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IDENTIFICATION AND QUANTIFICATION OF PHENOLIC COMPOUNDS IN POMEGRANATE JUICES FROM EIGHT MACEDONIAN CULTIVARS

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Punica granatum L. is one of the species enjoying growing interest due to its complex and unique chemical composition that encompasses the presence of anthocyanins, ellagic acid and ellagitannins, gallic acid and gallotannins, proanthocyanidins, flavanols and lignans. This combination is deemed responsible for a wide range of health-promoting biological activities.

This study was focused on the analysis of flavonoids, anthocyanins and phenolic acids in eight pomegranate varieties (*Punica granatum*) from Macedonia, in two consecutive years. Fruits from each cultivar were washed and manually peeled, and the juice was filtered. NaF (8.5 mg) was added to 100 ml juice as a stabilizer. The samples were centrifuged for 15 min at 3000 rpm and analyzed using an HPLC/DAD/MS^n method that was optimized for determination of their polyphenolic fingerprints.

The dominant anthocyanin in all pomegranate varieties was cyanidin-3-glucoside followed by cyanidin and delphinidin 3,5-diglucoside. From the results, it can be concluded that the content of anthocyanins was higher in 2016 compared to 2017. But in contrast, the total content of non-colored polyphenols was around 10 times lower in 2016 compared to the amount found in the same samples in 2017.

Keywords: pomegranate; polyphenols; HPLC/MS

ИДЕНТИФИКАЦИЈА И КВАНТИФИКАЦИЈА НА ФЕНОЛНИ СОЕДИНЕНИЈА ВО СОКОТ НА ОСУМ МАКЕДОНСКИ СОРТИ КАЛИНКИ

Punica granatum L. е еден од видовите што предизвикуваат голем интерес поради својот комплексен и уникатен хемиски состав кој опфаќа присуство на антоцијани, елагова киселина и елагитанини, гална киселина и галотанини, проантоцијанидини, флаваноли и лигнани. Оваа комбинација од соединенија се смета одговорна за широк спектар биолошки процеси поврзани со здравјето.

Истржувањето беше фокусирано на анализа на флавоноиди, антоцијани и фенолна киселина во осум сорти калинки (*Punica granatum*) од Македонија, од две последователни години. Зрната од овошјето беа измиени и рачно сепарирани од остатокот и сокот беше добиен со филтрирање. Кон секој примерок беше додадено 8,5 mg NaF на 100 ml сок како стабилизатор. Примероците беа центрифугирани во временски интервал од 15 минути на 3000 вртежи во минута и беа анализирани со употреба на HPLC/DAD/MS^n и овој метод беше оптимизиран за одредување на полифенолните маркери.

Доминантен антоцијанин кај сите сорти калинки беше цијанидин-3-глукозид, проследен со цијанидин и делфинидин 3,5-диглукозид. Од резултатите, може да се заклучи дека содржината на антоцијанини била повисока во 2016 година во однос на 2017 година. Но, спротивно на тоа, вкупната содржина на безбојните полифеноли беше околу 10 пати помала во 2016 година, во споредба со количината пронајдена во исти примероци во 2017 година.

Клучни зборови: калинки; полифеноли; HPLC/MS

1. INTRODUCTION

In recent years, there has been a growing interest in the consumption of healthy and functional foods due to their demonstrated health benefits. Epidemiological studies suggest that regular consumption of fruits reduces the risk of chronic and degenerative human diseases [1], and many of the health benefits have been attributed to the presence of polyphenolic compounds [2]. Fresh fruits and products thereof (e.g., fruit juices, fruit teas and jams) are valuable sources of polyphenols. Polyphenolic compounds form a large group of substances that differ in the number of aromatic rings, their linkage, and the presence of functional groups (e.g., hydroxylation and/or methoxylation) on the rings. Several hundred polyphenols have already been identified in plant-derived foods. Polyphenols are secondary metabolites. They are most frequently bound to sugar moieties, and therefore they are water soluble. According to their chemical structure, they can be divided into several groups (Fig. 1) including phenolic acids and flavonoids [3]. Polyphenolic substances show antioxidant activity by reacting as reducing substances, proton donors, and scavengers of free radicals, and by forming chelates with metal cations. In addition to their protective role against oxidative damage, polyphenols have been reported to exhibit anti-inflammatory, antiallergic, antiviral and vasodilator activities in humans [4].

Nowadays, *Punica granatum* L. is one of those species enjoying growing interest after a period of being an out-fashioned fruit with limited commercial appeal, mostly due to the time and patience needed to remove the rind and the tiny seeds. In particular, the juice obtained from pomegranates is experiencing a soaring success in the marketplace. This has been spurred by several features including a favorable combination of novelty, price, availability, color, unique taste, and health properties [5, 6].

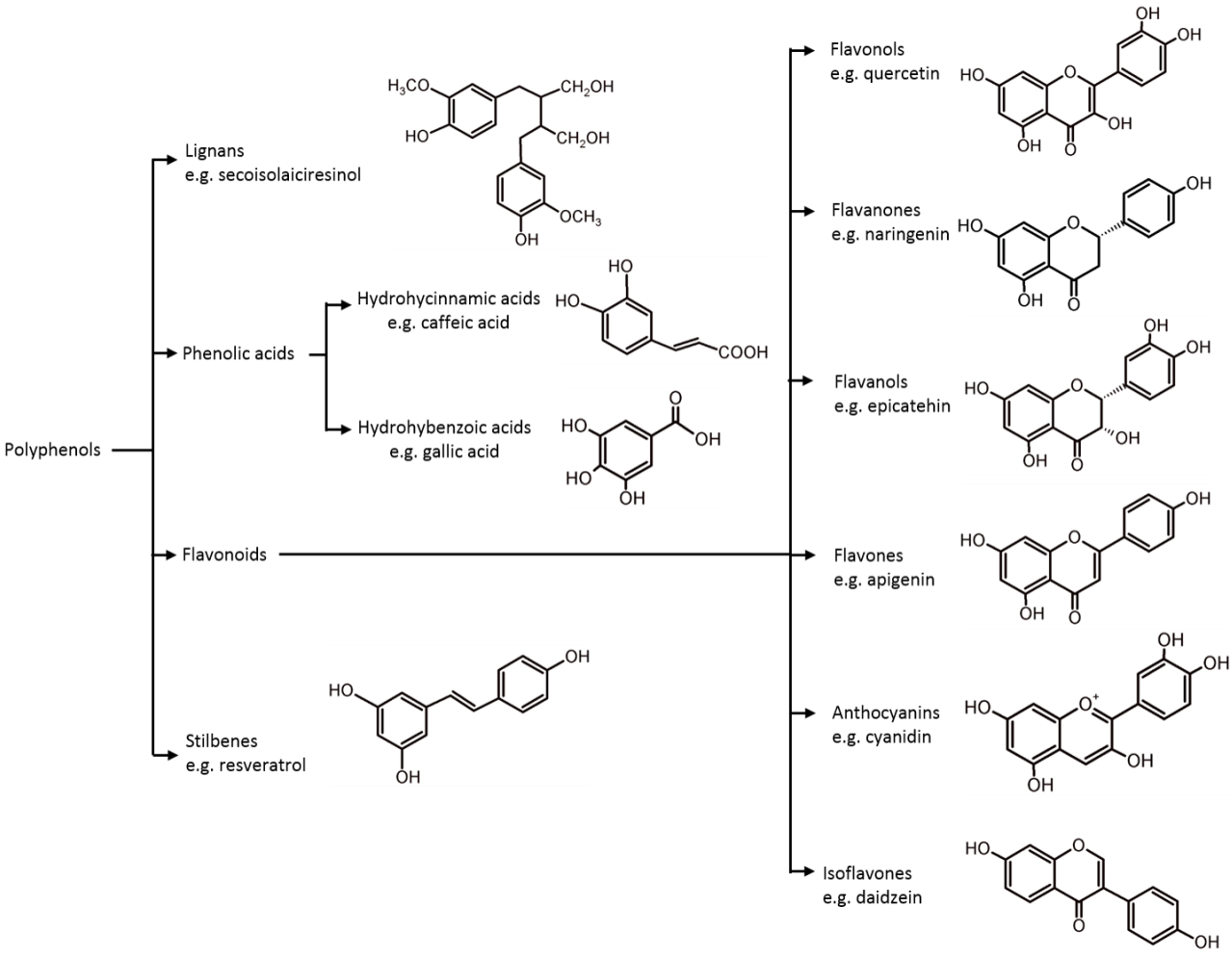


Fig. 1. Chemical structures of the main classes of polyphenols

The latter are strictly related to the phytochemical composition of this fruit, which is both complex and unique and encompasses the presence of anthocyanins (monoglycosides and diglycosides of cyanidin, delphinidin, and pelargonidin), ellagic acid and ellagitannins (mainly punicalagins and punicalins), gallic acid and gallotannins, proanthocyanidins, flavanols and lignans, whose combination is deemed responsible for a wide range of health-promoting biological activities exerted both directly or after an assimilation mediated through colonic biotransformation [7–10].

The aim of this work was to study the complex polyphenolic composition of eight pomegranate varieties (*Punica granatum*) from Macedonia, collected in two consecutive years, including both anthocyanin and non-anthocyanin compounds. For this purpose, a method using high-performance liquid chromatography with diode array detection and tandem mass spectrometry with positive and negative electrospray ionization (HPLC-DAD-ESI/MS^n) with an ion-trap mass analyzer was developed and optimized. The obtained results give an insight into the polyphenolic profile of the pomegranate fruits from the Balkan climate.

2. MATERIALS AND METHODS

2.1. *Reagents and chemicals*

Formic acid, methanol and water were purchased from Merck (Darmstadt, Germany). A standard of malvidin-3-glucoside chloride was purchased from Phytolab (Vestenbergsgreuth, Germany); (–)-epicatechin, gallic acid, ellagic acid, and quercetin were from Sigma (Darmstadt, Germany); caffeic acid was from Genay (Lyon, France).

2.2. *Plant material*

Eight autochthonic pomegranate cultivars (Zumnarija, Hidzas, Kisela, Bernarija, Limfonka, Ropkavac, Karamustafa, Valandovka) were selected from mature fruits grown in Valandovo, south part of Macedonia, in two consecutive years (2016, 2017). Ten kilograms of each cultivar were picked at maturity. According to Mirdehghan and Rahemi (2007) [11], the harvest maturity for pomegranate is achieved in September, when the arils' weight is greater than that of the peel. Fruits were transferred to a 4 °C storeroom on the same day as they were harvested. To avoid possible contamination of the juices with the metabolites produced by microorganisms, fruits with cracks, cuts, sunburn, and other defects in the husk were disposed of. During the experiments, only healthy fruits of uniform size and appearance were arranged in one row in wooden boxes containing packing material.

2.3. *Sample preparation*

Fruits for each cultivar were manually peeled, and juice was obtained from pomegranate arils by a hand press. The juice passed through a perforated plate and the seeds and pulp remained on the plate. Solid NaF (8.5 mg) was added to 100 ml juice (corresponding to 2 mM) to inactivate polyphenol oxidases and prevent phenolic degradation [12]), then the samples were centrifuged for 15 min at 3000 rpm at room temperature. The supernatant was filtered through a 0.45 μm nylon membrane before analysis using an HPLC/DAD/MS^n method that was optimized for determination of their polyphenolic fingerprints.

2.4. *LC/DAD/ESI-MS^n analysis*

Chromatographic separations were carried out on a 250 mm × 4.6 mm, 5 μm, Supelco C18 Discovery column (Sigma-Aldrich, Germany). The mobile phase consisted of water–formic acid (2 %, v/v) (A) and methanol (B). Gradient elution method was used (0–5 min, 5 % B; 5–20 min, 5–35 % B; 20–45 min, 35–50 % B; 45–55 min, 50–70 % B; 55–60 min, 70–100 % B and 60–65 min, 15 % B). The flow rate used was 0.35 ml/min. The injection volume was 10 μl.

The HPLC system was equipped with an Agilent 1100 series diode array detector and an ion trap mass detector in series (Agilent Technologies, Waldbronn, Germany). It consisted of a G1312A binary pump, a G1329A autosampler, a G1379B degasser and a G1315D photodiode array detector; it was controlled by ChemStation software (Agilent, v.08.03). Spectral data from all peaks were accumulated in the range 190–600 nm and chromatograms were recorded at 260 nm for hydrolysable tannins and gallocatehins, at 280 nm for hydroxybenzoic acids, at 320 nm for hydroxycinnamic acids, at 350 nm for flavonols, and at 520 nm for anthocyanins.

The mass detector was a G2449A ion-trap mass spectrometer equipped with an electrospray ionization (ESI) system and controlled by LCMSD software (Agilent, v.6.1.). Nitrogen was used as the nebulizing gas at a pressure of 50 psi, and the flow was adjusted to 12 l/min. The heated capillary and the voltage were maintained at 325 °C and 4 kV, respectively. The parameters for capillary exit off-

set, skimmer 1, and skimmer 2 were 100 V, 40 V and 6V, respectively, and compound stability was 100 %. MS data were acquired in the positive and negative ionization modes. The full scan covered the mass range at *m/z* 50–1200.

2.5. *Identification and quantification of polyphenolic compounds*

Liquid chromatography with diode-array detection (LC–DAD) was used for separation and quantification. Peak assignment of the various classes of polyphenols in the chromatograms was based on the comparison of their retention behavior and UV–Vis spectra to those of the authentic compounds and literature data. The conjugated forms of polyphenolic compounds were further characterized by electrospray ionization mass spectrometric detection in the positive ionization mode for anthocyanins and in the negative ionization mode for the other non-colored compounds.

Quantification was performed directly by HPLC/DAD using five-point regression curves ($R^2 \geq 0.999$) of authentic standards. Anthocyanins were quantified with cyanidin 3-*O*-glucoside chloride at 520 nm, flavonols were determined at 350 nm as quercetin equivalent, hydroxycinnamic acids were determined at 320 nm using caffeic acid as external standard, hydroxybenzoic acids were determined at 280 nm using gallic acid; hydrolysable tannins at 260 nm as ellagic acid, and gallocatehins at 260 nm as (–)-epicatechin.

In case of overlapping peaks in the DAD-chromatograms, separate quantitation was possible with the help of the extracted ion chromatograms (EICs) at the *m/z* values of the corresponding molecular ions of each overlapping compound: the EIC integral value was used to estimate the contribution of each individual overlapping compound to the joint DAD peaks. Total content is the sum of the contents of all individual phenolic compounds.

3. RESULTS AND DISCUSSION

3.1. *Characterization of phenolic compounds by HPLC/DAD/MS^n*

In total, 26 different polyphenolic compounds were detected in all studied samples and they were classified into the following groups: anthocyanins, hydrolysable tannins (gallotannins and ellagitannins), phenolic acids (hydroxybenzoic and hydroxycinnamic acids), flavan 3-ols and flavonols. The retention times, UV/Vis, and mass spectral characteristics as well as peak assignments for all compounds are specified in Table 1. The HPLC-DAD chromatograms are presented in Figure 2 (peak assignments as in Table 1).

Eight different anthocyanins were found in all analyzed samples. The anthocyanins revealed the typical UV and MS behavior in ESI (+)-experiments. From the UV and MS data, three anthocyanidins were detected. The $[Y_0]^+$ ions at *m/z* 303, 287, and 271 indicated delphinidin (aglycone of compounds **A1** and **A4**), cyanidin (**A2**, **A5**, **A7** and **A8**), and pelargonidin (**A3** and **A6**) (Fig. 3a). They also showed sequential loss of their sugar moieties, releasing the three aglycones in MS^2 experiments.

All anthocyanins were found in all analyzed samples in the two consecutive years except for cyanidin 3,5-pentoside-hexoside (**A5**), which was found only in 2016 in the samples from Bejnarija and Valandovka.

Eighteen different polyphenols belonging to six subclasses of non-colored polyphenols (gallotannins, ellagitannins, hydroxybenzoic and hydroxycinnamic acids, flavan-3-ols and flavonols) were tentatively identified.

As already reported in the literature [4, 13, 14], hydrolyzable tannins are the most abundant polyphenolic compounds in pomegranate juice and include gallotanins, ellagitannins and gallagyl esters such as punicalagin and punicalin.

The detected gallotannin showed a UV spectrum that is similar to that of gallic acid (λ_{max} = 269 nm). Galloyl hexose (**T1**) revealed an $[M–H]^–$ ion at *m/z* 331 and produced a fragment ion at *m/z* 169 (MS^2), indicating the loss of a hexose moiety and base ion characteristic for gallic acid.

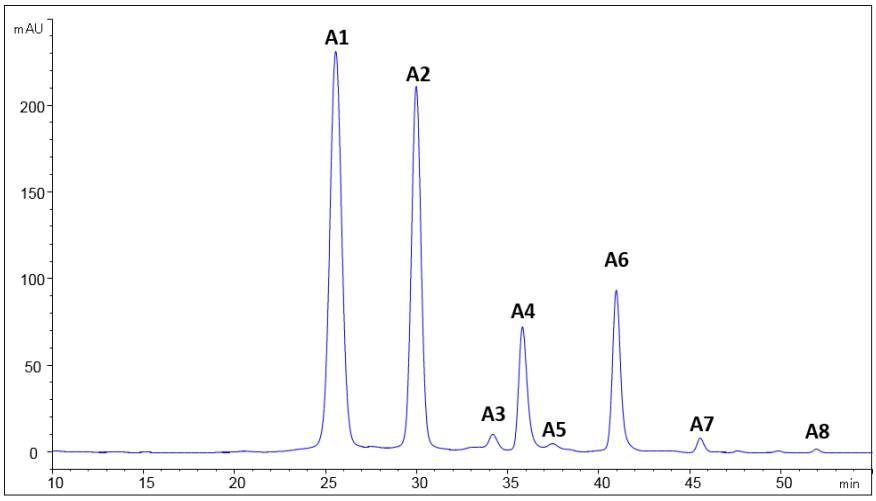
Ellagic acid (**T8**) (Fig. 3b) was previously found in pomegranate samples [8, 15, 16]. It exhibited UV maxima at 254 and 368 nm, a deprotonated molecular ion at *m/z* 301, and fragment ions produced in MS^2 at *m/z* 257 and 229.

Three compounds (**T5**, **T6** and **T7**) with $[M–H]^–$ at *m/z* 433, 447, and 463 were detected. In MS^2 all three produced fragment ions at *m/z* 301 indicating the presence of an ellagic acid moiety. The loss of 132, 146, and 162 indicated the presence of a pentose, rhamnose and hexose unit, respectively. Hence, these compounds were identified as ellagic acid pentoside, ellagic acid rhamnoside and ellagic acid hexoside, respectively.

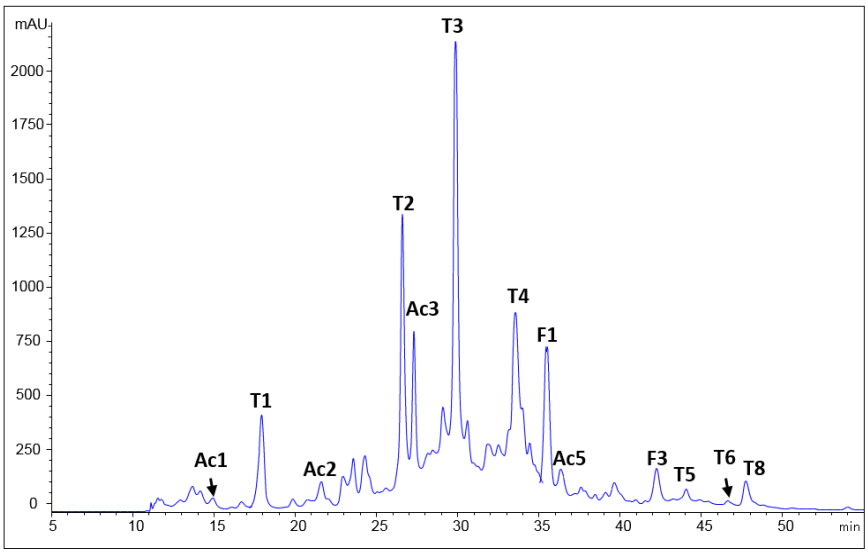
T a b l e 1

Retention times, UV/Vis spectra and characteristic ions of polyphenolic compounds of pomegranate

Peak no.	Compound	t_R/min	λ_{max}/nm	$[M]^+/[M-H]^-$	MS^2
	Anthocyanins				
A1	delphinidin-3,5-diglucoside	25.0	272, 300, 524	627^+	465, 303
A2	cyanidin-3,5-diglucoside	29.9	240, 276, 516	611^+	449, 287
A3	pelargonidin-3,5-diglucoside	34.2	276, 504	595^+	433, 271
A4	delphinidin-3-glucoside	35.8	240, 278, 528	465^+	447, 303
A5	cyanidin 3,5-pentoside-hexoside	37.5	278, 518	581^+	287
A6	cyanidin-3-glucoside	41.1	240, 280, 520	449^+	287
A7	pelargonidin-3-glucoside	45.4	276, 504	433^+	271
A8	cyanidin-3-pentoside	51.9	282, 520	419^+	287
	Hydrolysable tannins				
	Gallotannins				
T1	galloyl hexoside	17.9	266, 375	331	271, 169
	Ellagitannins				
T2	lagerstannin C (galloyl HHDP glucose)	26.5	260	649	497, 301
T3	pedunculagin I (bis-HHDP glucose)	30.5	256, 380	783	601, 301
T4	Punicalin	33.9	260, 380	781	721, 601, 301
T5	ellagic acid pentoside	44.8	254, 366	433	301
T6	ellagic acid rhamnoside	46.7	254, 360	447	301, 257
T7	ellagic acid hexoside	47.1	254, 360	463	301
T8	ellagic acid	49.0	254, 368	301	257
	Phenolic acids				
	Hydroxycinnamic acids				
Ac1	caffeic acid hexoside	15.0	332	341	179, 161, 135
Ac2	5-*O*-caffeoylquinic acid	21.8	316, 325	353	191, 163,145
Ac3	feruloylquinic acid	27.0	290, 326	367	191, 179, 144
Ac4	ferulic acid hexoside	35.2	280, 332	355	217, 193
Ac5	caffeic acid	36.5	290, 328	179	143, 119
	Hydroxybenzoic acids				
Ac6	gallic acid	24.3	270	169	125
Ac7	vanillic acid hexoside	37.3	254, 298	329	167
	Flavonoids				
	Flavan 3-ols				
F1	gallocatehin	36.0	264	305	219, 179
	Flavonols				
F2	kaempferol hexoside	40.1	284, 340	447	285
F3	quercetin	42.6	252, 360	301	163



(a)



(b)

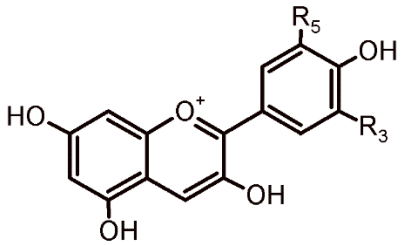
Fig. 2. HPLC-DAD chromatograms of pomegranate: **(a)** 520 nm, cultivar Valandovska, 2016, **(b)** 280 nm, cultivar Zumnarija, 2017

Due to their ability to yield urolithins after metabolism within the digestive tract, ellagitannins (polymeric structures including but not limited to different numbers of galloyl and hexahydroxydiphenoyl (HHDP) units esterified with glucose) are supposed to be the main bioactive phytochemicals of pomegranate juice. Seven ellagitannins were detected in the pomegranate juices assessed. They were distinguished by their characteristic fragment ion spectra yielding sequential losses of galloyl (*m/z* 152), gallate (*m/z* 170), and HHDP residues (*m/z* 301).

Compound **T3** exhibited an $[M–H]^-$ ion at *m/z* 783 and fragment ions in the MS^2 experiment at *m/z* 601 (gallagic acid) and *m/z* 301 (ellagic acid). Based on this fragmentation, compound **T3** was identified as bis-HHDP-hexoside (pedunculagin I, Fig. 3c). Compound **T3** occurred in three isomeric forms as can be deduced from the retention times (if any are present), and the isomers also differed in their fragmentation pathways as has been previously reported [17, 18].

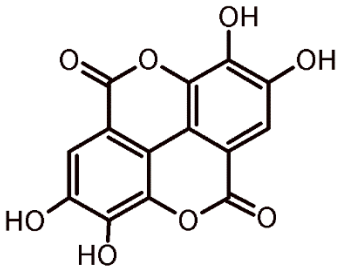
An ellagitannin with a gluconic acid core was also found in pomegranates. Compound **T2** was identified as galloyl-HHDP-gluconic acid (lagerstannin C, Fig. 3d). Its $[M–H]^-$ ion at *m/z* 649 released fragments at *m/z* 497 and 301 resulting from the loss of gallic acid (releasing HHDP gluconic acid) and ellagic acid, respectively. This compound has been previously found in pomegranate samples collected in Italy [14].

Compound **T4** was identified as punicalin (4,6-gallagyl-glucoside) (Fig. 3e) which revealed the loss of gallagic acid (*m/z* 601) and ellagic acid (*m/z* 301) moieties in the MS^2 experiment. It has also been identified in pomegranate samples collected from Italy and California [14, 15].

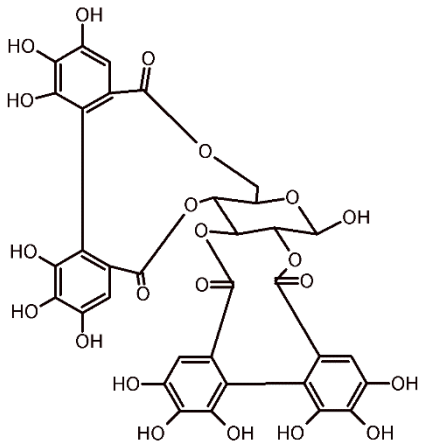


$R_3 = R_5 = H$, pelargonidin
$R_3 = H$, $R_5 = OH$, cyanidin
$R_3 = OH$, $R_5 = OH$, delphinidin

(a)



(b)



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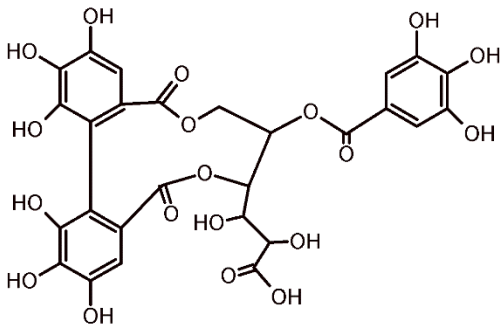

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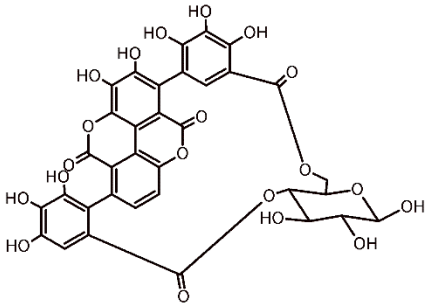

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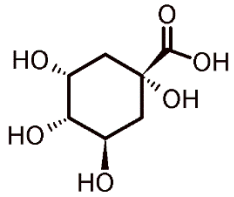

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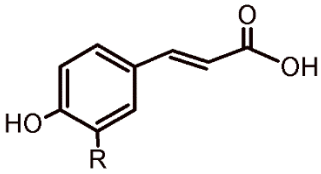
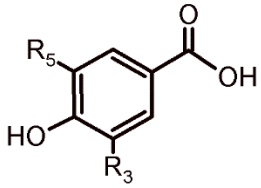


R=H coumaric acid
R=OH caffeic acid
R=OCH_3 ferulic acid

(g)



R_3=OH, R_5=OH gallic acid
R_3=OCH_3, R_5=H vanillic acid

(h)

Fig. 3. Chemical structures of **(a)** anthocyanidins, **(b)** ellagic acid, **(c)** pedunculagin I, **(d)** lagerstannin C, **(e)** punicalin, **(f)** quinic acid, **(g)** hydroxycinnamic acids, **(h)** hydroxybenzoic acids

Among hydroxycinnamic acids, compound **Ac1** was identified as caffeic acid hexoside (*m/z* 341), which showed a loss of a hexose moiety in the MS^2 experiment (162) and a partial decarboxylation of the caffeic acid moiety resulting in fragments at *m/z* 179 and 135, whereas compound **Ac4** was identified as ferulic acid hexoside after loss of 162 resulting in a fragment at *m/z* 193.

Furthermore, a compound with an $[M–H]^–$ ion at *m/z* 353 (**Ac2**) was detected and identified as chlorogenic acid (5-*O*-caffeoylquinic acid) by comparison of its retention time, UV and mass spectra with those of an authentic reference substance. Similarly, compound **Ac5** (*m/z* 179) was identified as caffeic acid, whereas **Ac3**, with $[M–H]^–$ at *m/z* 367 and fragment ion at *m/z* 191 (quinic acid moiety), was identified as a feruloylquinic acid isomer.

Only two hydroxybenzoic acids were detected, gallic acid (at *m/z* = 169) (**Ac6**) and vanillic acid hexoside (at *m/z* = 329) (**Ac7**).

From the group of flavonoids, only one flavan-3-ol was detected with an absorption maximum at 264 nm, a deprotonated molecular ion at *m/z* 305 and fragment ions in MS^2 at *m/z* 219, 179. This compound was identified as gallocatehin (**F1**). The last two compounds belong to the group of flavonols, and according to their UV and MS spectra they were identified as kaempferol hexoside (**F2**) and quercetin (**F3**).

3.2. *Quantification of phenolic compounds by HPLC/DAD*

All results for the content of every measured compound in all studied cultivars from two seasons of pomegranate samples are given in Table 2. The total phenolic content (TPC) of the eight pomegranate cultivars analyzed in the two years is given in the graph in Figure 4, where the polyphenolic patterns are given as distributed in the groups of anthocyanins (**A**), hydrolysable tannins (**T**), phenolic acids (**Ac**), and flavonoids (**F**). The relative abundance as the percent of total anthocyanins, total hydrolysable tannins, total phenolic acids, and total flavonoids compared to TPC is given in Table 3.

The total anthocyanin content was in range from 40.5 mg/l for Kisela (2017) to 465.2 mg/l for Hidzas (2016). The content of anthocyanins was notably higher in 2016 compared to 2017 for all varieties except for Zumnarija. The dominant anthocyanin in pomegranate was cyanidin-3-glucoside contributing 30 to 45 % of the total anthocyanin content, but also there was cyanidin and delphinidin 3,5-diglucoside with 20 to 40 % of the total anthocyanin content.

On the other hand, the total content of non-colored polyphenols was around 10 times lower in 2016 compared to the amount found in the samples from the same varieties in 2017.

Compounds **T1**–**T4** (galloyl hexose, lagerstannin C, pedunculagin I and punicalin) were detected only in the samples from 2017, whereas compound **T7** (ellagic acid hexoside) only in the samples from 2016. The total content of hydrolysable tannins in 2016 varied from 13 mg/l to 101 mg/l, which represents from 5–17 % of total the phenolic content, whereas in 2017 it was in range from 130.5 mg/l to 403.5 mg/l, which represents 37–64 % of the total phenolic content.

The group of phenolic acids contains hydroxycinnamic and hydroxybenzoic acid derivatives. The content of phenolic acid derivatives was in range of 13.19 mg/l to 77.3 mg/l. Hydroxybenzoic acid derivatives were found only in the samples from 2016 but not in 2017. From the hydroxycinnamic acid derivatives, caffeoyl and feruloylquinic acid derivatives were dominant in both years, whereas caffeic acid contributed 20–40 % to the total phenolic acid content but only in 2017, because it was not detected in 2016.

From the class of flavonoids, gallocatehin was found in 2017, whereas kaempferol hexoside in 2016. The content of quercetin in both years was similar and contributed 24 to 65 % to the total flavonoid content.

The total phenolic content (in mg/l), calculated as a sum of the content of the individual compounds, was in the range from 148.6 mg/l for Limfonka 2016 to 786.4 mg/l for Zumnarija 2017. In general, TPCs were higher in 2017 than in 2016. Anthocyanins were the dominant group in 2016 year, and their contribution to TPC was between 75 and 80 %, followed by hydrolysable tannins (5-15 % of TPC), phenolic acids (5–24 % of TPC), and flavonoids (1–3 %).

In contrast, in 2017 hydrolysable tannins were the dominant group, and they contributed with 37–65 % of the TPC, followed by anthocyanins (10–30 % of TPC), flavonoids (10–20 %) and phenolic acids (3–15 %).

Despite the great number of studies, comparison of the phenolic content with literature data is still aggravated due to the following: a) different analytical methodologies that measure individual or classes of polyphenolic compounds, and b) the variability of the polyphenol composition depending on many factors, including the cultivar, the growing region, the maturity stage, environmental conditions, processing steps and storage conditions. As our results demonstrate, differences are evident not only between different pomegranate varieties but also for the samples from the same variety collected in different years.

The polyphenolic contents in different types of juices also differ due to differing procedures for the preparation of juice and which part of the arils are present during the procedure. In our case, we prepared the juices only from the isolated arils around the seeds, and we did not use the peel, mesocarp, or seeds.

T a b l e 2

*Phenolic compound concentration (*mg/l*) in pomegranate juice from eight Punica granatum cultivars analyzed in two seasons (2016, 2017)*

Peak no.	Compound/Sample	**Z16**[a]	**Z17**	**H16**	**H17**	**B16**	**B17**	**LC16**	**L16**	**L17**	**R16**	**R17**	**KM16**	**KM17**	**V16**	**V17**	**KS17**
	Anthocyanins																
A1	del-3,5-diglc	10.04[b]	60.33	131.0	73.24	50.54	25.38	52.55	26.78	11.01	35.54	8.516	71.09	43.73	197.1	30.49	9.455
A2	cya-3,5-diglc	52.65	47.65	114.4	96.49	35.56	29.69	73.95	29.51	13.42	86.37	41.87	60.56	49.95	141.6	22.00	11.18
A3	pel-3,5-diglc	2.551	1.828	2.593	4.575	1.925	1.662	2.253	nd	nd	5.346	2.363	3.250	2.351	5.212	1.188	0.832
A4	del-3-glc	5.851	47.88	77.37	38.08	13.26	9.306	18.75	17.72	9.022	10.73	3.771	9.554	6.338	41.43	34.53	7.872
A5	cya-3,5-pent-hex	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	2.185	nd	nd
A6	cya-3-glu	51.58	69.60	127.9	60.54	19.05	16.27	47.95	38.04	18.48	60.67	28.28	15.94	9.361	53.74	33.87	9.672
A7	pel-3-glc	6.071	4.071	7.884	3.662	1.878	1.686	2.463	2.871	1.149	9.754	2.763	2.277	0.630	3.754	1.551	1.531
A8	cya-3-pent	nd	1.128	4.005	1.641	nd	nd	1.356	0.565	nd	nd	nd	nd	nd	1.217	0.669	nd
	Total	**128.7**	**232.5**	**465.2**	**278.2**	**122.2**	**83.99**	**199.3**	**115.5**	**53.08**	**208.41**	**87.56**	**162.7**	**112.4**	**446.2**	**124.3**	**40.54**
	Hydrolysable tannins																
	Gallotannins																
T1	galloyl hex	nd	49.20	nd	46.25	nd	27.65	nd	nd	16.26	nd	13.80	nd	24.20	nd	nd	28.39
	Ellagitannins																
T2	lagerstannin C	nd	66.26	nd	134.9	nd	62.69	nd	nd	128.8	nd	78.67	nd	67.35	nd	nd	66.26
T3	pedunculagin I	nd	168.2	nd	74.51	nd	19.30	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
T4	Punicalin	nd	106.8	nd	nd	nd	202.4	nd	nd	168.2	nd	92.80	nd	153.5	nd	305.4	31.87
T5	ellagic acid-pent	nd	3.573	5.248	3.681	2.703	2.398	5.958	nd	2.995	4.218	3.198	nd	2.134	5.361	nd	nd
T6	ellagic acid-rham	15.64	3.432	37.59	3.588	3.889	nd	18.497	8.540	nd	17.81	3.972	nd	nd	19.65	nd	nd
T7	ellagic acid-hex	2.493	nd	17.45	nd	3.383	nd	16.403	3.756	nd	11.95	nd	nd	nd	11.56	nd	nd
T8	ellagic acid	nd	5.973	42.67	5.987	2.984	15.35	5.360	1.452	6.890	1.732	4.521	9.202	7.327	35.48	11.10	3.992
	Total	**18.13**	**403.5**	**101.0**	**268.9**	**12.96**	**329.8**	**46.218**	**13.75**	**323.1**	**35.72**	**197.0**	**9.202**	**254.5**	**72.05**	**316.5**	**130.5**
	Phenolic acids																
	Hydroxycinnam-ic acids																
Ac1	caffeic hex	5.109	2.197	0.914	1.625	1.235	2.453	2.140	3.204	1.724	4.531	2.680	2.597	1.578	1.900	3.040	2.898
Ac2	5-O-caff-quinic acid	4.820	5.324	3.908	2.999	37.60	5.721	4.787	4.559	2.786	2.276	2.137	6.240	3.826	7.467	5.946	7.291
Ac3	feruloylquinic acid	0.328	11.22	0.395	19.95	0.349	2.547	0.379	0.123	23.97	0.321	9.475	0.168	9.939	0.505	0.869	14.08
Ac4	feryllic acid hex	2.778	nd	1.203	nd	3.009	nd	2.947	3.475	nd	3.747	nd	4.370	nd	3.070	nd	nd
Ac5	caffeic acid	nd	11.22	nd	20.99	nd	2.473	nd	nd	23.52	nd	8.772	nd	9.504	nd	nd	15.28
	Hydroxybenzoic acids																
Ac6	gallic acid	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	67.440	nd
Ac7	vanillic acid hex	2.602	nd	7.085	nd	2.982	nd	3.815	3.445	nd	4.366	nd	3.742	nd	5.099	nd	nd
	Total	**15.64**	**29.96**	**13.51**	**45.57**	**45.17**	**13.19**	**14.07**	**14.80**	**51.99**	**15.24**	**23.06**	**17.12**	**24.85**	**18.04**	**77.30**	**39.54**
	Flavonoids																
	Flavan 3-ols																
F1	gallocatehin	nd	81.62	nd	71.17	nd	54.68	nd	nd	21.69	nd	46.42	nd	30.32	nd	nd	90.75
	Flavonols																
F2	kaempferol hex	1.432	nd	3.056	nd	2.139	nd	5.573	2.101	nd	nd	nd	2.684	nd	4.538	nd	nd
F3	quercetin	nd	38.77	2.757	59.54	1.895	32.42	3.322	2.428	41.00	1.172	27.93	2.672	21.92	2.943	9.195	28.13
	Total	**1.432**	**120.39**	**5.813**	**130.7**	**4.034**	**87.10**	**8.895**	**4.529**	**62.70**	**1.172**	**74.35**	**5.356**	**52.24**	**7.481**	**9.195**	**118.9**
	Total phenolic content	**163.9**	**786.4**	**585.5**	**723.4**	**184.4**	**514.1**	**268.5**	**148.6**	**490.9**	**260.5**	**382.0**	**194.4**	**444.0**	**543.8**	**527.3**	**329.5**

[a]Z–Zumnarija, H–Hidzas, B–Bejnarija, L–Limfonka, LC–Limfonka clone, R–Ropkavac, KM–Karamustafa, V–Valandovka, KS–Kisela; 16 and 17, years of collections 2016 and 2017.
[b]Values represent the mean of three measurements (mean ± SD)

T a b l e 3

Relative abundance in percent of total anthocyanins, total hydrolysable tannins, total phenolic acids, and total flavonoids to TPC

Compounds	Z16	Z17	H16	H17	B16	B17	LC16	L16	L17	R16	R17	K16	K17	V16	V17	KS17
Anthocyanins	78.5	29.6	79.5	38.5	66.3	16.3	74.2	77.7	10.8	80.0	22.9	83.7	25.3	82.1	23.6	12.3
Hydrolysable tannins	11.1	51.3	17.2	37.2	7.0	64.2	17.2	9.3	65.8	13.7	51.6	4.7	57.3	13.3	60.0	39.6
Phenolic acids	9.5	3.8	2.3	6.3	24.5	2.6	5.2	10.0	10.6	5.8	6.0	8.8	5.6	3.3	14.7	12.0
Flavonoids	0.9	15.3	1.0	18.1	2.2	16.9	3.3	3.0	12.8	0.4	19.5	2.8	11.8	1.4	1.7	36.1

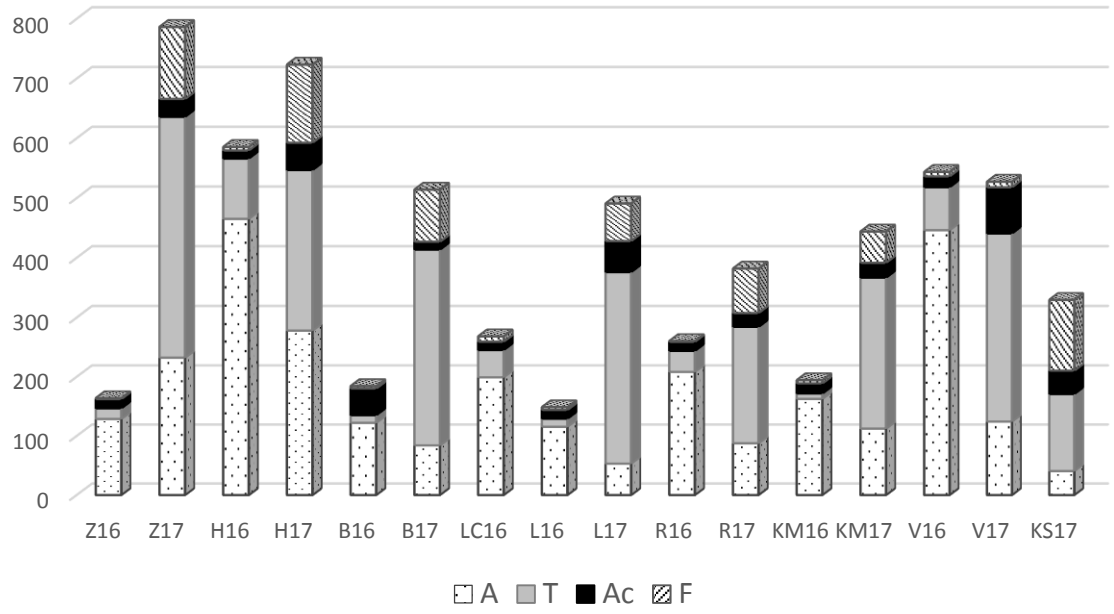


Fig. 4. Polyphenolic content (anthocyanins (A), hydrolysable tannins (T), phenolic acids (Ac) and flavonoids (F)) of pomegranate juice from eight *Punica granatum* cultivars collected and analyzed in two seasons (2016, 2017) (expressed in mg/l)

The anthocyanins patterns and contents were very similar to those found in the literature. In the study of Gil *et al*. (2000) [17], the total anthocyanin content in different types of juice varied from 161.9 mg/l to 387.4 mg/l. They concluded that when arils are frozen and stored prior to juice extraction, the anthocyanins are partly degraded and/or transformed into other products. The total anthocyanins content in 15 different Iranian pomegranate varieties (unprocessed juices, mg/l) [18] was in the range from 15.01 mg/l to 252.22 mg/l. Fischer *et al*. (2011) [8] prepared different types of juice and they found differences in the anthocyanin content. The juice prepared only from isolated arils which were coating the seeds had the highest anthocyanin content (557.7 mg/l) compared to the juice prepared from not separated fruits (198.3 mg/l).

The content of hydrolysable tannins in our study was lower compared to those found in the literature. In the results of Gil *et al*. (2000) [17], hydrolysable tannins were in range from 640.3 to 2699.4 mg/l, whereas in the study of Fischer *et al*. (2011) [8] they were in the range from 93.2 to 2074.4 mg/l. The total content of hydrolysable tannins is also in correlation with the procedure used for juice preparation, because it is known that the content of hydrolysable tannins is higher in the peel and mesocarp.

From the literature, it is also evident that when the total anthocyanin content was higher, the total content of hydrolysable tannins was lower.

The content of the other detected compounds was minor and comparable to those found in the literature [19]. The content of hydroxycinnamic acids was in the range from 6.42 mg/l to 52.0 mg/l. Compounds **Ac1-Ac3** were found in all analyzed samples, whereas compound **Ac4** was present only in the samples of 2016 and compound **Ac5** in the samples collected in 2017. Compound **Ac6** was found only in one sample (V17), whereas compound **Ac7** was in all samples collected in 2016.

Gallocatehin was not detected in the samples collected in 2016, and its content in the samples collected in 2017 ranged from 21.69 mg/l to 90.75 mg/l.

Kaemferol hexoside was detected in the samples from 2016, with the content from 1.432 mg/l to 5.573 mg/l, whereas quercetin was detected in all analyzed samples (except Z16) and its content was lower in the samples collected in 2016 (1.172–3.322 mg/l) compared to those in the samples collected in 2017 (9.195–59.4 mg/l).

From our results, but also from literature data, we can conclude that pomegranate samples collected from Macedonia present a very rich source of polyphenolic compounds, but the polyphenol composition variability (both pattern and content) depends considerably on many factors such as the cultivar, the maturity stage, the growing region, environmental conditions, processing steps and storage conditions.

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