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# Genistein isoflavone glycoconjugates in sour cherry cultivars (*Prunus cerasus* L.)

László Abrankó\*<sup>a</sup>, Ádám Nagy<sup>a</sup>, Blanka Szilvássy<sup>a</sup>, Éva Stefanovits-Bányai<sup>a</sup>, Attila  
Hegedűs<sup>b</sup>

<sup>a</sup>*Department of Applied Chemistry, Faculty of Food Science, Corvinus University of  
Budapest, 29-33 Villányi, 1118 Budapest, Hungary*

<sup>b</sup>*Department of Genetics and Plant Breeding, Faculty of Horticultural Science, Corvinus  
University of Budapest, Ménesi út 44., 1118 Budapest, Hungary*

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\*Corresponding author

Tel: +36 1482 6163

Fax: +36 1466 4272

e-mail: [laszlo.abranko@uni-corvinus.hu](mailto:laszlo.abranko@uni-corvinus.hu)

23 **Abstract**

24 **Although the isoflavone genistein has well-established health-beneficial effects, it is not a**  
25 **major component of Western diet, since soy consumption, the main dietary source of**  
26 **genistein, is low in these populations.** Genistein compounds were studied in twelve  
27 commercial sour cherry (*Prunus cerasus* L.) cultivars grown in Hungary. High performance  
28 liquid chromatography coupled to quadrupole/time-of-flight mass spectrometry, equipped  
29 with electrospray ion source (HPLC-ESI-qTOFMS) was used for screening and confirmatory  
30 analyses. Genistin and genistein were found in some Hungarian native sour cherry cultivars  
31 including ‘Pipacs1’, ‘Kántorjánosi’, ‘Debreceni bőtermő’ and ‘Éva’. Genistein content in  
32 fruits of the latter three cultivars ranged between 0.4 to 0.6 mg, while in ‘Pipacs1’ a total of  
33 4.4 mg genistein compounds (expressed as aglycone equivalents per 100 g of fresh fruit) was  
34 determined. These cultivars may play an important role as complementary genistein sources  
35 in the Western diet. Especially ‘Pipacs 1’, may be best utilized in functional food products.

36

37 **Keywords:** *Prunus cerasus* L., sour cherry, genistin, genistein, isoflavone, qTOFMS

38

## 39 **1. Introduction**

40 Polyphenols are plant secondary metabolites, which have drawn interest in food science, since  
41 an impressive list of health benefits was associated with these compounds when they  
42 consumed in various forms of polyphenol-rich plant foods (Crozier, Jaganath, & Clifford,  
43 2009; Del Rio, Rodriguez-Mateos, Spencer, Tognolini, Borges, & Crozier, 2013). Genistein –  
44 a polyphenol compound belonging to the subclass of isoflavones – also showed various  
45 health-beneficial effects in numerous experiments due to its multiple mechanisms of action.  
46 For instance, genistein can improve lipid profile and lower blood pressure and hence exert  
47 cardiovascular protection (Schwab, Stein, Scheler, & Theuring, 2012). Genistein was also  
48 proved to be a promising therapeutic agent at least for ameliorating diabetes and obesity states  
49 (Behloul & Wu, 2013). However, most studies on the health beneficial effects of genistein are  
50 focusing on its cancer preventive properties. It has been shown that genistein can induce  
51 apoptosis in haematological tumour cells through multiple mechanisms, while protecting  
52 normal cells from toxicity. Genistein is also a potent growth inhibitor of breast, prostate,  
53 pancreatic, melanoma, and kidney cancer cells *in vitro* (Li, Frame, Hirsch, & Cobos, 2010). In  
54 a recent study, daidzein and genistein were effectively induced apoptosis in HT-29 colon  
55 cancer cells (G. N. Kim, Song, Kim, Choi, & Jang, 2012).

56 In substantial amounts, genistein has been found almost exclusively in leguminous plants so  
57 far with the highest concentration occurring in soybean (*Glycine max*). Therefore, soybean is  
58 considered as the main dietary source of genistein (Liggins, Bluck, Runswick, Atkinson,  
59 Coward, & Bingham, 2000a, 2000b). Concentrations of genistein in soybean are inherently  
60 heterogeneous depending on type, climate, crop year, and location of the cultivation plot  
61 (Chan, Murphy, Ho, Kreiger, Darlington, So, et al., 2009). According to a comprehensive  
62 database for the isoflavone content of selected foods, published by the US Department of  
63 Agriculture, genistein varied in the range of 5.6-276 mg/100 g in raw mature soybeans

64 (Bhagwat, Haytowitz, & Holden, 2008). Soy is still a staple food in Asia, but it is a relative  
65 newcomer at the dinner table in other parts of the world, which means that human exposure to  
66 genistein varies widely because of cultural differences in diet (Li, Frame, Hirsch, & Cobos,  
67 2010). This might explain the conclusion achieved in a number of human studies i.e. risk of  
68 the cancers where genistein proved to have preventive actions is lower in Japan and China  
69 than in the US and Europe (Kurahashi, Iwasaki, Inoue, Sasazuki, & Tsugane, 2008; Lampe,  
70 Nishino, Ray, Wu, Li, Lin, et al., 2007; Yang, Shu, Chow, Zhang, Li, Ji, et al., 2012)

71 In contrast to its well-established health beneficial effects, the genistein isoflavone is not a  
72 major component of the Western diet, since soy food intake in these populations is typically  
73 low (Crozier, Del Rio, & Clifford, 2010; Crozier, Jaganath, & Clifford, 2009; Lampe, et al.,  
74 2007). However, in addition to soybean, genistein is also present in small quantities in a  
75 number of edible plants. According to the studies of Liggins *et al.* only legumes contained 0.2  
76 - 0.6 mg genistein and daidzein per 100 g of wet weight of food among foods commonly  
77 eaten in Europe (Liggins, Bluck, Runswick, Atkinson, Coward, & Bingham, 2000b). With  
78 respect to fruits, they found that currants and raisins were the richest sources of the  
79 isoflavones, containing around 0.2 mg of the genistein and daidzein combined per 100 g of  
80 wet weight of food (Liggins, Bluck, Runswick, Atkinson, Coward, & Bingham, 2000a). In  
81 this context peanut should be also mentioned containing genistein and daidzein around 0.03  
82 mg/100 g (Chukwumah, Walker, Vogler, & Verghese, 2012). In a recent study, it was shown  
83 that genistein occurred in groundnut among others in the form of genistein-7-O-  
84 genitiobioside, a glycoconjugate that has not been detected before (Nara, Nihei, Ogasawara,  
85 Koga, & Kato, 2011). Considering the genistein concentrations typical in plant foods, clearly  
86 the inclusion of even a small portion of a soy product in the diet will expose consumers to  
87 very significant concentrations of daidzein and genistein. Nevertheless, fruits, nuts, and

88 vegetables contain a broad range of concentrations of these compounds and will contribute to  
89 the daily dietary intake (Liggins, Bluck, Runswick, Atkinson, Coward, & Bingham, 2000a).

90 Sour or tart cherries (*Prunus cerasus* L.) are commercially important and consumed in a  
91 variety of ways, including fresh, frozen, canned, brined or dried fruits or as juice.  
92 Anthocyanins and other flavonoids, as well as melatonin, in various cultivars were analysed,  
93 and it has been known that sour cherries contain substantial amounts of anthocyanins and  
94 phenolic acids (Ficzek, Vegvari, Sandor, Steger-Mate, Kallay, Szugyi, et al., 2011;  
95 Kirakosyan, Seymour, Llanes, Kaufman, & Bolling, 2009). So far, studies focusing on the  
96 beneficial health-effects of sour cherry consumption are scarce; however, a few interesting  
97 papers are available reporting on the biological effects of sour cherry constituents present in  
98 fruit (Hevesi, Blázovics, Kállay, Végh, Stéger-Máté, Ficzek, et al., 2012; Khoo, Clausen,  
99 Pedersen, & Larsen, 2011; D. O. Kim, Heo, Kim, Yang, & Lee, 2005) and seed kernel (Bak,  
100 Lekli, Juhasz, Varga, Varga, Gesztelyi, et al., 2010). Data on the genistein content of sour  
101 cherries are very limited (Wang, Nair, Strasburg, Booren, & Gray, 1999). Its reason might be  
102 that most cultivars do not contain genistein in detectable amounts; however, natural variation  
103 among sour cherry cultivars might be considerable, as is known to occur in soya (Bhagwat,  
104 Haytowitz, & Holden, 2008; Chan, et al., 2009).

105 In this study, twelve sour cherry (*Prunus cerasus* L.) genotypes (including commercial  
106 cultivars and cultivar candidates) grown in Hungary were screened for genistein compounds  
107 in a non-target manner. High performance liquid chromatography coupled to  
108 quadrupole/time-of-flight mass spectrometry, equipped with electrospray ion source (HPLC-  
109 ESI-qTOFMS) was used for screening and confirmatory analyses of indicated genistein  
110 glycoconjugates. In addition, UV spectra of the indicated compounds were also provided for  
111 confirmatory purposes. The quantitative determination of genistein compounds was also  
112 performed.

113

## 114 **2. Materials and methods**

### 115 *2.1 Plant material*

116 Twelve sour cherry (*Prunus cerasus L.*) genotypes were tested in the present study, most of  
117 which are of Carpathian Basin origin (Hungary or Serbia). Studied genotypes are listed in  
118 **Table 2**. All cultivars were cultivated at the same germplasm collection in the Research and  
119 Extension Centre for Fruit Growing (Újfehértó, Eastern Hungary, 47 ° N latitude, 21° E  
120 longitude and 122 m altitude). Fruits were harvested in June-July 2009 and 2010 at  
121 consumption maturity stage.

122

### 123 *2.2. Chemicals and standards*

124 Acetonitrile and methanol (Prolabo HiPerSolv) used were super gradient grade. Formic acid  
125 (~98% for mass spectrometry) was obtained from Fluka. Crystalline reference substances of  
126 genistein aglycone and genistein-7-O- $\beta$ -D-glucoside (genistin) and daidzein were obtained  
127 from Extrasynthese (Genay, France). A Milli-Q ultrapure water system was used throughout  
128 the study to obtain high purity water.

129

### 130 *2.3. Sample preparation*

131 Sour cherry fruits were halved and pitted before lyophilisation. Lyophilized samples were  
132 pulverized and an amount of 200 mg was extracted for 40 min with 10 ml 60/39/1  
133 methanol/water/formic acid solution using an ultrasonic bath. Extracts were centrifuged and 4  
134 ml supernatant was evaporated approximately to 0.5-0.7 ml in a vacuum centrifuge.  
135 Afterwards 100  $\mu$ l acetonitrile 10  $\mu$ l 1:1 diluted formic acid was added and the samples were  
136 reconstituted to 1 ml with water and were thoroughly vortexed. For the standard addition  
137 calibration, aqueous methanolic (80/20) standard solutions of genistein aglycone and genistin

138 were added to aliquots of the reconstituted samples. Daidzein aglycone was added to all  
139 samples as internal standard. Spiked aliquots were further diluted with water (1:10 or 1:20  
140 depending on the sample) for quantitative MS measurements. All samples were filtered  
141 through a 0.45- $\mu$ m PTFE syringe filter before injecting to the HPLC.

142

#### 143 *2.4 Chromatographic separation*

144 Chromatographic separation was carried out on a Phenomenex Kinetex C18, 4.6 $\times$ 150 mm, 2.6  
145  $\mu$ m column using an Agilent 1200 series HPLC system. For the elution, 0.5% (v/v) formic  
146 acid in water (mobile phase A) and 0.5% (v/v) formic acid in acetonitrile (mobile phase B)  
147 were used as solvents at a flow rate of 500  $\mu$ l/min. The gradient program started at 8% B, and  
148 after 5 min of isocratic run, solvent B was increased linearly and reached 45% at 35 min and  
149 then 100% at 40 min. Finally, 100% B was kept constant for 5 min.

150

#### 151 *2.5 Screening of genistein compounds by HPLC-DAD-ESI-qTOFMS*

152 For qualitative (screening and confirmatory) analysis the HPLC system including a diode  
153 array detector (DAD) was coupled to an Agilent 6530 quadrupole – time-of-flight (q-TOF)  
154 hybrid mass spectrometer, equipped with a dual spray ESI source. Positive ion mode was used  
155 in all experiments. The q-TOFMS was used with the following operation parameters:  
156 capillary voltage, 4,000 V; nebulizer pressure, 40 psig; drying gas flow rate, 13 l/min; gas  
157 temperature, 350  $^{\circ}$ C. During these experiments, fragmentor voltage was triggered  
158 automatically between 160 V and 210 V. The lower value is representing mild conditions in  
159 order to minimize in-source fragmentation, while the higher one is to foster in-source  
160 fragmentation. Full-scan mass spectra in the range of  $m/z$  50-1100 were recorded at 1.5  
161 spectra/s scanning speed at all times during the chromatographic run. The instrument  
162 performed the internal mass calibration automatically, using an automated calibrant delivery

163 system, which introduces the flow from the outlet of the chromatograph together with a low  
164 flow (approximately 10  $\mu\text{l}/\text{min}$ ) of a calibrating solution. The solution contains the internal  
165 reference masses of HP-921 [hexakis-(1H,1H,3H-tetrafluoro-pentoxo)-phosphazene] and  
166 purine. Protonated molecules of purine ( $[\text{C}_5\text{H}_4\text{N}_4]^+$  at  $m/z$  121.050873) and HP-0921  
167 ( $[\text{C}_{18}\text{H}_{19}\text{O}_6\text{N}_3\text{P}_3\text{F}_{24}]^+$  at  $m/z$  922.009798) were used as reference masses. The DAD was  
168 acquiring data in the range of 200-800 nm in 2 nm steps at 0.5 spectra/s acquisition speed.

169

## 170 *2.6 Quantitation of genistein compounds by HPLC-DAD-ESI-MS/MS*

171 Quantification of found genistein compounds was carried out using the HPLC system  
172 including the DAD coupled to an Applied Biosystems (Foster City, CA, USA) 3200 Q-Trap  
173 hybrid triple quadrupole/linear ion trap MS/MS instrument equipped with a Turbo-V ESI ion  
174 source that was used in the positive ion mode. Multiple reaction monitoring (MRM) scan  
175 mode was used for mass spectrometric quantification of genistein and genistin using the  
176 standard addition calibration technique. The tentatively identified other genistein-hexoside  
177 compound was quantified based on UV absorbance signal at 260 nm using the calibration  
178 curve obtained for genistin at the same wavelength.

179

## 180 **3. Results and discussion**

### 181 *3.1. HPLC-DAD-ESI-TOFMS profiling of genistein glycoconjugates*

182 Sour cherry extracts were screened for flavonoid glycoconjugates using an HPLC-DAD-ESI-  
183 qTOFMS coupled analytical system. Compounds separated by HPLC were passed through the  
184 on-line coupled diode array detector (DAD) and then entering the ESI-MS system. First, for  
185 general-purpose flavonoid screening the qTOFMS system was used in TOF mode and  
186 quadrupole (q) was set to 'RF only' mode meaning that practically no mass filtering takes  
187 place in the quadrupole. During this preliminary screening step, in-source fragmentation was



188 utilized in order to provide structural information on the compounds. This approach offers the  
189 advantage that on the contrary to real tandem MS experiments, where precursor ion is  
190 selected and then subjected to fragmentation; here fragmentation information is obtained  
191 simultaneously on unlimited numbers of compounds without requiring any preliminary  
192 selection and isolation of the suspected ions.

193 It is typical to flavonoid-O-glycosides that MS fragmentation primarily gives rise to product  
194 ions, which are formed by the cleavage of interglycosidic linkage or the linkage between the  
195 glycan part and the aglycone. Among the formed diagnostic ions, the aglycone fragment  
196 (often referred to as  $Y_0$  fragment) can be used to indicate the presence of a certain type of  
197 flavonoid, since the aglycone fragment will generally be formed from O-glycoside derivatives  
198 of the given aglycone (Abrankó, Garcia-Reyes, & Molina-Diaz, 2012). Results of the  
199 performed preliminary general-purpose flavonoid screening indicated that besides the well-  
200 known flavonoid constituents of sour cherry (e.g. glycoconjugates of anthocyanidins such as  
201 cyanidin, and flavonols including quercetin and kaempferol) some analyzed samples seem to  
202 contain genistein compounds. This rather uncommon observation was further investigated.

203 In Fig. 1, results obtained in 'Pipacs1' cultivar is shown. In Fig. 1A, the UV signal recorded at  
204 260 nm is plotted, which wavelength is the typical absorption maximum of genistein  
205 compounds (see Fig. 4). In Fig. 1B, the extracted ion chromatogram (EIC) of  $m/z$  271.0601 is  
206 given, which is corresponding to  $[C_{15}H_{11}O_5]^+$ , representing the protonated genistein aglycone  
207 fragment ( $Y_0^+$ ). (Throughout all TOFMS experiments, a 5 mDa mass window was used to  
208 extract  $m/z$  values of interest.) The appropriate retention time matching between UV and MS  
209 signals was obtained in five cases regarding the observed peaks, namely for peaks eluting at  
210 18.33, 21.03, 21.60, 22.73, and 31.33 min. Out of these five compounds, the last eluting one  
211 at 31.33 min was confirmed with reference standard as genistein aglycone. The remaining  
212 four compounds are supposed to be genistein glycoconjugates and the ion signals observed in

213 Fig. 1B are corresponding to the aglycone fragments ( $Y_0^+$ ) cleaved from the original  
214 glycoconjugates during in-source fragmentation.

215 In order to reveal the sugar (glycan) part of these supposed genistein glycoconjugates, a series  
216 of  $m/z$  values, which represent theoretical combinations of genistein aglycone and typical  
217 glycan part constituents, were extracted automatically using a home-made exact mass  
218 database. The glycan residue most often consists of building blocks of various hexoses such  
219 as glucose or galactose. Deoxyhexose (e.g. rhamnose) and pentose units such as xylose and  
220 arabinose are also common. Disaccharides are also often found in association with flavonoids,  
221 the most common ones are rutinose (rhamnosyl-( $\alpha 1 \rightarrow 6$ )-glucose) and neohesperidose  
222 (rhamnosyl-( $\alpha 1 \rightarrow 2$ )-glucose), and occasionally, tri- or even tetrasaccharides are encountered  
223 (Abad-García, Berrueta, Garmón-Lobato, Gallo, & Vicente, 2009).

224 In Fig. 1C, EIC of  $m/z$  433.1129 is given, representing a diagnostic ion for protonated  
225 genistein-hexoside (Gen-H). In three cases, namely for compounds at 18.33, 21.03, 22.73  
226 min, nice peaks were observed for Gen-H. In Fig. 1D, the EIC of the sodiated Gen-H ( $m/z$   
227 455.0954) is shown. This diagnostic ion helped decide whether the supposed Gen-H peaks in  
228 Fig. 1C are only fragment ions of more complex genistein glycoconjugates or Gen-H is the  
229 sought intact compound. Since sodium adduct of a fragment will not be formed, once the  
230 sodiated ion occurs together with the protonated ion, there is no need to search more complex  
231 glycoconjugates. The appearance of sodium adduct is “capping” the molecule, and thus sets  
232 the endpoint of this bottom-up exploratory protocol (Abrankó, Garcia-Reyes, & Molina-Diaz,  
233 2012). The sodium adduct of Gen-H (Gen-H-Na) was not observed in case of the compound  
234 at 18.33 min indicating that this compound is a more complex glycolconjugate and only the  
235 formed Gen-H fragment of the intact compound produced the ion signal in Fig. 1C. Sodium  
236 caps of compounds at 21.03 and 22.73 min were found, which provide evidence that these  
237 compounds can be both tentatively identified as genistein-hexosides. The compound eluting at

238 21.03 min was confirmed with reference standard as genistein-7-O- $\beta$ -D-glucoside also  
239 referred to as genistin. The sodium adduct of Gen-H (Gen-H-Na) also appeared for compound  
240 at 21.60 min; however, the  $[M+H]^+$  ion of Gen-H ( $m/z$  433.1129) was not observed at this  
241 retention time. The missing Gen-H ion weakens the assumption that this compound also can  
242 be a genistein-hexoside, nonetheless UV signal at 260 nm and the available accurate mass  
243 spectral data for genistein aglycone fragment along with Gen-H-Na (see Fig. 1A, B and D)  
244 coherently support this assumption.

245 In Fig. 1E and 1F, EICs of  $m/z$  595.1658 and 617.1483 are shown; representing diagnostic  
246 ions for protonated genistein-dihexoside (Gen-H-H) and its sodium adduct (Gen-H-H-Na),  
247 respectively. As in case of the compound at 18.33 min both Gen-H-H and the sodium adduct  
248 appears, supporting that this compound can be tentatively identified as genistein-dihexoside.  
249 Diagnostic ions found for each compound along with the errors of MS identification of the  
250 ions of interest are summarized in **Table 1**.

251

252 *3.2 Additional confirmatory tandem MS and UV data relating to the detected genistein*  
253 *compounds.*

254 In addition to presented TOFMS results, further accurate mass tandem MS (qTOFMS)  
255 experiments along with UV spectrum acquisitions were carried out in order to provide  
256 additional confirmatory data strengthening the findings of profiling. Precursor ions  
257 corresponding to protonated Gen-H-H ( $[C_{27}H_{31}O_{15}]^+$ ,  $m/z$  595.1658) in the case of compound  
258 eluting at 18.33 min and Gen-H ( $[C_{21}H_{21}O_{10}]^+$ ,  $m/z$  433.1129) for compounds at 21.03, 21.60  
259 and 22.73 min were selected respectively for targeted qTOF tandem MS experiments. In the  
260 mass spectra given in Fig. 2,  $[C_{15}H_{11}O_5]^+$  fragment with the monoisotopic ion  $m/z$  271.0601  
261 appears for all investigated glycoconjugates (with less than 1.5 mDa or 5 ppm error) as a  
262 pronounced peak, which represents the genistein aglycone fragment ( $Y_0^+$ ) cleaved from the

263 original glycoconjugates. In the case of Gen-H-H at 18.33 min, the Gen-H fragment  
264 ( $[\text{C}_{21}\text{H}_{21}\text{O}_{10}]^+$ , monoisotopic ion  $m/z$  433.1129) also appears with 3.2 mDa or 7.4 ppm error  
265 (see Fig. 2A). It should be noted when qTOF tandem MS experiments were performed, the  
266 quadrupole (q) was working with a 4-Da-wide mass window to cover the isotopologue cluster  
267 of the selected precursor compound. That is why in Fig. 2A an interfering ion peak with  $m/z$   
268 594.2441 can be also seen closely next to the less abundant targeted one of  $m/z$  595.1658.  
269 (The latter is indicated with a diamond symbol.) The fragment with the monoisotopic mass of  
270  $m/z$  271.0601 appearing commonly for all four glycoconjugates was assumed to be the  
271 genistein aglycone fragment ( $\text{Y}_0^+$ ) cleaved from original compounds. However, this fragment  
272 with the supposed elemental composition of  $[\text{C}_{15}\text{H}_{11}\text{O}_5]^+$  can also fit an isomeric compound of  
273 genistein. For instance the flavonoid apigenin has the same elemental composition and can  
274 also form glycoconjugates. It is noted that genistein aglycone (at 31.33 min) and genistin  
275 (genistein-7-O- $\beta$ -D-glucoside) at 21.03 min were successfully confirmed with reference  
276 standards in ‘Pipacs1’ cultivar, as described before. It means that besides the accurate mass  
277 spectral data and UV signals at 260 nm, retention time matching with reference standards in  
278 case of these two compounds provided confirmatory data for the identification. With respect  
279 to the remaining three compounds at 18.33, 21.60 and 22.73 min, additional confirmatory data  
280 were collected in order to provide additional support to the hypothesis that the aglycone core  
281 of the compounds is genistein. In contrast with the previously described qTOFMS  
282 experiments, 210 V fragmentor voltage was used instead of 160 V, in order to encourage in-  
283 source fragmentation of the glycoconjugates, similarly to ‘TOF-only’ profiling experiments.  
284 In this qTOFMS experiment however,  $m/z$  271.0601 (the aglycone fragment) was chosen as  
285 the only precursor ion. The results are given in Fig. 3.

286 The observed ions of  $m/z$  253.0501  $[\text{C}_{15}\text{H}_9\text{O}_4]^+$ ,  $m/z$  243.0657  $[\text{C}_{14}\text{H}_{11}\text{O}_4]^+$ ,  $m/z$  197.0603  
287  $[\text{C}_{13}\text{H}_9\text{O}_2]^+$ ,  $m/z$  169.0650  $[\text{C}_{12}\text{H}_9\text{O}]^+$ ,  $m/z$  153.0188  $[\text{C}_7\text{H}_5\text{O}_4]^+$  ions are equally typical for

288 some flavonoids such as the isoflavone genistein and the flavone apigenin. Nevertheless, this  
289 fact supports the basic assumption that the studied compounds are most probably flavonoid  
290 derivatives. On the other hand, the ion  $[C_{13}H_{11}O_3]^+$  (with the monoisotopic  $m/z$  215.0703),  
291 which is a result of double elimination of CO from the aglycone is specific for isoflavones  
292 like genistein, and atypical for apigenin (Kuhn, Oehme, Romero, Abou-Mansour, &  
293 Tabacchi, 2003; March, Lewars, Stadey, Miao, Zhao, & Metcalfe, 2006; March, Miao,  
294 Metcalfe, Stobiecki, & Marczak, 2004). This ion was common in the spectra of all four  
295 compounds, and was observed with less than 1.5 mDa or 5 ppm error in all cases. This result  
296 provided support that the compounds detected are genistein glycoconjugates.

297 In order to provide data for additional confirmation, UV spectra of the compounds were also  
298 acquired using the diode array detector (DAD) inserted between the HPLC and TOFMS. The  
299 UV spectra of the compound eluting at 22.73 min and based on MS data was tentatively  
300 identified as genistein-hexoside, well matched those of genistin ( $t_R = 21.03$  min) and genistein  
301 ( $t_R = 31.33$  min). Spectra of these three compounds acquired in 'Pipacs1' sample are given in  
302 Fig. 4. In the case of the compound eluting at 21.60 min, contradictory results were obtained.  
303 The UV spectrum of this compound was substantially different from those shown in Fig. 4.  
304 Such differences can be partly explained with the presence of any UV absorbing interferences  
305 in the chromatographic peak. Nonetheless, in contrast with the obtained MS data presented  
306 earlier, the ambiguous UV spectrum observed for this compound did not give support for the  
307 assumption that this compound is a genistein glycoconjugate. With respect to the compound  
308 at 18.33 min, the sensitivity of the UV detector limited our success to acquire UV spectrum  
309 for this compound, which was present only in small amounts in all investigated samples.

310

311 *3.3. Quantification of genistein glycoconjugates*

312 Quantification was carried out using an HPLC-DAD instrument coupled to a tripe quadrupole  
313 ESI-MS/MS instrument. With this coupling quantification could be performed using both MS  
314 and UV data. Since reference substances were available only for genistein aglycone and  
315 genistin, only these two genistein compounds were quantified based on their MS data and  
316 using the standard addition calibration technique. The ESI-MS/MS was used in multiple  
317 reaction monitoring (MRM) mode. Two MRM transitions were selected for each compounds,  
318 namely 433/271 (quantifier) and 271/215 (qualifier) for genistin and 271/153 (quantifier) and  
319 271/215 (qualifier) for genistein aglycone, respectively. Genistein compound eluting at 22.73  
320 min provided pronounced UV signal in the samples where the two other genistein compounds  
321 were also present. Quantitation of this genistein-hexoside was also carried out based on UV  
322 chromatograms. Flavonoids absorb UV light effectively, which is a result of the  
323 chromophores found in the aglycone molecule. The aromatic A-ring of an aglycone, which is  
324 a common chromophore in all flavonoids, results UV absorptions in the 250 nm region of the  
325 absorbance spectrum. It should be noted that substituents without chromophores such as  
326 glycosyl moieties will not significantly change the absorbance spectrum of the given  
327 flavonoid (de Rijke, Out, Niessen, Ariese, Gooijer, & Brinkman, 2006). Therefore, the  
328 characteristic absorption band of genistein aglycone, which has a maximum at 260 nm could  
329 be used for quantitative measurements of the genistein glycoconjugate at 22.73 min.  
330 Concentrations of this genistein-hexoside were calculated using the calibration equation of  
331 genistin. Results are given in mg per 100 gram fresh weight in **Table 2**.

332 Out of twelve genotypes, in eight , ('Oblachiskha', 'VN-7', 'Érdi bőtermő', 'Csengődi',  
333 'Cigány404', 'Korai pipacs', 'VN-4' and 'Sárdy SF') both genistein and genistin were below  
334 the quantification limit of 0.02 mg per 100 g wet weight. However, traces of genistein  
335 compounds were detected in 'Érdi bőtermő' and 'Korai pipacs'. In these samples no UV peak  
336 was obtained for Gen-H (22.73 min). On the contrary, in 'Pipacs1', 'Kántorjánosi',

337 'Debreceni bőtermő' and 'Éva' genistein compounds was present in considerable amounts  
338 and 'Pipacs1' contained genistein compounds at an exceptional high concentration. These  
339 results show that natural variation in the concentration of genistein compounds among sour  
340 cherry cultivars is considerable. It is known to similarly occur in soya (Bhagwat, Haytowitz,  
341 & Holden, 2008; Chan, et al., 2009). As it was shown in a former study on sour cherries, it  
342 can be attributed to the considerable genetic diversity of the sour cherry genotypes native to  
343 the Carpathian Basin, which also display great phenotypic variability. Considerable variation  
344 in terms of fruit weigh, total polyphenol, total acidity, soluble solids, and total anthocyanin  
345 content was reported (Papp, Szilvássy, Abrankó, Szabó, Pfeiffer, Szabó, et al., 2010). The  
346 anthocyanin content of 'Pipacs1', 'Kántorjános', and 'Debreceni bőtermő' was measured to  
347 be similarly low, in particular in the range of 10-20 mg cyanidin-glucoside equivalent per 100  
348 g. 'Pipacs1' fruit can be characterized with bright red skin and yellow flesh. It is likely that in  
349 these cultivars the anthocyanin biosynthesis is blocked at a currently unidentified step in a  
350 way that down-regulation of the biosynthetic pathway will not result in complete absence of  
351 the end-products. Interestingly, experimental data clarified that key enzymes of polyphenol  
352 biosynthesis (PAL, CHS, DFR, ANS) were expressed even in the yellow fruit flesh of 'Pipacs  
353 1' (Papp, et al., 2010). This may result in the over-accumulation of some flavonoid  
354 intermediates including genistein in fruit flesh of 'Pipacs 1' and similar cultivars.

355

#### 356 **4. Conclusions**

357 When nutritional aspects of genistein are discussed, almost exclusively soy is considered as  
358 the only relevant dietary source. In this study, however, we reported convincing experimental  
359 data obtained by state-of-the-art techniques such as accurate mass tandem mass spectrometry,  
360 that certain sour cherry (*Prunus cerasus* L.) cultivars contain substantially high amounts of  
361 various genistein compounds. We unambiguously identified genistin and genistein aglycone

362 in four commercial sour cherry cultivars ‘Pipacs1’, ‘Kántorjánosi’, ‘Debreceni bőtermő’ and  
363 ‘Éva’, which are native to Hungary. The genistein content of ‘Kántorjánosi’, ‘Debreceni  
364 bőtermő’ and ‘Éva’ was in the range of 0.4-0.6 expressed as milligrams of genistein aglycone  
365 equivalents per 100 g of fresh weight. This is overlapping with the values reported in legumes  
366 (e.g. beans, beansprouts). Legumes are considered as potential alternative sources of genistein  
367 to soy products in the Western diet. For instance, in the study of Liggins *et al*, Mung  
368 beansprouts was reported to be the next richest investigated food following soy-related ones,  
369 with combined genistein and daidzein contents in the range of 0.3-0.6 mg/100g (Liggins,  
370 Bluck, Runswick, Atkinson, Coward, & Bingham, 2000b). In ‘Pipacs1’, a total of 4.4 mg/100  
371 g genistein compounds were measured expressed as milligrams of genistein aglycone  
372 equivalents per 100 g of wet weight. Interestingly, this amount is comparable to some  
373 amounts reported in soy-based foods such as soy cheese or soy drink. In raw mature soybeans  
374 genistein varied widely between 5.6 and 276 mg/100 g, providing an average content of 81  
375 mg/100g (Bhagwat, Haytowitz, & Holden, 2008). Genistein content of ‘Pipacs1’ is  
376 comparable to soy beans with low genistein concentration, produced typically in Europe and  
377 Taiwan.

378 According to a former study ‘Kántorjánosi’ can be considered as a cultivar with good eating  
379 quality, while ‘Debreceni bőtermő’ is less preferred for fresh consumption due to its lower  
380 soluble solid content. These sour cherry cultivars consumed as fresh products or in the form  
381 of juice may play an important role as complementary genistein sources in the Western diet.  
382 On the contrary, ‘Pipacs 1’ is not suitable for fresh consumption since it is sour and  
383 characteristically astringent due to its low pH and high acidity level as well as high  
384 polyphenolic contents (Papp, et al., 2010). ‘Pipacs 1’ fruits were formerly used for  
385 confectionary purposes and is currently non-utilized. However, due to its outstanding



386 genistein concentration compared to other assayed cultivars, its best future utilisation might  
387 be in functional food products.

388

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394

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494

495

## Figure Captions

**Fig. 1.** Overlaid TOFMS extracted ion chromatograms of selected ions used for tentative identification of genistein compounds.

**Fig. 2.** Accurate-mass qTOF mass spectra of  $m/z$  595.1658 at 18.33 min (A),  $m/z$  433.1129 at 21.03 min (B), 21.60 min (C), and 22.73 min (D). During the qTOFMS experiment the quadrupole (q) was working with a 4-Da-wide filtering mass window.

**Fig. 3.** Accurate-mass qTOF mass spectra of in-source formed fragment ion of  $m/z$  261.0601 at 18.33 min (A), 21.03 min (B), 21.60 min (C), and 22.73 min (D). Fragmentor voltage of 210 V was applied in order to encourage in-source fragmentation.

**Fig. 4.** UV spectra of genistein compounds in 'Pipacs1' fruit, obtained from HPLC-DAD acquisitions.