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

László Abrankó*[a], Ádám Nagy[a], Blanka Szilvássy[a], Éva Stefanovits-Bányai[a], Attila Hegedűs[b]

[a]*Department of Applied Chemistry, Faculty of Food Science, Corvinus University of Budapest, 29-33 Villányi, 1118 Budapest, Hungary*

[b]*Department of Genetics and Plant Breeding, Faculty of Horticultural Science, Corvinus University of Budapest, Ménesi út 44., 1118 Budapest, Hungary*

Prepared for: *Food Chemistry*

Date: 20.06.2013

*Corresponding author

Tel: +36 1482 6163

Fax: +36 1466 4272

e-mail: laszlo.abranko@uni-corvinus.hu

Abstract

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

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

1. Introduction

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

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

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

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

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

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

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

2. Materials and methods

2.1 Plant material

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

2.2. Chemicals and standards

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

2.3. Sample preparation

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

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

2.4 Chromatographic separation

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

2.5 Screening of genistein compounds by HPLC-DAD-ESI-qTOFMS

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

system, which introduces the flow from the outlet of the chromatograph together with a low flow (approximately 10 μl/min) of a calibrating solution. The solution contains the internal reference masses of HP-921 [hexakis-(1H,1H,3H-tetrafluoro-pentoxy)-phosphazene] and purine. Protonated molecules of purine ($[C_5H_4N_4]^+$ at *m/z* 121.050873) and HP-0921 ($[C_{18}H_{19}O_6N_3P_3F_{24}]^+$ at *m/z* 922.009798) were used as reference masses. The DAD was acquiring data in the range of 200-800 nm in 2 nm steps at 0.5 spectra/s acquisition speed.

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

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

3. Results and discussion

3.1. HPLC-DAD-ESI-TOFMS profiling of genistein glycoconjugates

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

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

In order to reveal the sugar (glycan) part of these supposed genistein glycoconjugates, a series of *m/z* values, which represent theoretical combinations of genistein aglycone and typical glycan part constituents, were extracted automatically using a home-made exact mass database. The glycan residue most often consists of building blocks of various hexoses such as glucose or galactose. Deoxyhexose (e.g. rhamnose) and pentose units such as xylose and arabinose are also common. Disaccharides are also often found in association with flavonoids, the most common ones are rutinose (rhamnosyl-(α1→6)-glucose) and neohesperidose (rhamnosyl-(α1→2)-glucose), and occasionally, tri- or even tetrasaccharides are encountered (Abad-García, Berrueta, Garmón-Lobato, Gallo, & Vicente, 2009).

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

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

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

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

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

original glycoconjugates. In the case of Gen-H-H at 18.33 min, the Gen-H fragment ($[C_{21}H_{21}O_{10}]^+$, monoisotopic ion *m/z* 433.1129) also appears with 3.2 mDa or 7.4 ppm error (see Fig. 2A). It should be noted when qTOF tandem MS experiments were performed, the quadrupole (q) was working with a 4-Da-wide mass window to cover the isotopolouge cluster of the selected precursor compound. That is why in Fig. 2A an interfering ion peak with *m/z* 594.2441 can be also seen closely next to the less abundant targeted one of *m/z* 595.1658. (The latter is indicated with a diamond symbol.) The fragment with the monoisotopic mass of *m/z* 271.0601 appearing commonly for all four glycoconjugates was assumed to be the genistein aglycone fragment (Y_0^+) cleaved from original compounds. However, this fragment with the supposed elemental composition of $[C_{15}H_{11}O_5]^+$ can also fit an isomeric compound of genistein. For instance the flavonoid apigenin has the same elemental composition and can also form glycoconjugates. It is noted that genistein aglycone (at 31.33 min) and genistin (genistein-7-O-β-D-glucoside) at 21.03 min were successfully confirmed with reference standards in 'Pipacs1' cultivar, as described before. It means that besides the accurate mass spectral data and UV signals at 260 nm, retention time matching with reference standards in case of these two compounds provided confirmatory data for the identification. With respect to the remaining three compounds at 18.33, 21.60 and 22.73 min, additional confirmatory data were collected in order to provide additional support to the hypothesis that the aglycone core of the compounds is genistein. In contrast with the previously described qTOFMS experiments, 210 V fragmentor voltage was used instead of 160 V, in order to encourage in-source fragmentation of the glycoconjugates, similarly to 'TOF-only' profiling experiments. In this qTOFMS experiment however, *m/z* 271.0601 (the aglycone fragment) was chosen as the only precursor ion. The results are given in Fig. 3.

The observed ions of *m/z* 253.0501 $[C_{15}H_9O_4]^+$, *m/z* 243.0657 $[C_{14}H_{11}O_4]^+$, *m/z* 197.0603 $[C_{13}H_9O_2]^+$, *m/z* 169.0650 $[C_{12}H_9O]^+$, *m/z* 153.0188 $[C_7H_5O_4]^+$ ions are equally typical for

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

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

3.3. Quantification of genistein glycoconjugates

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

Out of twelve genotypes, in eight , (‘Oblachiskha’, ‘VN-7’, ‘Érdi bőtermő’, ‘Csengődi’, ‘Cigány404’, ‘Korai pipacs’, ‘VN-4’ and ‘Sárdy SF’) both genistein and genistin were below the quantification limit of 0.02 mg per 100 g wet weight. However, traces of genistein compounds were detected in ‘Érdi bőtermő’ and ‘Korai pipacs’. In these samples no UV peak 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’, ‘Kántorjánosi’, 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
389 **Acknowledgment**
390 This research was co-financed by OTKA PD 100506 and OTKA K 84290 research grants.
391 L.A is grateful for the funding of the Corvinus Research Excellence Scholarship (Corvinus
392 Kutatói Kiválósági Ösztöndíj) and A.H. acknowledges a János Bolyai Scholarship, Hungarian
393 Academy of Sciences Thanks for Dr. Gábor Rak for his preliminary measurements.
394

References

Abad-García, B., Berrueta, L. A., Garmón-Lobato, S., Gallo, B., & Vicente, F. (2009). A general analytical strategy for the characterization of phenolic compounds in fruit juices by high-performance liquid chromatography with diode array detection coupled to electrospray ionization and triple quadrupole mass spectrometry. *Journal of Chromatography A, 1216*(28), 5398-5415.

Abrankó, L., Garcia-Reyes, J. F., & Molina-Diaz, A. (2012). Systematic Bottom-Up Approach for Flavonoid Derivatives Screening in Plant Material using Liquid Chromatography High Resolution Mass Spectrometry. *Analytical and Bioanalytical Chemistry*, *403*(4), 995-1006.

Bak, I., Lekli, I., Juhasz, B., Varga, E., Varga, B., Gesztelyi, R., Szendrei, L., & Tosaki, A. (2010). Isolation and Analysis of Bioactive Constituents of Sour Cherry (Prunus cerasus) Seed Kernel: An Emerging Functional Food. *Journal of Medicinal Food, 13*(4), 905-910.

Behloul, N., & Wu, G. (2013). Genistein: A promising therapeutic agent for obesity and diabetes treatment. *European Journal of Pharmacology, 698*(1–3), 31-38.

Bhagwat, S., Haytowitz, D. B., & Holden, J. M. (2008). USDA Database for the Isoflavone Content of Selected Foods. In A. R. S. U.S. Department of Agriculture (Ed.), 2.0 ed.). Beltsville, Maryland.

Chan, S. G., Murphy, P. A., Ho, S. C., Kreiger, N., Darlington, G., So, E. K. F., & Chong, P. Y. Y. (2009). Isoflavonoid Content of Hong Kong Soy Foods. *Journal of Agricultural and Food Chemistry, 57*(12), 5386-5390.

Chukwumah, Y., Walker, L., Vogler, B., & Verghese, M. (2012). Profiling of bioactive compounds in cultivars of Runner and Valencia peanut market-types using liquid chromatography/APCI mass spectrometry. *Food Chemistry*, *132*(1), 525-531.

Crozier, A., Del Rio, D., & Clifford, M. N. (2010). Bioavailability of dietary flavonoids and phenolic compounds. *Molecular Aspects of Medicine, 31*(6), 446-467.

Crozier, A., Jaganath, I. B., & Clifford, M. N. (2009). Dietary phenolics: Chemistry, bioavailability and effects on health. *Natural Product Reports, 26*(8), 1001-1043.

de Rijke, E., Out, P., Niessen, W. M. A., Ariese, F., Gooijer, C., & Brinkman, U. A. T. (2006). Analytical separation and detection methods for flavonoids. *Journal of Chromatography A, 1112*(1-2), 31-63.

Del Rio, D., Rodriguez-Mateos, A., Spencer, J. P. E., Tognolini, M., Borges, G., & Crozier, A. (2013). Dietary (poly)phenolics in human health: Structures, bioavailability, and evidence of protective effects against chronic diseases. *Antioxidants and Redox Signaling, 18*(14), 1818-1892.

Ficzek, G., Vegvari, G., Sandor, G., Steger-Mate, M., Kallay, E., Szugyi, S., & Toth, M. (2011). HPLC evaluation of anthocyanin components in the fruits of Hungarian sour cherry cultivars during ripening. *Journal of Food Agriculture & Environment, 9*(1), 30-35.

Hevesi, M., Blázovics, A., Kállay, E., Végh, A., Stéger-Máté, M., Ficzek, G., & Tóth, M. (2012). Biological activity of sour cherry fruit on the bacterial flora of human saliva in vitro. *Food Technology and Biotechnology, 50*(1), 117-122.

Khoo, G. M., Clausen, M. R., Pedersen, B. H., & Larsen, E. (2011). Bioactivity and total phenolic content of 34 sour cherry cultivars. *Journal of Food Composition and Analysis, 24*(6), 772-776.

Kim, D. O., Heo, H. J., Kim, Y. J., Yang, H. S., & Lee, C. Y. (2005). Sweet and sour cherry phenolics and their protective effects on neuronal cells. *Journal of Agricultural and Food Chemistry, 53*(26), 9921-9927.

444 Kim, G. N., Song, J. H., Kim, E. S., Choi, H. T., & Jang, H. D. (2012). Isoflavone content and
445 apoptotic effect in HT-29 cancer cells of a soy germ extract. *Food Chemistry, 130*(2),
446 404-407.
447 Kirakosyan, A., Seymour, E. M., Llanes, D. E. U., Kaufman, P. B., & Bolling, S. F. (2009).
448 Chemical profile and antioxidant capacities of tart cherry products. *Food Chemistry,*
449 *115*(1), 20-25.
450 Kuhn, F., Oehme, M., Romero, F., Abou-Mansour, E., & Tabacchi, R. (2003). Differentiation
451 of isomeric flavone/isoflavone aglycones by MS2 ion trap mass spectrometry and a
452 double neutral loss of CO. *Rapid Communications in Mass Spectrometry, 17*(17),
453 1941-1949.
454 Kurahashi, N., Iwasaki, M., Inoue, M., Sasazuki, S., & Tsugane, S. (2008). Plasma
455 Isoflavones and Subsequent Risk of Prostate Cancer in a Nested Case-Control Study:
456 The Japan Public Health Center. *Journal of Clinical Oncology, 26*(36), 5923-5929.
457 Lampe, J. W., Nishino, Y., Ray, R. M., Wu, C., Li, W., Lin, M. G., Gao, D. L., Hu, Y.,
458 Shannon, J., Stalsberg, H., Porter, P. L., Frankenfeld, C. L., Wahala, K., & Thomas,
459 D. B. (2007). Plasma isoflavones and fibrocystic breast conditions and breast cancer
460 among women in Shanghai, China. *Cancer Epidemiology Biomarkers & Prevention,*
461 *16*(12), 2579-2586.
462 Li, W., Frame, L. T., Hirsch, S., & Cobos, E. (2010). Genistein and hematological
463 malignancies. *Cancer Letters, 296*(1), 1-8.
464 Liggins, J., Bluck, L. J. C., Runswick, S., Atkinson, C., Coward, W. A., & Bingham, S. A.
465 (2000a). Daidzein and genistein content of fruits and nuts. *Journal of Nutritional*
466 *Biochemistry, 11*(6), 326-331.

Liggins, J., Bluck, L. J. C., Runswick, S., Atkinson, C., Coward, W. A., & Bingham, S. A. (2000b). Daidzein and genistein contents of vegetables. *British Journal of Nutrition, 84*(5), 717-725.

March, R. E., Lewars, E. G., Stadey, C. J., Miao, X. S., Zhao, X., & Metcalfe, C. D. (2006). A comparison of flavonoid glycosides by electrospray tandem mass spectrometry. *International Journal of Mass Spectrometry, 248*(1-2), 61-85.

March, R. E., Miao, X. S., Metcalfe, C. D., Stobiecki, M., & Marczak, L. (2004). A fragmentation study of an isoflavone glycoside, genistein-7-O-glucoside, using electrospray quadrupole time-of-flight mass spectrometry at high mass resolution. *International Journal of Mass Spectrometry, 232*(2), 171-183.

Nara, K., Nihei, K., Ogasawara, Y., Koga, H., & Kato, Y. (2011). Novel isoflavone diglycoside in groundnut (Apios americana Medik). *Food Chemistry, 124*(3), 703-710.

Papp, N., Szilvássy, B., Abrankó, L., Szabó, T., Pfeiffer, P., Szabó, Z., Nyéki, J., Ercisli, S., Stefanovits-Bányai, É., & Hegedűs, A. (2010). Main quality attributes and antioxidants in Hungarian sour cherries: identification of genotypes with enhanced functional properties. *International Journal of Food Science & Technology, 45*(2), 395-402.

Schwab, K., Stein, R., Scheler, C., & Theuring, F. (2012). Dietary genistein enhances phosphorylation of regulatory myosin light chain in the myocardium of ovariectomized mice. *Electrophoresis, 33*(12), 1795-1803.

Wang, H., Nair, M. G., Strasburg, G. M., Booren, A. M., & Gray, J. I. (1999). Antioxidant polyphenols from tart cherries (Prunus cerasus). *Journal of Agricultural and Food Chemistry, 47*(3), 840-844.

Yang, G., Shu, X. O., Chow, W. H., Zhang, X. L., Li, H. L., Ji, B. T., Cai, H., Wu, S. H., Gao, Y. T., & Zheng, W. (2012). Soy Food Intake and Risk of Lung Cancer: Evidence

From the Shanghai Womens Health Study and a Meta-Analysis. *American Journal of Epidemiology, 176*(10), 846-855.

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.