Macedonian Journal of Chemistry and Chemical Engineering. Vol. **29**, No. 2, pp. 181–194 (2010)
MJCCA9 - 563 ISSN 1857 - 5535
Received: March 31, 2010 UDC: 634:75:[547.562:543.42(497.7)
Accepted: July 8, 2010
Original scientific paper

HPLC-DAD-ESI-MS^n IDENTIFICATION OF PHENOLIC COMPOUNDS IN CULTIVATED STRAWBERRIES FROM MACEDONIA

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Strawberry fruits contain phenolic compounds that exhibit antioxidant, anticancer, antiatherosclerotic, antinflammatory and anti-neurodegenerative properties. High-performance liquid chromatography (HPLC) coupled to electrospray ionization mass spectrometric (ESI-MS) detection in the positive and negative ion mode has been used to identify the phenolic compounds in extracts from sixteen different strawberry cultivar fruits from Republic of Macedonia. Photodiode-array detection (DAD) has been used for screening of the different classes of phenolic compounds, whereas MS and MS^n fragmentation data were employed for their structural characterization. The phenolic compounds identified were grouped as: ellagic acid and ellagic acid conjugates with sugars, ellagitannins, anthocyanins, flavonols, flavanols, and acylated sugars (feruloyl, caffeoyl and coumaroyl hexoses). Quercetin and kaempferol were the major flavonols found as quercetin 3-*O*-glucoside, quercetin 3-*O*-glucuronide, kaempferol 3-*O*-glucoside, kaempferol 3-*O*-glucoronide, kaempferol 3-*O*-malonylglucoside, kaempferol 3-*O*-acetylglucoside and kaempferol 3-*O*-coumaroylglucoside. Pelargonidin-3-*O*-glucoside was the most abundant anthocyanin in all strawberry extracts. Proanthocyanidins were also identified by MS^n fragmentation as dimers, trimers and tetramers of (epi)catechin and (epi)afzelechin. This is the first assay of the phenolic profile of the strawberry cultivars in Macedonia, which can be further developed for characterization and evaluation of their quality with regards to their phenolic composition.

Key words: strawberries; HPLC-DAD-ESI-MS^n; phenolic compounds

ИДЕНТИФИКАЦИЈА СО HPLC-DAD-ESI-MS^n НА ФЕНОЛНИОТ ПРОФИЛ НА КУЛТИВИРАНИ ЈАГОДИ ОД МАКЕДОНИЈА

Јагодите имаат висок процент на фенолни компоненти кои имаат антиоксидантни, антиканцерогени, антиинфламаторни и антинеуродегенеративни својства. Високоефикасна течна хроматографија (HPLC) поврзана со масена спектрометрија (MS) со електроспреј јонизација (ESI) беше користена во позитивен и негативен јонски мод за идентификација на фенолните компоненти во екстракти од 16 култивирани јагоди од Република Македонија. Беше користен детектор со низа од диоди (DAD) за анализа на различните класи фенолни компоненти, додека со фрагментациите MS и MS^n беа користени за нивна структурна карактеризација. Фенолните компоненти беа идентификувани и групирани како слободна и конјугирани форми на елагова киселина, танини на елагова киселина, антоцијани, флавоноли, флаван-3-оли и ацилирани шеќери (ферулоил, кафеоил и кумароуил хексози). Кверцетин и кемферол беа основните флавоноли најдени како кверцетин-3-*O*-глукозид, кверцетин-3-*O*-глукуронид, кемферол-3-*O*-глукозид, кемферол-3-*O*-глукуронид, кемферол-3-*O*-малонилглукозид, кемферол-3-*O*-ацетилглукозид и кемферол-3-*O*-кумароилглукозид. Пеларгонидин-3-*O*-глукозид беше доминантен антоцијан во сите екстракти од јагоди. Проантоцијанидините беа исто така идентификувани со фрагментација со MS^n како димери, тримери и тетрамери на (епи)катехин и (епи)афзелехин. Ова е прво истражување на фенолниот профил на култивирани јагоди во Македонија, кое може понатаму да се употреби за нивна карактеризација и оцена на квалитетот врз основа на фенолниот состав.

Клучни зборови: јагоди; HPLC-DAD-ESI-MS^n; фенолни компоненти

1. INTRODUCTION

Fruits and fruit products, together with tea, red wine, cereals, chocolate and legumes, are regarded as major dietary sources of polyphenols [1]. The evaluation of berry fruits as a source of bioactive phenolic antioxidants involves studies of the nature and quantity of phenolic compounds, as well as their absorption, distribution and metabolism, which is related to the nature of the conjugated sugars in the phenolic compounds [2–4]. Phenolic compounds include structurally diverse classes of compounds with different kinds of conjugates. The major class of phenolic compounds in strawberries is represented by hydrolysable tannins with anthocyanins being the second most important class in pigmented berries whereas hydroxycinnamic acids, flavonols, flavan-3-ols and proanthocyanidins are the minor ones [5–8] (structures given in Figure 1).

The most frequently used analytical technique for the separation of phenolic compounds is reversed-phase high-performance liquid chromatography (RP-HPLC), with diode array detection (DAD) and mass spectrometry (MS) detection. This is currently the most widely available and commonly used combination of techniques for separation, identification and quantification of phenolic compounds [5, 9]. UV-Vis spectra are still valuable for a preliminary identification and for quantification using characteristic absorption maxima, whereas mass spectra contribute greatly to structural characterization. The polyphenolic compounds are classified on the basis of their characteristic UV-Vis spectra: 280 nm for flavanols, 300–320 nm for hydroxycinnamic acid derivatives, 500–520 nm for anthocyanins, etc. Mass spectrometry provides information about the molecular mass and fragmentation pattern of the analyte. The ionization may be performed in the positive and/or negative ion mode and anthocyanins are analyzed in the positive mode, whereas other groups are usually analyzed in the negative mode [10–13]. In the negative ionization mode, acids deprotonate easily, and in the positive mode, they form adducts with the cations in the sample or mobile phase, for example, sodium ions [14]. The recent applications of electrospray ionisation (ESI) for analysis of the polyphenolic compounds involve detection of the pseudomolecular ion in order to investigate the degree of polymerization. Multicharged species like $[M-2H]^{2-}$, $[M-H+Cl]^{2-}$, $[M+2Cl]^{2-}$ or $[M-3H]^{3-}$ are often observed [15, 16]. Their existence can be proved by calculating the isotopic distribution of the signals [17] and depends on concentration, desolvation potential and concentration of Cl^- in the system [16]. The MS results are valuable but still difficult to interpret without having any additional information about the compound. For that reason, both UV-Vis diode array and mass spectrometry coupled to RP-HPLC have proved as complementary and most convenient techniques.

The purpose of our study was to use the complementary information from HPLC with DAD detector in combination with mass spectrometry, to identify the structural characteristics of the conjugated forms of phenolic compounds in strawberry cultivars grown in Republic of Macedonia.

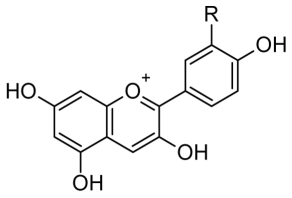
2. EXPERIMENTAL

2.1. *Plant material*

The species *Maya* (M), *Alba* (A) and *Roxana* (R) from Kočani (Češinovo, Teranci, Zrnovci) were harvested on May 8, 2009; *Maya*, *Alba*, *Roxana* and *Tudla* from Strumica (Smolari) were harvested on May 19, 2009; *Maya*, *Alba* and *Roxana* from Skopje (Dolno Lisiče) were harvested on May 21, 2009. Sixteen samples in total were collected for further analysis of the phenolic compounds.

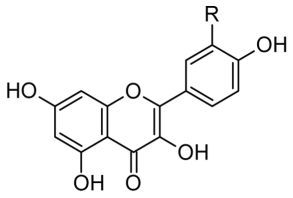
The strawberries were harvested at commercial ripeness, specifically when 80 % of the surface showed a red colour, which corresponds to stage 5 of ripeness in terms of commercial criterion.

With the purpose of modernization of the assortment, four promising strawberry varieties of the *Fragaria ananassa* species have been introduced: *Alba*, *Maya*, *Roxana*, and *Tudla*. Three demonstrative orchards have been established in the east, north and south part of R. Macedonia, in the region of Kočani (Češinovo, Teranci, Zrnovci), Strumica (Smolari), and Skopje (Dolno Lisiče), in



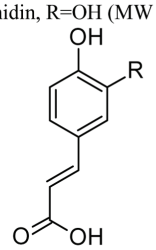
Anthocyanidins

Pelargonidin, R=H (MW 271)
Cyanidin, R=OH (MW 287)



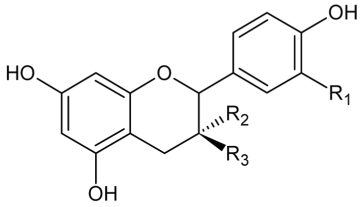
Flavonols

Kaempferol, R=H (MW 286)
Quercetin, R=OH (MW 302)



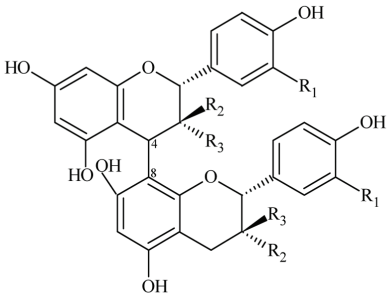
Hydroxycinnamic acid

p-Coumaric acid, R=H (MW 164)
Caffeic acid, R=OH (MW 180)
Ferulic acid, R=OCH_3 (MW 194)



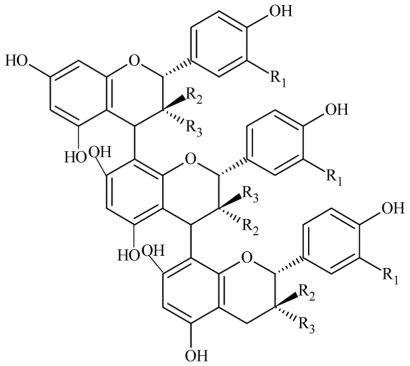
Flavan-3-ols

Afzelechin, R_1=H, R_2=H, R_3=OH (MW 274)
(epi)Afzelechin R_1=H, R_2=OH, R_3=H (MW 274)
Catechin R_1=OH, R_2=H, R_3=OH (MW 290)
(epi)Catechin R_1=OH, R_2=OH, R_3=H (MW 290)



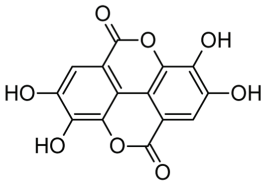
Proanthocyanidins dimers

(epi)Afzelechin - (epi)Catechin (MW 562)
(epi)Catechin - (epi)Catechin (MW 578)

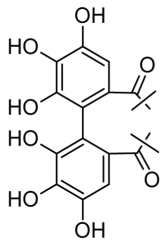


Proanthocyanidins trimers

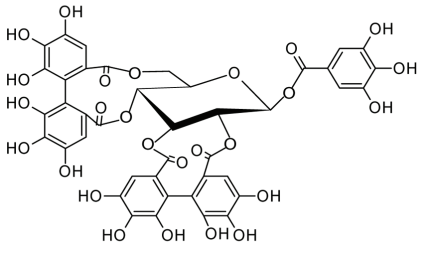
(epi)Catechin - (epi)Catechin - (epi)Afzelechin (MW 850)
(epi)Catechin - (epi)Catechin - (epi)Catechin (MW 866)



Ellagic acid (MW 302)



HHDP (MW 304)



Ellagitannin, galloyl-bis-HHDP-glucose, (MW 936)

Fig. 1. Structures of phenolic compounds found in strawberry fruits

September 2006, with imported green seedlings rooted in pots. The orchards have been established in a system of two-row beds with a width of 80 cm, mulched with black foil. The drip irrigation and fertirigation has been performed during the growing. The distance between seedlings in the beds was 40 × 30 cm, with an empty space of 60 cm between the beds. This growing system consisted of 47600 plants per hectare. In order to enforce the production of fruits, in spring, the orchard was covered with high polyethylene tunnels in Kočani and Strumica, and in Skopje the orchard was open.

500 g of each variety from each locality was randomly sampled. Samples were stored at –80 °C until analysis.

2.2. *Sample preparation*

Each frozen strawberry sample (5 g) was homogenized and extracted with 10 mL of two solvent mixtures: acetone and acetic acid (99:1, *V*/*V*), and methanol and acetic acid (99:1, *V*/*V*). The extracts were sonicated for 15 minutes on an ultrasonic bath (Branson model 3510, USA), centrifuged for 15 minutes at 3000 rpm, and the supernatants were concentrated in rotary evaporator at 37 °C. The volume of the extract (aqueous residue) was made up to 5 mL with 20 % methanol in water, and it was filtered through 0.45 μm pore-size polyethersulfone filter (Econofilter, 25/0.45 μm NL, Agilent Technologies, Germany) before analysis. All extracts were analyzed by HPLC-ESI-MS.

2.3. *Reagents and standards*

Formic acid of analytical grade and methanol of gradient grade for liquid chromatography were purchased from Merck KGaA (Darmstadt, Germany). Acetone and acetic acid of analytical grade were purchased from Alkaloid (Skopje, R. Macedonia).

Pelargonidin-3-glucoside and proanthocyanidin B2 were purchased from Phytolab (Germany), catechin and quercetin were purchased from Sigma (Germany), *p*-coumaric acid, ferulic acid and ellagic acid were purchased from Genay (France).

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

The HPLC system was equipped with an Agilent 1100 series diode array and ion trap mass detector in series (Agilent Technologies, Waldbronn, Germany). It consisted of a G1312A binary pump, a G1329A autosampler, a G1379B degasser, G1316A thermostat column and G1315B photo-diode array detector, controlled by ChemStation software (Agilent, v.01.03).

Chromatographic separations were carried out on 150 mm × 4.6 mm, 5 μm Eclipse XDB–C18 column (Agilent Technologies, Germany). The mobile phase consisted of two solvents: formic acid (1 %, *V*/*V*) in water (A) and methanol (B). A linear gradient starting with 5 % B, 5 % B at 5 min, was installed to reach 80 % B at 45 min, 100 % B at 50 to 60 min. The flow rate was 0.4 mL min^{-1} and the injection volume 20 μL and column temperature of 40 ºC.

Spectral data from all peaks were accumulated in the range 190–600 nm and chromatograms were recorded at 260 nm for ellagic acid, conjugated forms of ellagic acid and ellagitannins and hydroxybenzoic acid derivatives, at 280 nm for flavan-3-ols and their dimers, trimers and tetramers, at 320 nm for conjugated forms of hydroxycinnamic acid, at 360 nm for flavonols and at 520 nm for anthocyanins.

The mass detector was Agilent G2449A Ion-Trap Mass Spectrometer equipped with an electrospray ionization (ESI) system and controlled by LCMSD software (Agilent, v.6.2). Nitrogen was used as nebulizing gas at pressure of 50 psi and the flow rate was adjusted to 12 Lmin^{-1}. The temperature and the voltage of the capillary were maintained at 325 °C and 3,5 kV, respectively. MS data were acquired in positive and negative ionization mode. The full scan covered the mass range from *m/z* 100–2000. Collision – induced fragmentation experiments were performed in the ion trap using helium as collision gas with voltage ramping cycle from 0.3 up to 2 V. Maximum accumulation time of ion trap and the number of MS repetitions to obtain the MS average spectra wereset at 200 ms and 5, respectively.

3. RESULTS AND DISCUSSION

Extraction experiments of phenolic compounds from cultivated strawberries using 1 % acetic acid in methanol and 1 % acetic acid in acetone (*V/V*) showed that acetone extracts contained more polyphenols, especially conjugated forms of ellagic acid and condensed tannins, and B type proanthocyanidins, which are dimers, trimers and tetramers of (epi)catechin and (epi) afzelechin that linked in C4-C6 or C4-C8 [28]. This can be seen in the chromatograms obtained using both extraction solvents at two wavelengths, 260 and 280 nm, given in Figure 2.

The different classes of phenolic compounds exhibit absorbance maxima at different wavelengths, so five wavelengths were selected for detection: 260 nm for ellagic acid and ellagitannins, 280 nm for flavanols and proanthocyanidins, 320 nm for hydroxycinnamic acid derivatives, 360 nm for flavonols, and 520 nm for anthocyanins, for optimal selectivity and sensitivity. For a more systematic discussion, the phenolic compounds in strawberries were grouped according to their structure features, i.e. their UV-Vis spectra, as usually [20–23] into: anthocyanins, flavonols, flavanols, proanthocyanidins, derivatives of hydroxycinnamic and hydroxybenzoic acids, and ellagitannins (Figure 1).

LC-MS and MS^n detection in both positive and negative ionization modes were used to obtain more information on the structural features of the conjugated forms of phenolic compounds. Identification of most compounds was achieved by consecutive MS^2 and MS^3 analyses. However, high resolution "zoom scan" analyses were necessary for characterization of the charge of some ellagitannins. Zoom scan data were collected using slower scans in a narrow mass range to improve the resolution of the $^{12}C/^{13}C$ isotope ratio of an analyte [12].

Identification of the compounds within each class, based on chromatographic behaviour, UV-Vis and mass spectra, and comparison with literature, is discussed below and summarized in Tables 1 to 5, where the compounds are numbered according to their retention times in the obtained chromatograms (Figure 2).

3.1. *Identification of anthocyanins*

The red colour of strawberries is due to anthocyanins present in their flavylium forms. Anthocyanins in strawberries are mostly glycosides of pelargonidin and cyanidin. Identification of their derivatives is based on retention times, UV-Vis absorption maxima and mass spectra obtained in positive mode by using their fragmentation patterns and literature data [18, 21, 22].

Seven peaks with absorption maxima at 504 nm and MS fragmentation ions at *m/z* 271

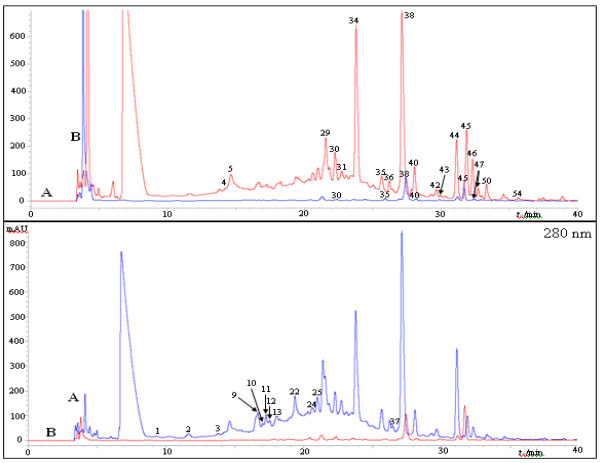


Fig. 2. Chromatograms of strawberry fruit (sample *Maya* from Teranci, MT) extracts: A acetone extract, and B methanol extract, detected at 260 nm and 280 nm. Peak numbers correspond to numbers in Tables 1–5 (missing numbers are for compounds identified in other samples)

were attributed to pelargonidin derivatives (Table 1). The peak **16** was confirmed as pelargonidin-3-*O*-diglucoside with *m/z* = 595 and MS^2 ion at 271 due to a loss of 324 amu corresponding to two glucose moieties. The fact that the ion at 271 is the most abundant one in MS^2 indicates that the two glucose moieties are linked to each other i.e. it is a diglucoside derivative of pelargonidin. Peak **38** with $[M]^+$ at *m/z* 433 and a fragment at 271 obtained after loss of 162 amu (hexose moiety) was identified as pelargonidin-3-*O*-glucoside, the major anthocyanin in strawberries. Peak **40** at *m/z* 579 and MS^2 fragments at 433 and 271 due to loss of rhamnose (–146 amu) and rutinose (–308 amu), respectively, was identified as due to pelargonidin-3-*O*-rutinoside. Peak **44** was attributed to pelargonidin-3-*O*-malonylglucoside, by HPLC-

DAD and HPLC-MS (*m*/z 519, MS^2 fragments at 433 and 271, corresponding to a first loss of 86 amu: malonyl moiety, and then 162 amu: hexose moiety). The latter three types of conjugated forms of pelargonidin were found in all sixteen studied strawberries samples. Peak **49** was identified as pelargonidin-3-*O*-acetylglucoside (*m/z* = 475, MS^2 = 271, loss of 204: acetyl-glucose moiety). Peak **55** with *m/z* 503 and MS^2 = 271 after loss of 232 amu, which can be due to either malonyldeoxyhexose or succinylpentose suggesting the presence of either pelargonidin-3-succinylarabinoside or pelargonidin-3-malonylrhamnoside, as indicated in a recent publication [23].

Finally, peak **43** with absorption maximum at a lower wavelength, 492 nm, and $[M]^+$ at *m*/z 501, with MS^2 ion at *m/z* 339 (loss of hexose moiety) and MS^3 ion at *m/z* 295 (loss of CO_2), was assigned as the pyrano form of pelargonidin-3-*O*-glucoside, isolated from strawberries [11]. The extraction solvent in our study was acetone, which has been shown to react with anthocyanins to produce pyranoanthocyanins [24].

The MS/MS analyses of anthocyanins containing cyanidin aglycone would give a characteristic fragment at *m*/z 287. The peaks giving this fragment in our study were **35** and **42** (Table 1). Both peaks exhibited absorption maxima at 518 nm, and peak **35** had molecular ion at *m/z* 449, with MS^2 fragment at *m/z* 287 (loss of hexose moiety), and was identified as cyanidin-3-*O*-glucoside. This conjugated form of cyanidin was found in all cultivated strawberries. Peak **42** was identified as cyanidin-3-*O*-malonylglucoside (*m/z* = 535, MS^2 = 449, 287, first loss of 86 amu, and then 162 amu, corresponding to malonyl and glucose moiety, respectively).

T a b l e 1

Identification of anthocyanins in strawberry fruits by HPLC–DAD, LC–MS, LC–MS/MS and LC–MS/MS/MS data

Peak	t_R (min)	λ_{max} [a] (nm)	MW	MS (*m/z*)	MS^2 ions (*m/z*)[b]	MS^3 ions (*m/z*)[c]	Compounds	Species[d]
16	18.5	504, 430 sh, 330 sh, 278	595	**595** $[M]^+$	433, **271**		pelargonidin-3-*O*-diglucoside	MT, MZ, MS, MC, AC, AL, AS, RZ, RT, RL, RS, TS
35	24.6	518, 282	449	**449** $[M]^+$	**287**		cyanidin-3-*O*-glucoside	all
38	26.3	504, 430 sh, 330 sh, 278	433	**433** $[M]^+$	**271**		pelargonidin-3-*O*-glucoside	all
40	27.1	504, 428 sh, 332 sh, 278	579	**579** $[M]^+$	433, **271**		pelargonidin-3-*O*-rutinoside	all
42	29.3	518, 282	535	**535** $[M]^+$	449, **287**		cyanidin-3-*O*-malonylglucoside	MT, MS, ML, AZ, AT, AC, AL, AS, RT, RL, TS
43	29.9	492, 358, 282	501	**501** $[M]^+$	**339**, 295	**295**	5- pyranopelargonidin-3-*O*-glucoside	MT, AZ, AT, AC, AS, RZ, RT
44	30.4	504, 426 sh, 332 sh, 270 sh	519	**519** $[M]^+$	433, **271**		pelargonidin-3-*O*-malonylglucoside	all
49	33.1	506, 430 sh, 332 sh, 278	475	**475** $[M]^+$	**271**		pelargonidin-3-*O*-acetylglucoside	ML, MC, AZ, AT, AC, AL, AS, RZ, RT, RC, RS, TS
55	36.8	508, 432 sh, 334 sh, 280	503	**503** $[M]^+$	**271**		pelargonidin-succinyl-arabinoside or pelargonidin-malonylrhamnoside	MC, AZ, AT, AC, AS, RZ, RT, RC

[a] sh, shoulder in the spectrum; [b,c] the most abundant ions are shown in bold;
[d] MT, Maya from Teranci; MZ, Maya from Zrnovci; MC, Maya from Češinovo; ML, Maya from Dolno Lisiče; MS, Maya from Smolari; AT, Alba from Teranci; AZ, Alba from Zrnovci; AC, Alba from Češinovo; AL, Alba from Dolno Lisiče; AS, Alba from Smolari; RZ, Roxana from Zrnovci; RT, Roxana from Teranci; RC, Roxana from Češinovo; RS, Roxana from Smolari; RL, Roxana from Dolno Lisiče; TS, Tudla from Smolari.

3.2. *Identification of flavonols*

Quercetin and kaempferol derivatives are the major flavonols found in strawberries [18–21, 29]. They exhibit UV-Vis absorption maxima at about 356 nm for quercetin and 348 nm for kaempferol derivatives (Table 2). Peaks **45** and **48** had max absorption at 356 nm and a characteristic MS^2 fragment at *m*/z 301 in negative mode, which together with the MS^3 specific fragments at *m/z* 179 and 151 confirms the identity of quercetin. Peak **45** was identified as quercetin-3-*O*-glucuronide with *m*/z 477 and MS^2 fragment 301 after elimination of a glucurone unit (176 amu), which has previously been reported as major flavonol in strawberries [20, 25]. Peak **48** with *m*/*z* 463 giving the 301 fragment, i.e. quercetin after losing a hexose unit (162 amu) was identified as quercetin-3-*O*-glucoside.

Five peaks were identified as kaempferol derivatives according to their UV spectra and MS fragmentation leading to the kaempferol aglycone at *m/z* 285 in negative mode. Peak **51**, was identified as kaempferol-3-*O*-glucuronide with *m/z* 461 and MS^2 fragment at 285 obtained after loss of 176 amu (glucurone unit). Peak **53** had $[M–H]^–$ at *m/z* 447 with fragment at *m/z* 285 (loss 162 amu: hexose moiety), and was identified as kaempferol-3-*O*-glucoside. Peak **54** had $[M–H]^-$ ion at *m/z* 533 in the negative mode, which is consistent with a kaempferol-3-*O*-malonylglucoside. The molecular ion fragmentation yielded fragment ions corresponding to kaempferol glucoside (*m/z* 447) after loss of the malonyl moiety (– 86 amu), and finally to kaempferol after losing a hexose moiety (–162 amu). Peak **56** had $[M–H]^–$ at *m/z* 489 with main fragment at *m/z* 285 (after loss 204 amu: acetylhexose) and was identified as kaempferol-3-*O*-acetylglucoside.

Peak **52** was also identified as kaempferol derivative according to the mass spectrum with a distinctive fragment at *m/z* 285 even though it had a maximum absorption at shorter wavelength (314 nm), which on the other hand indicated acylation of the sugar moiety on this flavonol with hydroxycinnamic acid [26]. MS in negative mode gave the base peak at *m/z* 593 and MS^2 main fragment at *m/z* 285 (loss 308: coumaroylglucoside moiety) corresponding to kaempferol-3-*O*-coumaroylglucoside, which has previously been reported in strawberries [21].

T a b l e 2

Identification of flavonols in strawberry fruits by HPLC–DAD, LC–MS, LC–MS/MS and LC–MS/MS/MS data

Peak	t_R (min)	λ_{max}[a] (nm)	MW	MS (*m/z*)	MS^2 ions (*m/z*)[b]	MS^3 ions (*m/z*)[c]	Compounds	Species[d]
45	32.2	356, 300 sh, 256	478	**477** $[M–H]^–$	**301**	**179**, 151	quercetin-3-*O*-glucuronide	all
48	32.9	356, 290 sh, 256	464	**463** $[M–H]^–$	**301**	**179**, 151	quercetin-3-*O*-glucoside	AT, AL, AS, RL, RS, TS
51	34.9	348, 266	462	**461** $[M–H]^–$	**285**		kaempferol-3-*O*-glucuronide	MS, MC, AZ, AT, AL, AS, RL, RS, TS
52	35.1	314, 266	594	**593** $[M–H]^–$	**285**		kaempferol-3-*O*-coumaroylglucoside	AZ, AT, AC, AS, RZ, RT, RC, TS
53	35.2	348, 290 sh, 266	448	**447** $[M–H]^–$	**285**		kaempferol-3-*O*-glucoside	MC, ML, MS, MZ, MT, AT, AC, AL, AS, RZ, RT, RC, RS
54	36.0	348, 290 sh, 266, 234	534	**533** $[M–H]^–$	**447**, 285	**285**	kaempferol-3-*O*-malonylglucoside	MT, MZ, MS, ML, AZ, RL, TS
56	37.5	352, 266	490	**489** $[M–H]^–$	**285**		kaempferol-3-*O*-acetylglucoside	AL, AS, RZ, RT, RC

[a,b,c,d] as in Table 1

3.3. *Identification of flavan-3-ols and proanthocyanidins*

LC-DAD, LC-MS and MS-MS data (retention and spectral data given in Table 3 were used for identification of the (+)catechin (peak **23**), B type proanthocyanidin dimers (peaks **11, 13, 25, 26, 27, 32**), B type proanthocyanidin trimers (peaks **1, 2, 3, 12, 15, 17, 22, 28, 37**) and B type proanthocyanidins tetramers (peaks **8, 9, 10, 14, 18, 21, 24**). These compounds have been described in several previous studies [20–23, 30–33].

The mass spectrum in full scan mode showed the deprotonated molecule $[M - H]^-$ of catechin at *m/z* 289 (peak **23**) with the characteristic MS^2 ions at *m/z* 245, 205 and UV maximum at 278 nm.

Peaks **11, 13, 26, 27** and **32** had $[M - H]^-$ at *m/z* 577 and main fragmentation with loss of 152 amu (characteristic fragmentation pathway by retro Diels-Alder reaction) [27] were recognized as proanthocyanidin dimers of the type (epi) catechin-(epi)catechin. On the other hand, peak **25** had $[M - H]^-$ at *m/z* 561 and MS^2 ions at *m/z* 543, 435 (loss of 126 amu indicating 1,3,5-trihydroxybenzene structure in ring A of the extension unit) and 289 [loss of (epi)afzelechin, 272 amu]. The sequence in this dimer was identified as (epi) afzelechin-(epi)catechin. The fragmentation pathway of this dimer detected in strawberries was also described in a previous study [28].

Peaks **2**, **3**, **22**, **37**, had $[M - H]^-$ at *m/z* 849, characteristic MS^2 ions at *m/z* 801, 697, 577 (base peak obtained after loss of 272 amu), 559, 425, 407 and 287, and MS^3 spectra of the base peak at *m/z* 577 gave ions at 425, 407 and 289 (losing (epi)catechin, 288 amu) corresponding to B type proanthocyanidin trimer of the type (epi)afzelechin-(epi)catechin-(epi)catechin [28].

Peaks **1**, **12**, **15**, **17**, **28** were identified as B type proanthocyanidin trimers, due to pseudomolecular $[M - H]^-$ ion at *m/z* 865, which yielded MS^2 ions at *m/z* 739, 695, 577, 425 and 287 and MS^3 spectra of the ion at *m/z* 695 gave ions at *m/z* 525, 407 and 289. The sequence in this trimer was identified as (epi)catechin-(epi) catechin-(epi)catechin since the consecutive losses of 288 amu were detected.

Peaks **8**, **9**, **10**, **14** and **18**, had $[M - H]^-$ ion at *m/z* 1153 and were tentatively identified as isomers of a B type proanthocyanidin tetramer with characteristic MS^2 ions at *m/z* 983, 865, 695 and 577 [32] and composed of 4 (epi) catechin units.

Peaks **21** had $[M - H]^-$ ion at *m/z* 1137 with MS^2 fragmentation at *m/z* 847, 577 and 407 was identified as proanthocyanidin tetramer composed of 3 (epi)catechin and one (epi) afzelechin unit, which has been described in a previous study [28].

Peak **24** with $[M - H]^-$ ion at *m/z* 1121 yielded MS^2 ions at *m/z* 849, 831, 577 and 407, and was also attributed to a proanthocyanidin tetramer, but composed of 2 (epi)catechin and 2 (epi)afzelechin units. The exact sequence of flavonol units, however, is not possible to be elucidated using mass spectrometry [28]. NMR can give the sequence, the regiochemistry and stereochemistry of the inter flavanic bond but the structure elucidation is very complex after the trimer [30].

No free epicatechin and epiafzelechin were found in the samples in spite of the fact that epicatechin was the major unit in the proanthocyanidins, which is also found in the work of Seeram [21].

3.4. *Identification of hydroxybenzoic and hydroxycinnamic acid derivatives*

The common hydroxycinnamic acids found in cultivated strawberries are *p*-coumaric, ferulic and caffeic acid, which are found in their conjugated forms usually linked to sugars [10, 18–21]. *p*-Coumaric acid has maximum absorption at 311 nm, caffeic and ferulic acid have maximum absorption at 326 nm. Esterification with sugars causes a bathochromic shift, whereas glycosylation of the hydroxyl group in *p*-coumaric acid causes a hypsochromic shift [10].

Similar consideration applies to hydroxybenzoic acid and its derivatives. Bathochromic shift was observed for hydroxybenzoic acid derivatives from 256 nm to 262 nm.

T a b l e 3 *Identification of flavan-3-ols in strawberry fruits by HPLC–DAD, LC–MS, LC–MS/MS and LC–MS/MS/MS data*

Peak	t_R (min)	λ_{max} [a] (nm)	MW	MS (*m/z*)	MS^2 ions (*m/z*)[b]	MS^3 ions (*m/z*)[c]	Compounds	Species[d]
23	20.1	278, 234	290	289 [M – H]$^-$	**245**, 205	**203**, 187	catechin	all
Proanthocyanidin dimers								
11	17.2	278, 234	578	577 [M – H]$^-$	**425**, 407, 289	**285**	proanthocyanidin dimer	MT, MZ,RZ, RT, RC, TS
13	18.0	278, 234	578	577 [M – H]$^-$	425, 407, **289**	**285**	proanthocyanidin dimer	MT, MZ, MS, ML, MC, AS, RZ, RT, RL, RS
25	20.7	234, 278	562	561 [M – H]$^-$	543, 435, **289**	**245**, 205	proanthocyanidin dimer	MZ, MT, ML, MS, MC, RC, RZ, RT, RL, RS, AZ, AT, AL, AS, TS,
26	21.0	278, 234	578	577 [M – H]$^-$	**425**, 407, 289	**285**	proanthocyanidin dimer	MT, MZ, AT, AL, AS, RC
27	21.2	278, 234	578	577 [M – H]$^-$	**425**, 407, 289	**285**	proanthocyanidin dimer	ML, AZ, RL, TS
32	23.0	278, 234	578	577 [M – H]$^-$	**425**, 407, 289	**285**	proanthocyanidin dimer	MT, MZ, RS
Proanthocyanidin trimers								
1	9.8	278, 234	866	865 [M – H]$^-$	739, **695**, 577, 425, 287	**525**, 407, 289	proanthocyanidin trimer	MT, MZ, MS, ML, MC, AL, RZ, RT, RC, TS
2	13.1	278, 234	850	849 [M – H]$^-$	801, 697, **577**, 559, 425, 407, 287	425, **407**, 289	proanthocyanidin trimer	MT, AL, AS,
3	13.3	278, 234	850	849 [M – H]$^-$	801, 697, **577**, 559, 425, 407, 287	425, **407**, 289	proanthocyanidin trimer	MT
12	17.6	278, 234	866	865 [M – H]$^-$	739, **695**, 577, 425, 287	**525**, 407, 289	proanthocyanidin trimer	MT, MZ, AZ, AT,RZ, RT, RC, TS
15	18.3	278, 234	866	865 [M – H]$^-$	739, **695**, 577, 425, 287	**525**, 407, 289	proanthocyanidin trimer	MC, MS, MZ, MT, ML, AZ, AT, AL, AS, RZ, RT, RC, RL, RS, TS
17	18.6	278, 234	866	865 [M – H]$^-$	739, **695**, 577, 425, 287	**525**, 407, 289	proanthocyanidin trimer	MT, MZ, MC
22	19.9	278, 234	850	849 [M – H]$^-$	801, 697, **577**, 559, 425, 407, 287	425, **407**, 289	proanthocyanidin trimer	MT, MZ, MS, ML, MC, AZ, AL, AS, RZ, RT, RL, RS, TS
28	21.6	278, 234	866	865 [M – H]$^-$	739, **695**, 577, 425, 287	**525**, 407, 289	proanthocyanidin trimer	MT, MZ, MS, RC, RL
37	25.3	278, 234	850	849 [M – H]$^-$	801, 697, **577**, 559, 425, 407, 287	425, **407**, 289	proanthocyanidin trimer	MT, AS
Proanthocyanidin tetramers								
8	16.3	278, 234	1154	1153 [M – H]$^-$	983, **865**, 695, 577	**695**, 575, 525, 407, 287	proanthocyanidin tetramer	TS
9	16.6	278, 234	1154	1153 [M – H]$^-$	**865**, 577, 451, 407	575, 451, 407, 287	proanthocyanidin tetramer	MT, RL, RS
10	17.0	278, 234	1154	1153 [M – H]$^-$	**865**, 577, 451, 407	575, 451, 407, 287	proanthocyanidin tetramer	MT, MZ, AZ, AT,RZ, RT, RC, TS
14	18.2	278, 234	1154	1153 [M – H]$^-$	983, **865**, 577, 407	695, 575, 451	proanthocyanidin tetramer	AS, MT, TS, AZ, MC, AT, RS, AL, RZ, MZ
18	18.7	278, 234	1154	1153 [M – H]$^-$	983, **865**, 577, 407	577, 287	proanthocyanidin tetramer	RC, MT
21	19.2	278, 234	1138	1137 [M – H]$^-$	**847**, 577, 407	425, **407**, 289	proanthocyanidin tetramer	AT, RL, MZ,
24	20.4	278, 234	1122	1121 [M – H]$^-$	849, 831, **577**, 407		proanthocyanidin tetramer	MS, MZ, TS, AS, AT

Table 4

Identification of hydroxycinnamic acid and hydroxybenzoic acid derivatives in strawberry fruits by HPLC–DAD, LC–MS, LC–MS/MS and LC–MS/MS/MS data

Peak	t_R (min)	λ_{max} [a] (nm)	MW	MS (*m/z*)	MS^2 ions (*m/z*)[b]	MS^3 ions (*m/z*)[c]	Compounds	Species[d]
6	15.9	262	300	**299** $[M-H]^-$	239, 179, **137**		hydroxybenzoylhexsose	MT,MZ, AZ, RL, TS
19	18.9	328, 302 sh, 248	342	**341** $[M-H]^-$	**179**, 161	**135**	caffeoylhexose	MC, AZ, AC
30	22.1	316, 236	326	**325** $[M-H]^-$	265, 187, 163, **145**		*p*-coumaroylhexose	all
33	23.5	316, 236	326	**325** $[M-H]^-$	265, 187, 163, **145**		*p*-coumaroylhexose	all
39	26.7	448, 352 sh, 312 sh, 248 sh	450	**449** $[M-H]^-$	431, **355**, 329, 287, 269, 193	**193**	ferulic acid hexose derivative	all

[a,b,c,d] as in Table 1

Peak **6** (Table 4) was identified as hydroxybenzoylhexose with MS^2 ions at *m/z* 239, 179 and 137 (loss of hexose), as reported in literature [18].

In the negative LC-MS mode, caffeoylhexose (peak **19**) and isomeric forms of *p*-coumaroylhexose (peaks **30** and **33**) exhibited deprotonated ions at *m/z* 341 and 325, respectively. Peak **19** had main MS^2 fragment ion at *m/z* 179 due to loss 162 amu (hexose moiety). Peaks **30** and **33**, had $[M-H]^-$ at *m/z* 325 and λ_{max} at 316 nm. These two *p*-coumaroylhexose isomers produced MS^2 ions at *m/z* 265, 187, 163 and 145 (base peak) by losing hexose and they were found in all samples.

Peak **39**, was identified as ferulic acid hexose derivative with $[M-H]^-$ at *m/z* 449 giving MS^2 ions at *m/z* 431, 355, 329, 287, 269 and 193, (base peak 355) and MS^3 spectra of the base ion (*m/z* 355) gave ions at *m/z* 193 (by loss of hexose unit). These fragmentation ions are in agreement with the previously published data [18]. This compound was also detected in all sixteen samples of cultivated strawberries. Free caffeic, ferulic *p*-coumaric acid were not etected in our samples.

3.5. Identification of free and conjugated forms of ellagic acid

The free ellagic acid in the samples was confirmed by its retention time, MS data and MS/MS fragmentation peak 50, peaks **41**, **46** and **47** were distinguished as ellagic acid derivatives and peaks **4**, **5**, **7**, **20**, **29**, **31**, **34** and **36** were identified as ellagitannins on the basis of almost identical UV-Vis spectra (Table 5) as reported in literature [20].

Peak **50** had $[M-H]^-$ at *m/z* 301 and MS^2 fragmentation ions in negative mode at *m/z* 257, 229 and 185, typical fragments of ellagic acid [12]. UV-Vis spectra of the peaks **41**, **46** and **47** suggested glycosylated forms of ellagic acid [33]. On the basis of LC-MS, MS/MS and MS/MS/MS data, peaks **41**, **46** and **47** were identified as glycosylated forms of ellagic acid. Peaks **46** and **47** had $[M-H]^-$ at *m/z* 447 and MS^2 products for both ions were at *m/z* 301, with MS^3 fragmentation at *m/z* 257, 229 and 185, corresponding to ellagic acid. The loss of 146 amu could be attributed to a deoxyhexoside unit. Previously, ellagic acid deoxyhexoside have been identified in strawberries [18]. Peak **41** with $[M-H]^-$ at *m/z* 463 with fragmentation pattern of *m/z* 301 (loss of a hexose) was tentatively identified as ellagic acid hexoside. It is interesting to point out here that ellagic acid hexose has previously not been reported in strawberry fruits, but there are reports for it in

strawberry flowers [34] and in blackberry fruits as ellagic acid glucoside [35].

Ellagitannins are hydrolysable tannins since they are esters of hexahydroxydiphenic acid (HHDP: 6,6′-dicarbonyl-2,2′,3,3′,4,4′-hexahydroxybiphenyl moiety) and a polyol, usually glucose, and in some cases gallic acid [2, 9]. High resolution "zoom scan" analyses were performed for determination of the charge state of some ellagitannins.

Peaks **4** and **5** with $[M - H]^-$ at *m/z* 783 and MS^2 fragments: 481 (loss of HHDP, 302 amu), and 301 (loss of HHDP-glucose, 482 amu) as a base peak, were identified as bis-HHDP-glucose, previously found in strawberries [18, 21]. Peaks **7** and **20** had same $[M - H]^-$ at *m/z* 633 but slightly different fragmentation ions. Peak **7** produced MS^2 fragments at *m/z* 481 by loss of galloyl unit, (152 amu) and at *m/z* 301 as main fragment (loss of galloylglucose, 332 amu). Unlike, peak **20** had MS^2 fragments at *m/z* 463 (loss of gallic acid, 170 amu) and 301 (again as the main fragment) after loss of galloylglucose (332 amu). These peaks were identified as HHDP-galloyl-glucose isomers differing in the position of the galloyl unit on glucose [18].

Peaks **29**, **31** and **36** were identified as galloyl-bis-HHDP-glucose with $[M - H]^-$ at *m/z* 935 and MS^2 fragment ions at *m/z* 633 (loss of HHDP, 302 amu) and 301 (loss of galloylglucose, 332 amu). Galloyl-bis-HHDP-glucose (Figure 1) is a basic unit of many ellagitannins, for example, sanguiin H-6 and lambertianin C, containing 2 and 3 units, respectively [20]. Peak **29** was found in all strawberries extracts.

Peak **34** had $[M - 2H]^{2-}$ at *m/z* 934 as a main peak, which was shown by zoom scan analysis to be a double-charged implying a true mass of 1870 and $[M - H]^-$ at *m/z* 1869 in negative mode. The double-charged ion was identified according to the spacing between the main peaks equal to 0.5 (at *m/z* 934, 934.5 and 935), which demonstrates the doubly-charged state of ions [12]. Fragmentation of double-charged ions gave single-charged products at *m/z* 1567, 1265, 1085, 897, 783, 633, 451 and 301. This peak was identified as sanguiin H-6, by comparison of its LC-MS/MS data with literature reports [21] and it was detected in all studied strawberries samples.

This study represents the first chemical investigation of cultivated strawberries from R. Macedonia and demonstrates the presence of a variety of anthocyanins, flavonols, flavan-3-ols, proanthocyanidins, conjugated forms of hydroxycinnamic acids, free and conjugated forms of ellagic acid and ellagitannins separated and identified by HPLC-DAD-MS^n in the acetone extracts. For that purpose, HPLC gradient elution profile has bee n optimized, which coupled to diode array and mass detection, enabled identification of 56 phenolic compounds in cultivated strawberries extracts.

Anthocyanins (especially pelargonidin-3-*O*-glucoside) and ellagitannins were the major compounds, which is typical for strawberries. *p*-Coumaroylhexose was the predominant hydroxycinnamic acid derivative. Oligomers of (epi)catechin and (epi)afzelechin have also been identified, the latter one being more specific since it is not commonly detected in strawberries. Catechin, galloyl-bis-HHDP-glucose, sanguiin H-6, *p*-coumaroylhexose, ferulic acid hexose derivative, quercetin-3-*O*-glucuronide, cyanidin-3-glucoside, pelargonidin-3-glucoside, pelargonidin-3-rutinoside and pelargonidin-3-malonylglucoside were identified in all sixteen acetone extracts.

The identification of phenolic compounds revealed some interesting differences in the analyzed samples in correlation to the variety and place of growing. Higher variety of the phenolic compounds was identified in the varieties *Tudla* and *Roxana*, whereas significantly lower was found in the varieties *Maya* and *Alba*.

Table 5

Identification of free and conjugated forms of ellagic acid in strawberry fruits by HPLC–DAD, LC–MS, LC–MS/MS and LC–MS/MS/MS data

Peak	t_R (min)	λ_{max} [a] (nm)	MW	MS (*m/z*)	MS^2 ions (*m/z*)[b]	MS^3 ions (*m/z*)[c]	Compounds	Species[d]
4	13.9	234	784	**783** $[M-H]^-$	481, **301**	**257**, 229, 185	bis-HHDP- glucose	MT, MC, AZ, RL, TS
5	15.2	234	784	**783** $[M-H]^-$	481, **301**	**257**, 229, 185	bis-HHDP- glucose	MT, MC, AZ, RL, TS
7	16.0	286	634	**633** $[M-H]^-$	481, **301**	**257**, 229, 185	HHDP-galloyl-glucose	RL, TS
20	19.0	286	634	**633** $[M-H]^-$	463, **301**	**257**, 229, 185	HHDP-galloyl-glucose	AZ, AT, AL, AS, AR, RT, RC, RL, RS, TS
29	21.9	236, 256 sh	936	**935** $[M-H]^-$	633, **301**	**301**	galloyl-bis-HHDP-glucose	all
31	22.5	256 sh, 236	936	**935** $[M-H]^-$	633, **301**	**301**	galloyl-bis-HHDP-glucose	MT, MZ, MS, MC, AS
34	24.1	266 sh, 234	1870	**934** $[M-2H]^{2-}$	1567, 1265, 1085, 897, 783, 633, 451, **301**	**257**, 229, 185	dimer of galloyl-bis-HHDP-glucose (sanguiin H-6)	all
36	24.9	256 sh, 236,	936	**935** $[M-H]^-$	**633**, 301	**301**	galloyl-bis-HHDP-glucose	MT, AL, RL, RS
41	28.6	368, 252	464	**463** $[M-H]^-$	**301**	**257**, 229, 185	ellagic acid hexoside	MT, MS, AT, AL, AS, RL, RS
46	32.4	372, 250	448	**447** $[M-H]^-$	**301**	**257**, 229, 185	ellagic acid deoxyhexoside	MT, MZ, ML, RZ, RT, RC, RL, RS, TS
47	32.7	372, 250	448	**447** $[M-H]^-$	**301**	**257**, 229, 185	ellagic acid deoxyhexoside	MT, MS, ML, MC, AZ, AL, RL
50	33.5	366, 254	302	**301** $[M-H]^-$	**257**, 229, 185		ellagic acid	MT, MZ, MS, ML, AZ, AT, AL, AS, RZ, RT, RC, RS, TS

[a,b,c,d] as in Table 1

As for the locality of growing, the most diverse group of phenolic compounds were identified in the samples cultivated in Smolari (south part of R. Macedonia), and the least was found in the samples from Dolno Lisiče (north part of R. Macedonia), which emphasizes the role of the local climate conditions apart from the genetic ones characteristic for the variety.

The high diversity of the phenolic compounds found in the studied strawberries samples implies their potential beneficial effects for human health and further studies should be carried out for quantification of all these compounds, measurement of their antioxidant potential and finally study the evolution of the phenolic profile during developing and ripening of the strawberries in order to establish most suitable conditions for obtaining fruits with optimal phenolic composition.

Acknowledgement: The work performed within this study was supported by the capacities project CHROMLAB-ANTIOXIDANT (GA 204756) financed under the Research Potential of the 7th Framework Program of the European Commission.

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