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COMPARISON OF DIFFERENT EXTRACTION SOLVENTS FOR ASSAY OF THE POLYPHENOL CONTENT IN THE PEEL AND PULP OF APPLE CULTIVARS FROM MACEDONIA

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Apples (*Malus domestica* Borkh.) contain a large array of phenolic compounds belonging to flavonoids and non-flavonoids. This study systematically evaluates the polyphenolic content of 21 apple cultivars from the Republic of Macedonia, both commercial and autochthonous, applying spectrophotometric methods for the determination of total polyphenolic compounds, total flavonoids, total anthocyanins and total flavan-3-ols. The reliability of these methods was checked by confirming the method linearity and accuracy with standards and spiked samples. The efficiency of acetone, water, methanol and a mixture of methanol/water (90 : 10, *V/V*) as extraction solvents was compared. Evident differences between extracts obtained from freeze-dried apple peel and pulp, extracted with different solvents, and analyzed with the four spectrophotometric tests, were observed and discussed. The most satisfactory extraction efficiency was achieved using methanol/water (90 : 10, *V/V*) mixture. The results obtained from the methanol/water extracts illustrate evident differences between polyphenol contents and reveal the diversity in polyphenols, total flavonoid and flavanol composition between the assayed commercial and autochthonous cultivars. The maximum abundance of polyphenols was noticed in the peel of Tetovka and Livadarka cultivars (15.63 mg/g GAE and 14.85 mg/g GAE, respectively) and in the pulp of Pasalma, Tetovka and Livadarka cultivars (12.55 mg/g GAE, 11.45 mg/g GAE and 11.22 mg/g GAE, respectively).

Keywords: apples; polyphenols; flavonoids; anthocyanins; flavan-3-ols; spectrophotometry; extraction

СПОРЕДБА НА РАЗЛИЧНИ РАСТВОРУВАЧИ ЗА ОПРЕДЕЛУВАЊЕ НА СОДРЖИНАТА НА ПОЛИФЕНОЛИ ВО ЛУШПАТА И ПУЛПАТА НА ЈАБОЛКО ОД РАЗЛИЧНИ СОРТИ ОД МАКЕДОНИЈА

Плодовите од јаболко (*Malus domestica* Borkh.) содржат различни фенолни соединенија кои се од групите на флавоноиди и не-флавоноиди. Ова истражување систематски ја оценува содржината на полифеноли во 21 сорта јаболко од Македонија, комерцијални и автохтони, со употреба на спектрофотометриски методи за определување на вкупни полифеноли, вкупни флавоноиди, вкупни антоцијани и вкупни флаван-3-оли. Веродостојноста на методите е проверена и потврдена е линеарноста и точноста со употреба на стандарди и стандардни додатоци. Споредена е ефикасноста на растворувачите ацетон, вода, метанол и смеса метанол/вода (90 : 10, *V/V*). Воочени и дискутирани се разлики помеѓу екстрактите добиени од лиофилизирани лушпи и пулпа од јаболко со различни растворувачи и анализирани со четирите спектрофотометриски методи. Највисока ефикасност на екстракцијата е добиена со употреба на смеса метанол/вода

(90:10, *V/V*). Добиените резултати за екстрактите во метанол/вода покажуваат разлики во содржината на полифенолите и укажуваат на различност на полифенолниот, флавоноидниот и флаванолниот состав на испитуваните комерцијални и автохтони сорти. Најголема застапеност на полифеноли е утврдена во лушпата од сортите тетовка и ливадарка (15,63 mg/g GAE и 14,85 mg/g GAE, соодветно) и во пулпата од пашалма, тетовка и ливадарка (12,55 mg/g GAE, 11,45 mg/g GAE и 11,22 mg/g GAE, соодветно).

Клучни зборови: јаболко; полифеноли**;** флавоноиди; антоцијани; флаван-3-оли; спектрофотометрија; екстракција

1. INTRODUCTION

The cultivated apple, *Malus domestica* Borkh. (family *Rosaceae*, subfamily *Pomoideae*), is known to play an important role in many worldwide cultures not only with its nutritional values as a fruit, but because the modern apple varieties and autochthonous cultivars are an essential part of the inheritance of the regions, with economic, mythological, ethnological, and cultural significance as well. The health benefits of the apples come primarily from pectin, fibers and vitamins but, as recently discovered, in many fruit crops polyphenols, especially flavonoids, play an important role as potent agents preventing inflammation, coronary diseases and showing anticarcinogenic and antineurodegenerative behavior [1, 2].

Apples contain five major groups of polyphenolic compounds, namely hydroxycinnamic acids, flavan-3-ols/procyanidins (flavanols), anthocyanins, flavonols and dihydrochalcones [3–5]. Chlorogenic acid, quercetin 3-glycosides, catechin, epicatechin, phloridzin, phloretin and cyanidin 3-glycosides are the major individual polyphenols in apples [5, 6]. These compounds, located in the plastids/vacuoles of the plant cell, contribute to the physical characteristics of the fruit such as the color of the peel (green, red and yellow), the flavor and astringency of the pulp and therefore to the differences between different varieties of apples [6].

Many authors have studied the polyphenolic compounds in apples using HPLC [6–8] as the most suitable analytical method for separation and quantification of the individual polyphenolic compounds. However, this technique is not as applicable for fast routine analysis compared to spectrophotometric methods, which are more affordable techniques with lower cost, reagent consumption and rapid measurements output. These methods can be used for analysis during the storage process of the fruits as well as in the field. Solvent extractions are the most commonly used procedures to prepare extracts from plant materials due to their ease of use, efficiency, and wide applicability. It is generally known that the yield of extraction depends on the type of solvents with varying polarities, extraction time and temperature, sample-to-solvent ratio, as well as on the chemical composition and physical characteristics of the samples. Most of the procedures for the determination of polyphenols in apples use aqueous methanol or acetone as the extraction solvent [9, 10]. In sample preparation, fruits are usually used fresh or freeze-dried. Asami *et al.* [11] showed that freeze-dried marionberries, strawberries and corn consistently had a higher total polyphenolic content level compared to air-dried fruits. Therefore in this study apples were lyophilized before extraction.

Apples are adaptable to various climates, but can be considered best adapted to the cool temperate zone (sub-Mediterranean climate) such as the Republic of Macedonia. The annual average apple fruit production in R. Macedonia is about 95 000 tonnes [12], which is over 50% of the total annual fruit production, so that apples represent leading fruit cultures. In spite of these facts, the data on the contents of polyphenolic compounds from this climate is still scarce.

In this work, the most commonly used spectrophotometric methods for determination of the total polyphenols, total anthocyanins, total flavan-3-ols and total flavonoids were employed to evaluate the polyphenolic composition of twenty-one apple cultivars grown in the Republic of Macedonia: Golden Delicious, Red Delicious, Cadel, Braeburn, Granny Smith, Idared, Gala, Mutsu, Jonathan, Fuji, Melrose, Srcika, Parmenka (Adam's Pearmain), Kojce, Livadarka, Karapasa, Mislimka, Pasalma, Kozara (Belle de Boskoop), Shareno Blago and Tetovka. The results from the spectrophotometric assays were also used to select the most efficient extraction solvent/solvent mixture for extraction of polyphenols, flavonoids, flavan-3-ols and anthocyanins from apple peel and pulp. Due to the deficiency of data for Macedonian apple cultivars, the purpose of this research was also to start the process of establishing a preliminary database for the polyphenolic content of selected commercial, domesticated and autochthonous apple cultivars grown in R. Macedonia.

2. EXPERIMENTAL

2.1. *Samples, reagents and equipment*

Analyses were made on the apple samples of 21 apple cultivars given in Table 1 and characterized at the Institute of Agriculture, Ss. Cyril and Methodius University, Skopje. The following apple cultivars were analyzed during this study (Table 1): Golden Delicious (S1), Red Delicious (S2), Cadel (S3), Braeburn (S4), Granny Smith (S5), Idared (S6), Gala (S7), Mutsu (S8), Jonathan (S9), Fuji (S10), Melrose (S11), Srcika (S12), Parmenka (Adam's Pearmain) (S13), Kojce (S14), Livadarka (S15), Karapasa (S16), Mislimka (S17), Pasalma (S18), Kozara (Belle de Boskoop) (S19), Shareno Blago (S20) and Tetovka (S21). Eight of these cultivars are autochthonous (Srcika, Kojce, Livadarka, Karapasa, Mislimka, Pasalma, Shareno Blago and Tetovka), two are domesticated (Parmenka (Adam's Pearmain) and Kozara (Belle de Boskoop), and eleven are commercial cultivars (Golden Delicious, Red Delicious, Cadel, Braeburn, Granny Smith. Idared, Gala, Mutsu, Jonathan, Fuji and Melrose). Only Granny Smith and Fuji were grown in the region of Skopje, R. Macedonia. All other cultivars used in this study were grown in the region of Resen, R. Macedonia, the most famous region in the country for apple production.

T a b l e 1

Code number, variety, type and region of apple cultivars analyzed in the study

Code	Variety	Type	Region
S1	Golden Delicious	commercial	Resen
S2	Red Delicious	commercial	Resen
S3	Cadel	commercial	Resen
S4	Braeburn	commercial	Resen
S5	Granny Smith	commercial	Skopje
S6	Idared	commercial	Resen
S7	Gala	commercial	Resen
S8	Mutsu	commercial	Resen
S9	Jonatan	commercial	Resen
S10	Fuji	commercial	Skopje
S11	Melorose	commercial	Resen
S12	Srcika	autochthonous	Resen
S13	Parmenka (Adam's Pearmain)	domesticated	Resen
S14	Kojce	autochthonous	Resen
S15	Livadarka	autochthonous	Resen
S16	Karapasa	autochthonous	Resen
S17	Mislimka	autochthonous	Resen
S18	Pasalma	autochthonous	Resen
S19	Kozara (Belle de Boskoop)	domesticated	Resen
S20	Shareno Blago	autochthonous	Resen
S21	Tetovka	autochthonous	Resen

Fruits were collected at full maturity. Maturity was determined on the basis of the color characteristics of each cultivar. The sampling was randomly made by picking fruits from different parts of the trees to avoid any fruit position effect.

Standards used in this work such as gallic acid and catechin were purchased from Sigma (St. Louis, USA). Acetone, methanol, Folin–Ciocalteu reagent, Na_2CO_3, $NaNO_2$, $AlCl_3$, NaOH, ethanol, and *p*-DMACA (*p*-(dimethylamino)cinnamaldehyde) were purchased from Merck (Darmstadt, Germany) and concentrated HCl was purchased from Alkaloid AD Skopje. For spectrophotometric analysis, the spectrophotometer Varian Cary Win 50 was used. A micropipette 100–1000 μl (Isolab, Germany), sensitive balance to 0.1 mg (Sartorius, Germany), ultrasonic bath Elmasonic S100H, freeze-dryer Freezone 2.5 (Labconco, USA), blender from Bosch and centrifuge Harrier 15/80 MSE were also used for sample preparation.

2.2. *Sample preparation*

Fifteen to twenty apple fruits were collected from each apple tree cultivar. All fruits were washed with distilled water and brushed while washing so that additional contamination was avoided. Stalks were removed and the fruits were manually skinned with a skinning knife. The peel was separated from the pulp. Peels were double-checked and blotted on paper to remove any residual pulp. Averaged samples from peels and pulps were prepared by collecting the peels and the pulps from 15 apples of each cultivar. Peel and pulps were then cut into a size suitable for the lyophilization plates as fast as possible so that browning could be avoided. Peels and pulps were placed in five separate plastic plates for lyophilization (containing about 20 g of fresh sample each) and were lyophilized for 24 h at a temperature of –45 °C and pressure of 0.055 mbar. After 24 h lyophilization, the residues from each apple cultivar were combined and ground with a blender at 400 W (peels and pulps separately). After blending, samples were kept at –20 °C till analysis. Four separate extractions of the peel and the pulp of the 21 apple cultivars were made with the following extraction solvents: water, acetone, methanol and methanol/water (90 : 10, *V/V*). About 0.5 g pulp/peel were extracted with two different portions (2.5 ml) of each solvent using ultrasound at room temperature for 1 h. After centrifugation (3000 rpm/min) for 10 min the supernatants from both extractions were combined and made up to a final volume of 5 ml with suitable solvent, as described in the test

procedures below. All samples were prepared in triplicate. The extracts were filtered twice through 0.45 μm membrane filters (PTFE, Agilent), once before applying the test reagents and again immediately before spectrophotometric measurement.

2.3. *Spectrophotometric methods*

2.3.1. *Total polyphenols assay*

The Folin-Ciocalteu method was used for determination of the total polyphenols in the apple cultivars, as proposed by Ivanova [13]. In short, 1 mL of the apple peel or pulp extract was added to a 10 ml volumetric flask, containing 5 ml distilled water followed by addition of 0.5 ml Folin-Ciocalteu reagent. After 3 minutes, 1.5 ml Na_2CO_3 solution (5 g/l) was added to the mixture and a total volume of 10 ml was made with distilled water. Samples prepared in this manner were kept in a water bath at 50 ºC for 16 minutes in sealed flasks and after subsequent cooling their absorbance was read at 765 nm against distilled water as a blank. The concentration of total phenolics in the samples is expressed as the gallic acid equivalent (GAE) per g of the lyophilized sample (mg GAE/g). All samples were prepared in triplicate.

2.3.2. *Total flavonoids assay*

The total flavonoid content was estimated according to a colorimetric assay with aluminum chloride [13, 14]. An aliquot of 1 ml apple pulp/peel extract was added to a 10 ml volumetric flask containing 4 ml of distilled water followed by addition of 0.3 ml of solution of $NaNO_2$ (0.5 g/l). After 5 min, 0.3 ml of 1 g/l solution of $AlCl_3$ was added and 6 min later, 2 ml NaOH (1 mol/l) was added to the mixture. The total volume was made up to 10 ml with distilled water, the solution was mixed and the absorbance was measured at 510 nm against distilled water as a blank sample. Catechin was used as a standard for the construction of the calibration curve and the concentrations were expressed as catechin equivalents per lyophilized material (mg CE/g). All samples were prepared in triplicate.

2.3.3. *The total anthocyanins assay*

As proposed by Di Stefano [13, 15], determination of the total anthocyanins was made by dilution of apple peel and pulp extracts with a solution consisting of 70/30/1 (*V/V/V*) ethanol/water/HCl (concentrated) and the absorbance was measured at 540 nm. The total anthocyanins content was expressed as cyanidin-3-glucoside equivalents and calculated using the equation $TA_{540\ nm}$ (mg/l) = $A_{540\ nm}$ 16.7 *d*, where $A_{540\ nm}$ is the absorbance at 540 nm and *d* is the dilution.

2.3.4. *Total flavan-3-ols assay*

The content of flavan-3-ols in apple peel and pulp expressed as catechin equivalents (CE mg/g) was measured using the *p*-(dimethylamino)cinnamaldehyde (*p*-DMACA) [13, 16]. An aliquot (1 ml) of previously prepared extracts was added to a 10 ml volumetric flask followed by the addition of 3 drops of glycerol and 5 ml of *p*-DMACA reagent. The total volume was made up to 10 ml with methanol. After 7 minutes incubation time, the absorbance was recorded at 640 nm against a methanol blank sample. The *p*-DMACA reagent was prepared immediately before use, and contained 1 % (*w/V*) *p*-DMACA in a cold mixture of methanol: HCl (4:1, *V/V*).

3. RESULTS AND DISCUSSION

Spectrophotometric methods for the determination of total polyphenols, total flavonoids, total flavan-3-ols and total anthocyanins contents have been widely used in fruit analysis and pomology. In this study, procedures based on standardized spectrophotometric measurements [13] were used in order to test the most efficient extraction solvent system for lyophilized apple peel and pulp and then perform the analysis of total polyphenols as well as different classes of polyphenols. The Folin–Ciocalteu method is based on oxidation–reduction reactions in which phenolics are oxidized and form a blue chromophore constituted by a tungstic-phosphomolybdenum complex with a maximum absorbance in the wavelength region between 725 nm and 765 nm. A calibration curve for total polyphenols assay was constructed using gallic acid standard solutions (0–200 μg/ml) with a calibration curve equation $y = 0.00477x + 0.00149$ and a correlation coefficient of 0.99985. In order to check the linearity of the method, control samples with standards at concentration levels of 7.5, 40 and 125 μg/ml were made and relative errors of 4.45, 2.26 and 2.23% were calculated for every test level, respectively. Furthermore, to confirm the specificity and selectivity of the method, spiked samples at the same three concentration levels were analyzed and satisfactory recoveries were obtained (95.67%, 102.64% and 103.28%, respectively).

The method for total flavonoids determination is based on the formation of stable complexes

with the C4 carbonyl group and either the C3 or C5 hydroxyl group of flavones and flavonols with an absorbance maximum at 510 nm [13]. The obtained calibration curve equation $y = 0.00449x + 0.02050$ with a correlation coefficient of 0.99958 for this test confirmed the linear dependence in the concentration range 0–200 μg/ml of catechin. Accuracy was confirmed with tests with standards of catechin with concentrations of 7.5 μg/ml, 40 μg/ml and 125 μg/ml that showed relative errors of 3.91%, 0.71% and 0.59%, respectively, and recoveries of 104.57%, 96.23% and 104.48% obtained for spiked samples. On the other hand, the determination of flavan-3-ols by the total flavan-3-ols assay is based on the formation of colored products in the reaction of *p*-(dimethylamino)cynnamaldehyde (*p*-DMACA) reagent with tannins [13]. The absorbance of the resulting products of this reaction involving monomeric flavan-3-ols was plotted against the catechin concentration in the range from 0–200 μg/ml and the following equation was obtained: $y = 0.00449x - 0.0014$ with a correlation coefficient of 0.99995. The accuracy of the method was verified by analyzing standards at concentration levels of 7.5, 40 and 125 μg/ml and relative errors of 3.25%, 3.10% and 2.43% were obtained, whereas recoveries of 97.25%, 101%, and 100% were obtained for the corresponding spiked samples. All the standards and spike samples were prepared in triplicate and measurements were averaged. Averaged results from the method verification such as calibration curve equations, correlation coefficients, relative errors and recoveries are summarized in Table 2.

T a b l e 2

Calibration curve equations, correlation coefficients, relative errors and recoveries for testing linearity and accuracy of the three spectrophotometric assays

Tested compounds	**Calibration curve**	**Correlation coefficient**	**Standards (RE*) (%)**			**RSD* (%) 3 replicates**			**CI* (%) 3 replicates**		
			7.5 μg/ml	**40.0 μg/ml**	**125.0 μg/ml**	**7.5 μg/ml**	**40.0 μg/ml**	**125.0 μg/ml**	**7.5 μg/ml**	**40.0 μg/ml**	**125.0 μg/ml**
Total polyphenols	$y = 0.00477x + 0.00149$	0.99985	4.45	2.26	2.34	3.28	1.83	2.25	1.32	4.15	2.14
Total flavonoids	$y = 0.00449x + 0.02050$	0.9958	3.91	0.71	0.59	5.32	6.32	3.84	2.38	3.25	4.16
Total flavan3-ols	$y = 0.00490x + 0.00140$	0.99995	3.25	3.1	2.43	4.48	3.33	1.97	5.37	1.28	1.44

*RE – relative error, RSD – relative standard deviation, CI – confidence interval

¥ Spiked samples were prepared in triplicate

All the results obtained for total polyphenols, total flavonoids, total flavan-3-ols and total anthocyanins in the studied 21 apple cultivars (peel and pulp separately) with all extraction solvents are given in the supplementary table S1. For better observation, the results obtained for the polyphenol content in the peel and pulp of all samples with the four extraction solvents are presented in Figure 1. Phenolic compounds in plants are polar compounds, which are usually extracted with polar solvents such as aqueous acetone and/or methanol. Since four different groups of compounds were analyzed, the phenolic profiles are expected to differ when different extraction solvents are used. It is obvious from Figure 1a and b that an almost twice higher concentration of polyphenols is found in the peel extracts in comparison with the pulp extracts, except for sample S1 (Golden Delicious) and S19 (Kozara–Belle de Boskoop), for which comparable content was measured in the peel and pulp.

The graphs in Figure 1 clearly demonstrate that 90% methanol in water is the most efficient solvent, giving the highest yield for all samples. Methanol and acetone showed a slightly lower efficiency than 90% methanol in water that could be attributed to the presence of more hydrophilic flavonoid glycosides that require water in the extraction solvent. On the other hand, poor water extraction results for the lyophilized apple samples, especially pulp, are most probably related to the content of sugars in the pulp, which causes difficulties in sample manipulation, especially in sample filtration (some of the sample is lost by sticking to filters and containers). In this manner, acetone, as a less polar solvent than water, was found to be more efficient, as has been demonstrated for the extraction of anthocyanins and fla-

van- 3-ols for strawberries by Kajdžanoska et al. [17, 18]. The charts also indicate more variable recovery of the water extracts in comparison with extracts obtained with methanol, acetone and the methanol/water mixture, which follow similar tendencies. Comparison of the results obtained with all the extraction agents creates a very different picture about the polyphenol contents, which especially counts for the water extracts, implying that the water extract results do not give the real picture of the polyphenolic content, most probably due to the loss of the sample during sample preparation.

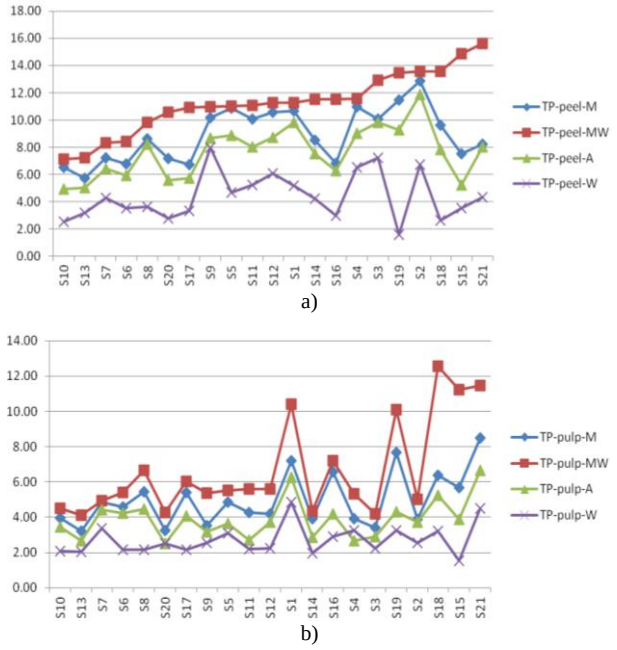

Fig. 1. Total polyphenols (TP) content in 21 apple cultivars from Macedonia extracted from **a)** peel, and **b)** pulp with methanol (M), 90% methanol in water, *V*/*V* (MW), acetone (A) and water (W), and expressed as mg gallic acid/g of freeze-dried material

While it was previously known that apples contain more of the lower molecular weight polyphenols, unlike anthocyanins [6–8], where methanol has been generally found to be a more efficient extraction agent, another set of extraction results was obtained using methanol. At this point, substantial differences between the peel and pulp extract were noticed, but no significant changes in comparison with the extracts of acetone were noticed. Namely, peel methanol extracts in all 21 apple cultivars show similar results when extracted with acetone and methanol, even though in all cases a slightly higher concentration of polyphenols was measured in the methanol extracts. For example, the pulp acetone polyphenol extracts for Gala, Srcika, Red Delicious and Jonathan contain 4.44, 3.73, 3.71, 3.16 and 4.25 mg/g GAE polyphenols respectively, whereas their methanol extracts show mass concentrations of 4.84, 4.20, 3.88, 3.52 and 4.58 mg/g GAE, respectively. Knowing that apple cultivars are more abundant in lower molecular weight polyphenols, this indicates that methanol has the potency to also extract higher molecular weight flavonols as well, contributing to this slightly higher yield. The results for total polyphenols obtained with acetone as the extraction agent show that the cultivars Cadel S3 (9.85 mg/g), Red Delicious S2 (11.85 mg/g), Kojce S14 (7.55 mg/g) and Mutsu S8 (8.22 mg/g) have a significantly higher concentration of polyphenols in the peel than in the pulp (Cadel 2.89 mg/g, Red Delicious 5.02 mg/g, Kojce 4.36 mg/g and Mutsu 4.46 mg/g). These cultivars are characterized by colored peel originating from anthocyanins, which are known to be extractable with acetone as solvent for higher molecular weight flavan-3-ols [17, 18].

The highest yields obtained with 90% methanol clearly demonstrate that this is the most effective extraction agent, giving the following highest polyphenol contents in the peel extracts of Tetovka S21 (15.63 mg/g), Livadarka S15 (14.85 mg/g), Pasalma S18 (13.55 mg/g), Red Delicious S2 (13.55 mg/g), Kozara (Belle de Boskoop) S19 (13.46 mg/g) and Cadel S3 (12.94 mg/g). This solvent system improves the extraction efficiency from lyophilized apple material most probably because methanol denaturates cell membranes, simultaneously dissolves the polyphenols, and stabilizes them. On the other hand, water improves the ability of the solvent mixture to enter plant cells. Excess of water of more than 10% would have the opposite effect, since the cell is already lyophilized. Selecting the right solvent affects the amount and rate of polyphenols extracted [19]. Selection of the methanol/water mixture as the most efficient solvent was confirmed by the results of the highest polyphenol contents in pulp extracts of: Pasalma S18 (12.55 mg/g), Tetovka S21 (11.45 mg/g), Livadarka S15 (11.22 mg/g), Golden Delicious S1 (10.40 mg/g) and Kozara (Belle de Boskoop) S19 (10.08 mg/g); see Figure 1b.

The results obtained for the total flavonoids content in the peel and pulp extract from 21 apple cultivars follow an analogous pattern to that discussed above for the polyphenols contents. The results for the peel and pulp extracts are shown in Figure 2a and b, arranged in order from the lowest to highest yield obtained by the most efficient solvent, 90% methanol. Water extracts data show irregular behavior, implying that even though most apple flavonoids are water-soluble glycosides, simple extraction with water is not suitable for

their analysis because of the nature of the lyophilized apple sample. With these results, the similarity of acetone and methanol as solvents is confirmed for flavonoids, especially for pulp extracts, even though for the peel extracts methanol is more efficient than acetone. Again, the methanol/water (90%, *V/V*) solvent mixture provided the best yield for total flavonoid content so that the most abundant apple cultivars in flavonoids are: Tetovka cultivar S21 with the highest concentration of flavonoids in the peel and the pulp (7.87 mg/g and 4.27 mg/g respectively), Kozara (Belle de Boskoop) S19 (peel 6.78 mg/g and pulp 3.43 mg/g) and Pasalma S18 (peel 6.23 mg/g and pulp 4.38 mg/g).

Flavan-3-ols follow almost the same extraction patterns as flavonoids. It was previously known that most flavan-3-ols are located in the apple pulp, from which the most abundant is (–)-epicatechin (6). From the results obtained in this study (suppl. material S1), the flavan-3-ols content in the pulp and the peel is very similar. Yet again, water extracts showed the lowest concentration levels, acetone and methanol show higher, but in favour of methanol, and the highest yields were obtained when using methanol/water mixture as the extraction agent. The most abundant in flavan-3-ols are the peel of Shareno Blago S20 with 4.22 mg/g (pulp 2.32 mg/g), Tetovka S21 with 4.19 mg/g (pulp: 3.22 mg/g), and Kojce S14 with 4.09 mg/g (pulp 3.58 mg/g).

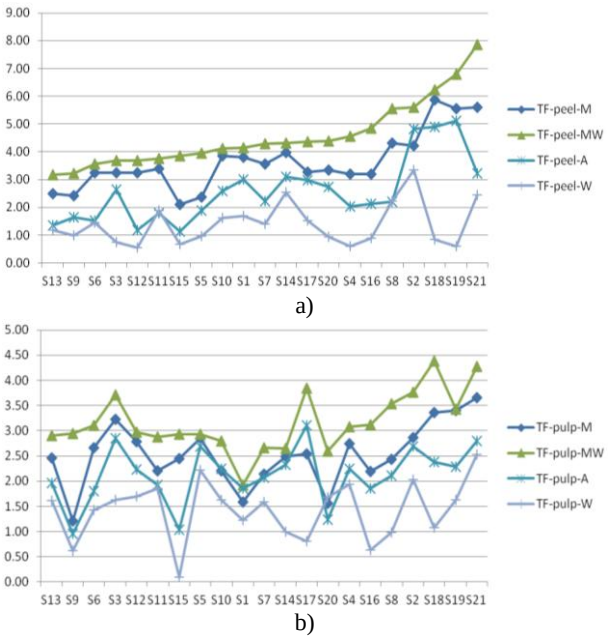

Fig. 2. Total flavonoids (TF) content in 21 apple cultivars from Macedonia extracted from **a)** peel, and **b)** pulp with methanol (M), 90% methanol in water, *V/V* (MW), acetone (A) and water (W) and expressed as mg catechin/g of freeze-dried material

Examination of the results obtained for total anthocyanins content revealed the very low presence of anthocyanins in the pulp (concentration range 0.01–2.09 mg/g), as found by all the extraction solvents used. Again, acetone and methanol showed similar behavior when red apples are observed, slightly in favor of methanol. From the studied cultivars only Red Delicious S2 apples were uniformly red and these had the highest content of anthocyanins, with 5.55 mg/g in the methanol/water extract, whereas other cultivars with reddish color contained less than 3 mg/g anthocyanins in the peel, down to 0.09 mg/g for Golden Delicious S1 (yellow peel). The highest content of anthocyanins in the pulp was measured for the Tetovka cultivar S21 (2.09 mg/g) and Braeburn S4 (2.02 mg/g) and the others were significantly lower.

All the results for the polyphenols, flavonoids, flavan-3-ols and anthocyanins obtained with the most efficient solvent methanol/water mixture for all the studied samples are summarized in Fig. 3 and the minimal and maximal values are given in Table 3. In Figure 3a the results for total polyphenols reveal the three autochthonous varieties Livadarka (S15), Pasalma (S18) and Tetovka (S21) with 14.85, 13.55 and 15.63 mg gallic acid equivalents in the peel and 11.22, 12.55 and 11.45 mg/g total polyphenols in the pulp, respectively. The domesticated variety Kozara (Belle de Boskoop) (S19) with 13.46 and 10.08 mg /g in the peel and pulp has also shown high polyphenols content followed by the commercial variety Golden Delicious (S1) with 11.25 and 10.40 mg/g polyphenols in the peel/pulp, respectively. Besides that, this graph reveals that these species with the highest total polyphenols content contain similar concentrations of polyphenols in the peel and the pulp, which is not the case for the other varieties with lower polyphenols content, where a higher amount is measured in the peel.

The results clearly demonstrate that the autochthonous cultivars, especially Livadarka (S15), Mislimka (S17), Pasalma (S18) and Tetovka (21) have higher polyphenol content in both peel and pulp when compared to commercial cultivars such as Idared (S6), Gala (S7) and Jonathan (S9). The domesticated Kozara (Belle de Boskoop) (S19) cultivar also shows a higher polyphenol content when compared to Fuji (S10) and Granny Smith (S5). It is obvious that commercial cultivars compared to autochthonous cultivar contain less polyphenols in the pulp (Fig. 3a).

The results for flavonoids, flavan-3-ols and anthocyanins presented in Figure 3b show that the most abundant in flavonoids are the peel of Tetov-

ka (7.87 mg/g) and the pulp of Pasalma (4.22 mg/g). The content of flavonols is very similar for all the analyzed cultivars and the maximum concentration is found in the peel of the Shareno Blago cultivar (4.38 mg/g) and in the pulp of Kojce (3.58 mg/g), whereas anthocyanins are mostly present in the peel of red apples such as Red Delicious (5.55 mg/g) and are below 2.85 mg/g in the peel of the other samples and below 2.1 mg/g in the pulp of all samples.

These results correspond to results found in the literature as analyzed by spectrophotometric methods [19] and are higher than the results obtained by some HPLC methods [6, 7, 8]. Marinova et al. [19] studied the polyphenol and flavonoid content in fruits and vegetables and found 99.7 and 125 mg gallic acid equivalents/100 g of fresh unpeeled yellow and red apples, which corresponds to around 6–8 mg gallic acid/g dry sample if the average 84% water content is taken into account. They also found that around one third of the total polyphenols can be attributed to flavonoids according to the spectrophotometric measurements.

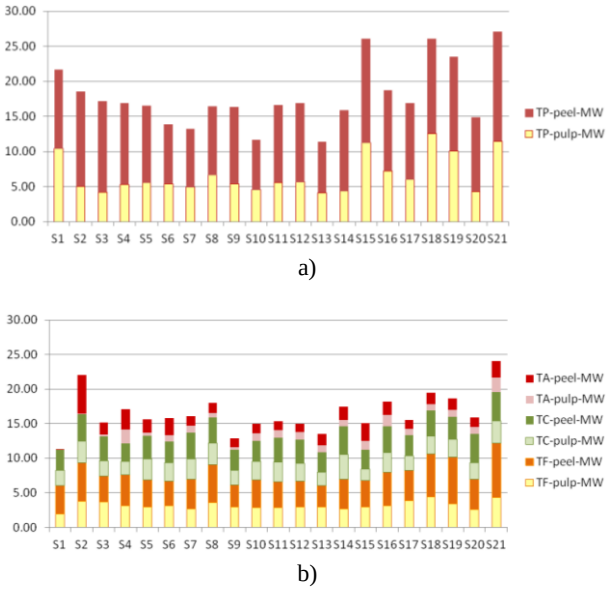

Fig. 3. a) TP – Total polyphenols expressed as mg gallic acid/g, **b)** TF – total flavonoids (mg of catechin/g), TC – total flavan-3-ols (mg of catechin/g) and TA – total anthocyanins (mg of cyanidin-3-glucoside/g) in the peel and pulp of 21 apple samples extracted with 90% methanol in water

T a b l e 3

Minimal and maximal concentration of the four tested groups of compounds of 21 apple cultivars using methanol/water as extraction agent

Results	Cultivar	Min (mg/g)	Cultivar	Max (mg/g)	Cultivar	Min (mg/g)	Cultivar	Max (mg/g)
	Pulp				Peel			
Polyphenols	Pramenka S13	4.10	Pasalma S18	12.55	Fuji S10	7.11	Tetovka S21	15.63
Flavonoids	Golden Delicious S1	1.92	Pasalma S18	4.38	Pramenka S13	3.17	Tetovka S21	7.87
Catechins	Livadarka S15	1.63	Kojce S14	3.58	Braeburn S4	2.69	Sareno Blago S20	4.22
Antocyanins	Golden Delicious S1	0.03	Tetovka S21	2.09	Golden Delicious S1	0.09	Red Delicious S2	5.55

Ceymann et al. [20] have compared the results for individual polyphenols (flavonoids and phenolic acids) measured by UHPLC-MS and total polyphenols measured by the Folin–Ciocalteu method in four apple cultivars and found that the latter gives 3–4 times higher results than the sum of polyphenols calculated from UHPLC-MS data. This difference was explained by losses due to solubility changes on dilution with water and by filtration before HPLC-MS analysis (10% losses due to mono- and dimeric polyphenol standards have been confirmed). Large amounts of oligomeric procyanidins have been reported in apples [21] and, according to Vrhovsek et al. [22], the oligomeric procyanidins represent around 64% of the total polyphenol value as measured by the Folin–Ciocalteu method. To confirm this, they used 3 different chromatographic runs (one normal-phase and two reversed-phase) to explain the complete composition of the total polyphenol content as measured by the Folin–Ciocalteu method by quantifying additionally the oligomeric procyanidins in the apple samples. This work demonstrated the applicability of the Folin–Ciocalteu method for the measurement of total polyphenols, accounting for all major polyphenols groups including the oligomeric procyanidins in the group of flavan-3-ols, as a group of compounds that are not easily detected and quantified in HPLC methods. The average content of total polyphenols found by Vrhovsek et al. [22] was 110.2 mg/100 g fresh fruit, which corresponds to approximately 7 mg/g dry apple, rang-

ing from around 4 mg/g for the Fuji cultivar, 5.4 mg/g for Golden Delicious, 8.2 mg/g for Red Delicious, up to around 13 mg/g for the Renetta cultivar (traditional for France and Italy).

4. CONCLUSION

Examination of commercial, domesticated and autochthonous apple cultivars with fast, applicable techniques that are ready for routine analysis is needed since apple fruits represent a very important part of everyday diets as well as of the economy of many countries. Thus, in this work, spectrophotometric methods for the determination of the total polyphenols, total anthocyanins, total flavan-3-ols and total flavonoids were optimized in terms of extraction solvent and employed to evaluate the polyphenol composition of 21 apple cultivars from the Republic of Macedonia. Simple sample preparation and a two-step extraction procedure are demonstrated as an efficient extraction method for all the measured polyphenols. Although the methods used here were previously developed and used for the determination of polyphenols in different fruits, all the methods were checked for linearity and accuracy, where very good correlation and recovery was confirmed. The results obtained from polyphenols analysis in 21 apple cultivars (peel and pulp separately) with different extraction solvents indicated that extraction with water leads to the poorest results and that methanol/water in the proportion (90:10, *V/V*) is the most efficient extraction agent in comparison to water, pure methanol and pure acetone when lyophilized apple peel and pulp are separately analyzed. The obtained results demonstrate that some autochthonous cultivars contain higher contents of total polyphenolic compounds and flavonoids, and further studies are needed in order to confirm the nature and content of the specific polyphenolic compounds. Data collected during this research can be used to create databases for polyphenol content regarding the quality of commercial, domesticated and autochthonous apple cultivars from the Republic of Macedonia.

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