11* quercetin derivative, *12* phloridzin, *13** quercetin-3-xyloside, *14* quercetin-3-rhamnoside, *15* quercetin (*tentatively identified according to literature data), *U* unidentified

2012; Jakobek et al. 2013). Some papers reported low amounts of flavonols in the flesh (van der Sluis et al. 2001; Tsao et al. 2003; Lamperi et al. 2008; Balázs et al. 2012; Jakobek et al. 2013) usually quercetin-3-rhamnoside (van der Sluis et al. 2001; Tsao et al. 2003; Lamperi et al. 2008; Jakobek et al. 2013) although quercetin-3-xyloside, quercetin-3-arabinoside, quercetin-3-galactoside, and quercetin-3-glucoside were also found (van der Sluis et al. 2001; Lamperi et al. 2008). These flavonols were not identified in this study. Furthermore, polymeric procyanidins were found in apples in high content (Jakobek et al. 2013). Since they do not give good, well-resolved peaks in RP-HPLC chromatograms due to many isomers that elute together, they were not determined in this study. They are usually determined by the use of phloroglucinolysis (Wojdylo et al. 2008; Jakobek et al. 2013) or normal-phase chromatography (Vrhovsek et al. 2004).

The content of individual polyphenols (Tables 3 and 4) are similar to other literature data (Escarpa and González 1998; Tsao et al. 2003; Khanizadeh et al. 2008; Lamperi et al. 2008) with somewhat lower content of some flavan-3-ols ((−)-epicatechin, and procyanidins). Lower content can be the result of the analysis of the first extract (in 5 ml of extraction solutions), without additional second extraction of the residue (in 2 ml of solvent). The values of the total polyphenols are also similar to literature data (flesh 177 to 1060 mg kg^{-1} FW; peel 1012 to 5760 mg kg^{-1} FW) (Tsao et al. 2003; Khanizadeh et al. 2008; Lamperi et al. 2008). In our earlier study, the amount of total polyphenols (without polymeric procyanidins) was from 525 to 1611 mg kg^{-1} FW in the flesh and from 672 to 3150 mg kg^{-1} FW in the peel (Jakobek et al. 2013). The differences between the total polyphenols obtained with Folin-Ciocalteau method (Tables 1 and 2) and HPLC method (Tables 3 and 4) could be observed. Folin-Ciocalteau method determines not only polyphenols but also some other compounds (sugars, amino acids, etc.). In order to obtain the results closer to the ones with HPLC, the extracts should be precleaned to remove all interferences. This step was not done in this study, so the results for total polyphenols obtained with Folin-Ciocalteau methods are higher. Moreover, apples contain high amount of polymeric procyanidins which were not determined with RP-HPLC-PDA method. This too could

Table 3 The amount of individual polyphenolic compounds determined with RP-HPLC-PDA in extracts of peel obtained by different extraction solvents and ultrasonic bath for 15 min

Phenolic compound	Polyphenols									
	(mg kg^{-1} FW)									
	40 % methanol		60 % methanol		80 % methanol		100 % methanol		Acidified methanol	
	Lještarka	Idared	Lještarka	Idared	Lještarka	Idared	Lještarka	Idared	Lještarka	Idared
Flavonols										
Quercetin-3-galactoside	399.2±6.6	237.1±10.4	345.1±17.7	205.1±12.6	370.9±9.1	254.3±8.5	305.6±4.7	237.1±10.1	347.7±7.6	268.6±14.6
Quercetin-3-glucoside	103.1±6.5	106.6±8.4	78.2±2.2	74.0±9.1	87.6±4.1	105.0±4.7	79.2±2.3	86.9±18.9	64.5±5.9	92.1±0.2
Quercetin derivative 1	19.9±1.8	12.1±1.1	24.7±3.4	6.9±0.7	27.9±1.4	12.3±5.0	21.1±2.2	10.0±1.7	28.1±0.1	14.2±2.4
Quercetin-xyloside[a]	48.7±5.9	35.8±3.9	58.4±4.2	30.9±1.3	67.9±3.5	39.6±8.4	53.1±4.2	36.8±4.8	48.8±4.6	31.7±6.6
Quercetin-3-rhamnoside	48.4±1.8	46.4±2.1	72.5±1.7	40.1±0.0	90.3±7.4	49.1±7.5	74.4±0.2	46.2±6.6	81.9±2.3	56.8±8.4
Quercetin	bdl	nd	bdl	nd	bdl	nd	bdl	nd	29.0±3.3	11.3±1.2
Dihydrochalcones										
Phloridzin	9.0±0.5	12.1±0	10.7±0.7	12.1±1.3	11.4±1.1	15.7±3.4	9.9±0.5	13.3±1.5	9.8±1.3	24.7±6.1
Anthocyanins										
Cyanidin-3-galactoside	1.4±0.3	7.0±0.7	6.8±0.4	9.3±0.4	7.7±0.6	44.3±8.6	1.0±0.4	22.5±7.3	4.0±2.1	33.5±4.6
Flavan-3-ols										
Procyanidin B1	nd	12.6±1.4	nd	7.3±1.0	nd	7.4±0.8	nd	7.2±2.2	nd	19.4±1.3
(+)-Catechin	bdl	14.4±0.5	bdl	13.9±0.4	bdl	11.3±1.5	bdl	10.4±2.9	22.2±2.0	18.0±2.8
Procyanidin B2	22.1±9.9	29.5±1.8	28.7±3.6	32.0±0.5	27.2±2.5	38.1±4.2	nd	33.0±0.8	22.9±1.8	37.3±2.2
(−)-Epicatechin	9.4±1.1	5.0±0.7	6.4±1.3	9.5±1.3	6.1±0.4	13.3±2.0	3.4±0.1	9.2±2.6	47.2±2.3	42.0±8.6
Hydroxycinnamic acids										
Chlorogenic acid	3.1±0.1	7.9±0.2	3.2±0.1	8.9±0.5	3.7±0.2	12.8±7.3	3.9±0.3	8.9±1.0	3.8±0.1	14.7±0.2
Total	664.6±33.9	526.5±31.3	634.7±23.1	449.9±29.2	700.7±29.9	603.2±61.8	551.6±13.1	521.6±60.5	681.0±25.7	653.2±58.4

bdl below detection limit, *nd* not detected

[a] Tentatively identified

Table 4 The amount of individual polyphenolic compounds determined with RP-HPLC-PDA in extracts of flesh obtained by different extraction solvents and ultrasonic bath for 15 min

Phenolic compound	Polyphenols									
	mg kg^{-1} FW									
	40 % methanol		60 % methanol		80 % methanol		100 % methanol		Acidified methanol	
	Lještarka	Idared	Lještarka	Idared	Lještarka	Idared	Lještarka	Idared	Lještarka	Idared
Flavan-3-ols										
Procyanidin B1	7.0±0.1	5.4±0.7	7.7±0.3	5.1±1.1	13.9±1.1	4.4±0.3	9.9±1.2	4.0±0.2	6.6±0.6	5.6±0.7
(+)-Catechin	9.8±0.8	6.8±1.4	4.3±0.6	4.4±0.8	14.8±1.6	10.9±2.1	10.4±0.9	3.5±0.9	18.1±0.8	13.7±2.3
Procyanidin B2	nd	31.0±2.3	nd	28.5±2.6	28.1±1.7	31.0±5.8	23.0±0.5	23.3±1.6	24.7±0.6	26.9±1.8
(−)-Epicatechin	8.8±0.7	2.4±0.3	4.6±0.4	3.3±0.4	7.9±0.9	5.7±1.6	4.9±0.9	2.3±1.0	28.9±0.5	3.4±1.2
Dihydrochalcones										
Phloretin-2′-xyloglucoside[a]	27.1±3.5	16.3±2.0	31.3±1.0	16.3±2.7	33.7±1.2	16.3±2.9	27.2±0.9	14.6±0.9	29.7±0.2	15.8±1.0
Phloridzin	22.8±2.1	10.4±2.4	23.7±4.4	11.0±2.9	26.5±1.3	12.1±1.7	21.8±2.1	12.5±2.7	24.0±2.3	16.7±5.1
Hydroxycinnamic acids										
Chlorogenic acid	158.3±4.3	5.6±2.7	212.0±4.8	9.1±0.6	242.9±9.4	12.2±4.0	192.1±6.4	10.4±3.0	162.4±10.7	6.8±1.0
p-Coumaroylquinic acid[a]	9.0±0.7		8.8±0.1		10.3±0.4		7.8±0.5		5.6±0.7	
Total	242.8±12.2	77.8±11.7	291.7±11.6	77.7±11.2	378.1±17.6	92.6±18.4	297.1±13.4	70.5±10.3	300.0±16.4	88.9±13.1

nd not detected

[a] Tentatively identified

affect somewhat lower content of total polyphenols determined with HPLC method.

The Influence of the Extraction Solvent on Individual Polyphenols

The influence of the solvent on the extractability of individual polyphenols was studied in two varieties: Lještarka and Idared. In the apple peel (Table 3), somewhat higher content of individual polyphenols was extracted with 80 % methanol (quercetin derivatives, phloridzin, cyanidin-3-glucoside, epicatechin) when different ratios of water and methanol were compared. In acidified methanol, the content of most polyphenols was similar to 80 % methanol, but the content of monomeric polyphenols such as (+)-catechin, (−)-epicatechin, and quercetin increased significantly. This is probably due to hydrolysis reactions which occur in acidic environment. Furthermore, total polyphenols obtained by 80 % methanol and acidified methanol show the highest values. According to these results, 80 % methanol could be a good choice to extract polyphenols from apple peel because it gives high amount of polyphenols but does not cause hydrolysis reactions as acidified one. Since peel contains anthocyanins as well, which are more stable in acidified methanol (Giusti and Wrolstad 2001), the solvent of choice could be acidified methanol as well. It could be mentioned that the peak area of unidentified flavonol derivatives also increased as the methanol percentage increased and is the highest when methanol is acidified (Fig. 2). This could mean that for the extraction of flavonols, acidified methanol could also be a good choice due to better diversity of flavonols extracted. In the case of the extraction with acidified methanol, it should be known that a hydrolysis reaction will cause the breakdown of some polymeric molecules which will cause higher amount of aglycons like catechins or quercetin.

In the flesh (Table 4), 80 % methanol gave higher content of some individual polyphenols (procyanidin B1 and B2, chlorogenic acid, and (+)-catechin) and total polyphenols in comparison to other methanol/water solvents. Acidified methanol, on the other hand, extracted significantly higher content of compounds like (+)-catechin and (−)-epicatechin. Higher content of these compounds could be the result of the hydrolysis of oligomeric and polymeric proanthocyanidins in an acidic environment. Namely, proanthocyanidins are more susceptible to hydrolysis due to labile nature of interflavonoid bonds toward acids (Hellström and Mattila 2008). The result of hydrolysis in an acidic environment is the higher content of monomeric flavan-3-ols. Because apple flesh contains high amount of flavan-3-ols (monomeric flavan-3-ols, oligomeric and polymeric procyanidins), the solvent of choice could be the mixture of methanol and water (80 % methanol) which does not influence the hydrolysis of the major polyphenolic compounds. Finally, according to the HPLC results, polyphenols from the flesh could be efficiently extracted by using 80 % methanol and polyphenols from the peel by using 80 % or acidified methanol. This is in accordance with the literature data (Arts and Hollman 1998). Van der Sluis et al. (2001) examined the extraction of polyphenols from apples with water, 50 % methanol, 100 % methanol, and methanol with 15 % acetic acid. Water was not suitable for the extraction of compounds, and there was no difference between 50 %, 100 % methanol, and acidified methanol for the majority of compounds (flavonols, anthocyanins, catechins, and phloridzin) (van der Sluis et al. 2001). But, epicatechin and chlorogenic acid were extracted better with 100 % methanol or acidified methanol. This is in agreement with our study considering the fact that they did not examine other ratios of water/methanol, between 50 to 100 %.

Statistical Model

For the HPLC data, a four-factor regression model was set up with suitable contrasts to test which sets of solvent types are best overall (Table 5). The three most important factors (explanatory variables) are the solvent type, the type of phenolic compound, and a factor specifying the part of apple (peel vs flesh). A fourth factor that plays a minor role specifies whether it was apple Lještarka or Idared. These factors were carefully encoded so that individually they provide orthogonal variables for the regression and they remain orthogonal when multiplied together to account for statistical interactions.

With two observations for each case, there are a total of 370 potential polyphenol measurements analyzed in these data. There are three cases of missing pairs of procyanidin-B2 at specific methanol levels (without acidification), in Lještarka. For these cases, estimates of the missing values from iterating the regression fit (initialized at zero estimates) were imputed, with the understanding that properly analyzed, there is a slight corresponding adjustment (by three) to the degrees of freedom in the estimation of the standard errors, to retain statistical validity. The estimated missing values for these absent procyanidin-B2 cases are 24.6 for 100 % methanol, in the peel, and 20.0 with 40 % methanol, and again 20.0 with 60 % methanol, in the flesh. By using these imputed missing values for procyanidin-B2, rather than zeros, disruption to the quality of the additive fits in to the rest of the measured cases was avoided.

The cases of compounds with larger concentrations exhibit larger variability of measurements. For these data, the standard deviation increases approximately linearly with the square root of the mean. Accordingly, to stabilize the variance, the square root of the polyphenol concentration was modeled. (In contrast, if the standard deviation were proportional to the mean, the case of constant coefficient of variation, then we would model the logarithm of the concentrations). Only if the standard deviations were approximately constant

Table 5 Regression fit to square root of individual polyphenol concentration obtained by RP-HPLC-PDA

Term	Coefficient	Standard error coefficient	*T* value	*P* value
Contrasts for solvent type[a]				
80 %, 100 %, acidified vs 40 %, 60 %	0.0578	0.0111	5.19	0.000
80 %, acidified vs 100 %	0.1523	0.0242	6.29	0.000
80 % vs acidified—flesh	0.1358	0.0675	2.01	0.045
80 % vs acidified—peel	(No significant difference so not included in final fit)			
40 % vs 60 %	(No significant difference so not included in final fit)			
Contributions to the mean from type of phenolic compound				
Quercetin-3-galactoside	16.627	0.131	127.24	0.000
Quercetin-3-glucoside	8.724	0.137	63.88	0.000
Quercetin-3-rhamnoside	7.371	0.125	59.14	0.000
Quercetin-3-xyloside	6.320	0.125	50.72	0.000
Quercetin derivative	3.763	0.125	30.19	0.000
Cyanidin-3-galactoside	2.656	0.132	20.15	0.000
(+)-Catechin	3.740	0.101	37.14	0.000
(−)-Epicatechin	0.912	0.228	4.00	0.000
Procyanidin B1	3.250	0.100	32.37	0.000
Procyanidin B2	5.2264	0.0775	67.48	0.000
p-Coumaroylquinic acid	2.715	0.173	15.67	0.000
Phloretin-2′-xyloglucoside	5.055	0.125	40.56	0.000
Phloridzin	3.8678	0.0775	49.94	0.000
Chlorogenic acid	5.4723	0.0775	70.65	0.000
Effects with contributions from differences of peel vs flesh[b]				
Phloridzin—peel vs flesh	−0.6661	0.0976	−6.82	0.000
Procyanidin B1—peel vs flesh	0.361	0.109	3.30	0.001
Chlorogenic acid—peel vs flesh	−3.2627	0.0976	−33.42	0.000
(−)-Epicatechin—peel vs flesh	1.624	0.214	7.59	0.000
Peel vs flesh Lještarka	0.4672	0.0769	6.08	0.000
Peel vs flesh Idared	0.2135	0.0655	3.26	0.001
Effects with contributions from differences between the two apples[c]				
Apple	−0.6184	0.0395	−15.65	0.000
Chlorogenic acid—flesh—apple	−4.978	0.121	−41.30	0.000
Chlorogenic acid—peel—apple	1.420	0.125	11.32	0.000
(−)-Epicatechin—peel—apple	0.908	0.125	7.24	0.000
Phloridzin—peel—apple	1.105	0.125	8.81	0.000
Procyanidin B2—peel—apple	1.162	0.125	9.26	0.000
Procyanidin B2—flesh—apple	0.736	0.121	6.10	0.000
Quercetin-3-galactoside—apple	−0.734	0.131	−5.59	0.000
Quercetin-3-glucoside—apple	1.023	0.125	8.16	0.000
Cyanidin-3-galactoside—apple	2.313	0.133	17.45	0.000
Specialized effects to capture high levels of epicatechin with acidification				
(−)-Epicatechin—acidified	3.227	0.226	14.25	0.000
(−)-Epicatechin—flesh—acidiified—Lještarka	3.176	0.423	7.51	0.000
Specialized effects to capture different response to solvents with the anthocyanin				
Anthocyanin—80 %, 100 %, acidified vs 40 %, 60 %	0.3514	0.0508	6.91	0.000
Anthocyanin—80 %, acidified vs 100 %	0.324	0.103	3.15	0.002
Anthocyanin—60 % Lještarka	2.406	0.430	5.60	0.000
Specialized effects for cases that would not fit (yet do not change the interpretations)				
(+)-Catechin—flesh 60 % methanol	−1.143	0.270	−4.23	0.000
Quercetin-3-galactoside—Lještarka—40 % methanol	1.707	0.390	4.38	0.000

Table 5 (continued)

Term	Coefficient	Standard error coefficient	*T* value	*P* value
Quercetin-3-glucoside—peel—40 % methanol	1.344	0.277	4.85	0.000
Chlorogenic acid—flesh—Lještarka—80 % vs acidified	1.286	0.254	5.06	0.000

[a] The solvent contrasts are chosen to be orthogonal, with the regressor 80, 100 %, acidified vs 40 %, 60 % set to +2 and −3, respectively, for the {80, 100 %, acidified} methanol cases and the {40, 60 %}% methanol cases. Likewise 80 %, acidified vs 100 % is set to +2 and −1 for its respective cases, and 80 % vs acidified and 40 vs 60 % are set to +1, −1 for their respective cases

[b] The peel vs flesh factor is set to +1 for peel and −1 for flesh. It multiplies the indicator of polyphenol type to create contrasts that test for differences in effect between the two parts of the apple

[c] The apple factor is set to +1 for the second apple and −1 for the first apple. It multiplies the other factors to account for differences in them between the two apples

(homoschedastic) would it be statistically appropriate to not do a transformation.

The data could also be analyzed by separate regressions for the peel and for the flesh for Lještarka and for Idared. Fortunately, there are enough statistical similarities between these that a suitable combined model captures the means with only a few regressors needed to explain what differences there are between peel and flesh and between Lještarka and Idared.

The regression proceeded by first including all main effects and sensible pairwise interactions, examining the strongly significant residuals to suggest additional interactions for inclusion, and then, removing all contributions to the model that are not statistically significant. Except where explicitly indicated, only the strongly statistically significant terms ($p<0.01$) were included, and by the indicated procedure, we arrange all the residuals to be less than 3 standard errors away from 0. Table 5 provides the resulting regression fit to the square root of the concentration individual polyphenols.

This is a comparatively parsimonious model with 44 parameters (coefficients) fit, with mostly main effects and interpretable low-order statistical interactions, compared to using 162 fit means. All of the coefficients in this model are strongly statistically significant. The one exception is the coefficient for the contrast testing 80 % methanol vs acidified methanol, for the flesh. These two solvent solutions perform similarly. Statistically, the 80 % is significantly better ($p<0.05$) but not strongly so. This model confirms what was suggested from Tables 3 and 4 that 80 % methanol gave the highest amount of polyphenols in the flesh with HPLC method through experiments. Moreover, 80 % methanol would still be suggested for the extraction from the flesh due to hydrolysis reaction in acidified methanol. As for peel, the statistical model shows no significant difference between the two best solvent solutions: 80 % methanol and acidified methanol. This is also a statistical confirmation of the experimental results for the peel.

In the Table 6, a formal test of type of solvent based on the data from Tables 1 and 2 (analysis of total polyphenols in peel and flesh, and total anthocyanins in peel, 15-min extraction) is provided. A similar linear model can be constructed, which again allows a composite analysis, rather than separate analysis for each of these three cases. Again for variance stabilization, the square root of the concentration is taken as the response variable. This model reaches the same conclusion that 80 % and acidified methanol are statistically significantly higher than the other cases. Moreover, 80 % and acidified produce comparable results except that acidified yields higher concentration in peel than in flesh. According to experiments and statistical model, it can be suggested to use 80 % methanol for the extraction of polyphenols from the flesh and 80 % or acidified for the extraction from the peel.

Table 6 Regression fit to square root of polyphenol concentration obtained by Folin-Ciocalteau and pH differential methods (15-min extraction)

Term	Coefficient	Standard error coefficient	*T* value	*P* value
Constant	5.637	0.384	14.68	0.000
Contrasts for testing types of solvent				
80, 100 %, Acidified vs 40, 60 %	0.01963	0.00811	2.42	0.019
80, Acidified vs 100 %	0.0775	0.0194	3.98	0.000
80 % vs Acidified	No significant effect			
40 vs 60 %	No significant effect			
Acidified polyphenol peel-flesh	0.786	0.143	5.49	0.000
Effect due to types of compounds				
Polyphenol vs anthocyanin	0.7631	0.0128	59.71	0.000
Effect due to peel vs flesh				
Polyphenol peel-flesh	0.6031	0.0172	35.11	0.000

Conclusions

In conclusion, various mixtures of methanol and water were used in order to extract polyphenolic compounds from peel and flesh of apples by the help of ultrasonic bath. Additionally, acidified methanol was also used as an extraction solvent (0.1 % HCl in methanol). Extraction process was conducted in various time periods. The results showed that an efficient liquid–solid extraction could be performed with 80 % methanol to extract flavonols, anthocyanins, dihydrochalcones, and flavan-3-ols from the peel. Acidified methanol could also be useful for the peel due to better stability of anthocyanins in acidified environment and higher diversity of flavonols. In case of using acidified methanol, it should be noticed that the appearance of monomers could be seen, due to hydrolysis of bigger, dimeric, oligomeric, and polymeric molecules. For the extraction of polyphenols from the flesh, 80 % methanol is the solvent of choice for monomeric and oligomeric flavan-3-ols, dihydrochalcones, and hydroxycinnamic acids.

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Compliance with Ethical Standards

Conflict of Interest Authors Jakobek Lidija, Boc Martina, Barron R. Andrew declare that they have no conflict of interest.

Research Involving Human Participants and/or Animals This article does not contain any studies with human participants or animals performed by any of the authors.

Informed Consent Informed consent was obtained from all individual participants included in the study.

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