Polish Journal of Food and Nutrition Sciences

Pol. J. Food Nutr. Sci., 2013, Vol. 63, No. 3, pp. 173-177
DOI: 10.2478/v10222-012-0081-5
http://journal.pan.olsztyn.pl

Original article
Section: Food Chemistry

Flavonols and Flavones in Some Bulgarian Plant Foods

Silvia Tsanova-Savova, Fanny Ribarova*

Medical University, Sofia, Medical College "Yordanka Filaretova", Yordanka Filaretova Str. 3, Sofia 1606, Bulgaria

Key words: flavonols, flavones, plant foods, HPLC

Flavonols and flavones are antioxidants of plant origin with a beneficial role in the prevention of different diseases. Therefore it is important for their content in various foods to be measured in order to be able to judge on their potential for disease prevention.

The aim of the study is to present precise and representative data for flavonols and flavones content of Bulgarian foods from *Malvaceae* and *Umbiliferae* plant families, based on a validated analytical HPLC procedure.

The content of flavonols and flavones was determined in one representative belonging to *Malvaceae* plant family used as food – okra, and four representatives of *Umbiliferae* plant family – dill, parsley, celery, and carrots. An HPLC method for simultaneous determination of flavonols myricetin, quercetin, kaempferol and flavones luteolin and apigenin was applied and validated.

The results showed that dill was particularly rich in the flavonol quercetin (403.0 mg/kg) followed by okra (200.3 mg/kg). The other analysed samples contained only flavones with the highest amount noted for luteolin in celery leaves (228.9 mg/kg) and for apigenin in parsley (747.9 mg/kg). Those data outlined the ranking of green leaf herbs among the greatest sources of flavonols and flavones among Bulgarian foods. They could be used to characterise various biological species and, what is more, they could be successfully applied in practice to formulate preventive antioxidant diets to be administered in case of various contemporary diseases.

INTRODUCTION

Flavonols and flavones are representatives of the large class of phenolic compounds – flavonoids. They are known as powerful antioxidants. The difference in the extent of their antioxidant activity is due to one hydroxyl group at the third position in the phenyl-benzo-γ-pyrone nucleus in flavonols [Rice-Evance *et al.*, 1996]. The best studied flavonols representative is quercetin. Flavones are comparatively less prevalent and are characteristic for vegetable composition rather than fruit composition. Luteolin and apigenin are the most frequently detected.

Initially the interest in those compounds was due mainly to their pigment properties in plants [Harborne, 1960; Von Elbe & Schwartz, 1996]. Those early studies did not address their antioxidant potential.

A change in the knowledge on flavonoids occurred in 1993, triggered by the publishing of the first epidemiological survey ("Zutphen Elderly Study") that revealed a reverse relationship between the high intake of flavonoids (flavonols and flavones) and cardiovascular risk (CVD) [Hertog *et al.,* 1993]. This survey was supported by numerous studies outlining the protective role of flavonoids concerning CVD [Geleijnse *et al.,* 1999; Knekt *et al.*, 2002; Mulvihill & Huff, 2010], cancer [Knekt *et al.*, 1997; Garcia-Closas *et al.*, 1999; Le Marchand *et al.*, 2000; Kozic, *et al.,* 2011] and neurodegenerative diseases like Parkinson and Alzheimer's disease [Gao *et al.*, 2012; Rossi *et al.*, 2008].

The first assessment of flavonoids dietary intake was made by Kuhnau in the USA who calculated the total flavonol and flavone-aglycone to amount 115 mg/day [Kuhnau *et al.*, 1976]. Hertog *et al.* [1992b], by referring to own accurate studies on the content of the basic flavonols: quercetin, myricetin and kaempferol and of the major flavones: apigenin and luteolin in the most often consumed foods in the Netherlands, evaluated an average daily intake of flavonoids at 23 mg/day. In 2002 in the USA Sampson *et al*. [2002] established an average daily intake of flavonols and flavones at 20–22 mg/day. The comparative assessment of the results of various studies needs the construction of a precise database for the composition and content of flavonoids in foods.

The most detailed database for flavonoid content is the USDA Database for the Flavonoid Content of Selected Foods, prepared by Nutrient Data Laboratory, U.S. Department of Agriculture. The current third edition of the database was elaborated in 2011 and covers data for flavonoid content in 500 foods presented in 300 international publications, and includes only data complying with the requirements for sample representativeness and accuracy of the analytical procedure [Bhagwat *et al.,* 2011; Haytowitz *et al*., 2009]. It should be noted that numerous researches on flavonoid content in foods have been published but not all of them comply with the set requirements.

* Corresponding Author: Tel: +3598887250138
E-mail: silvia_tsanova@abv.bg (S.Tsanova-Savova)

© Copyright by Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences

Therefore the aim of this study was, on the basis of a validated analytical HPLC procedure, to present precise data for flavonols and flavones content of Bulgarian foods from *Malvaceae* and *Umbiliferae* plant families. Our interest was focused on these two plant families as they are an integral part of the daily and traditional Bulgarian diet.

MATERIALS AND METHODS

Plant material

Plant foods from the *Malvaceae* family: okra (*Abelmoschus esculentus*, raw); and plant foods from the *Umbiliferae* family: carrot (*Daucus carota*, root), celery (*Apium graveolens*, root), celery (*Apium graveolens*, leaves), parsley (*Petroselium sativum*, leaves), and dill (*Anethum gravelens*, leaves) were the focus of the study.

The food samples were collected over a 2-year period within their harvesting stage. In order to ensure representative samples each laboratory sample was a composite of 3 individual market samples, purchased from 3 different locations in Sofia. A minimum of 1 kg or three bands for the herbs (dill, parsley, celery leaves) was sampled per location. The individual food samples were combined per product and after removing the non-edible parts, were chopped into small pieces and freeze-dried. The lyophilized samples were stored in hermetically sealed packages at 4°C. Right before analysis the lyophilized sample was grinded to fine powder, sieved through a 0.5 mm pore size sieve and homogenized, and 0.5 mg of this material were taken for analytical sample.

Standard substances and chemicals

Flavonols: quercetin dihydrate (3,3',4',5,7-pentahydroxyflavone); myricetin (3,3',4',5,5',7-hexahydroxyflavone); kaempferol (3,5,7–trihydroxy-2[4-hydroxyphenyl]-4H-1–1-benzopyran--4-one), as well as flavones: luteolin (3',4',5,7-tetrahydroxyflavone); apigenin (4',5,7-trihydroxyflavone) and Internal Standard – morine (2',3,4',5,7-pentahydroxyflavone) were used in the study. All standard substances were purchased from Sigma Chemicals Co., M-4008.

Tert-butylhydroquinone (TBHQ) ≥98.0% was from Fluka, Sigma-Aldrich Chemie GmbH, USA. Hydrochloric acid, ascorbic acid and methanol, used as a solvent, were of analytical grade and were purchased from Merck (Darmstadt, Germany). Methanol used for HPLC was gradient grade for liquid chromatography (Merck, Darmstadt, Germany).

Apparatus

Hewlett Packard Liquid Chromatograph with HP pump 1050; thermostat: HP 1100; UV detector: HP 1050; injector: Rheodyne 750; and ChemStation Software for data handling (Agilent Technology) was used in experiments.

Analytical method

The extraction and hydrolysis of the flavonoids from the plant material was performed with 1.2 mol/L HCl in 50% methanol, refluxing 0.50 g of the lyophilized sample for 2 h at 90°C. After hydrolysis 1 mL of ascorbic acid solution was added (1 mg/mL). The extract was homogenised and aliquot of 2 mL was ultracentrifuged. The supernatant was filtrated through 0.2 μm membrane filter and 50 μL were injected into the liquid chromatograph.

The stock solutions of the flavonols (myricetin, quercetin, and kaempferol) of the flavones (luteolin and apigenin) and of the Internal Standard (morine) were made at 500 μg/mL concentration level in methanol and stored at -18°C.

The calibration standard solutions were prepared within the concentration range of 0.25–25.0 μg/mL right before each series of analyses. These solutions were prepared as follows: 2.5 mL of TBHQ solution (0.500 mg/mL); 5–500 μL of the stock solution of individual compounds, and 50 μL of the Internal Standard solution was added to test tube of 10 mL. Then 1.8 mL of water, 0.6 mL of 10 mol/L HCl and 50 μL of ascorbic acid solution were added and the volume was made to 10 mL with methanol.

The chromatographic separation was performed by using Alltima (100 × 4.6 mm i.d., 3 μm) C18 analytical column, connected to pre-column Alltima (4 × 4.6 mm i.d., 3 μm) C18, Alltech Association Inc. An isocratic elution with 53% MeOH in 2% acetic acid was applied, with a flow rate of 0.8 mL/min, resulting to a working pressure of 18.0–18.5 MPa. For determination of the selected flavonoids a fixed UV detection at 365 nm was used.

Statistical analysis

In the present work the results are reported as an average value in mg/kg fresh weight of edible portion of food. In order to assess the biological diversity the standard deviation of the average (±SD) was also calculated according the formula:

$$SD = \sqrt{\frac{\sum_i \left(x_i - \overline{x}\right)^2}{n-1}},$$

where: x_i is the individual result of each food sample analysed, $\overline{x}$ is the average value of all samples analysed per food item, and n is the number of samples.

RESULTS AND DISCUSSION

As the aim of this study outlines two directions – validation of a method for analysis of flavonols and flavones in foods and presenting data for their content in the selected Bulgarian plant vegetables, firstly we start with the characteristics of the applied method and secondly with the obtained results. Figure 1 presents a chromatogram of the analysed flavonoids. It can be seen that a baseline separation of 6 individual compounds – myricetin, quercetin, kaempferol, luteolin, apigenin and the Internal Standard – morine was achieved within 14-min isocratic elution. We managed to achieve this result using methanol as an organic modifier of the mobile phase in the chromatographic system, while no separation of quercetin and luteolin was achieved when using acetonitrile, in accordance with the method, reported by Hertog *et al.* [1992a].

The parameters of the validation of the analytical method are as follows: Limit of detection – 0.05 μg/mL for myricetin and quercetin, corresponding to 0.3 mg/kg fresh weight (f.w.); 0.1 μg/mL for kaempferol, luteolin and apigenin, corresponding to 0.7 mg/kg f.w. Limit of quantitation – 1 mg/kg f.w. for

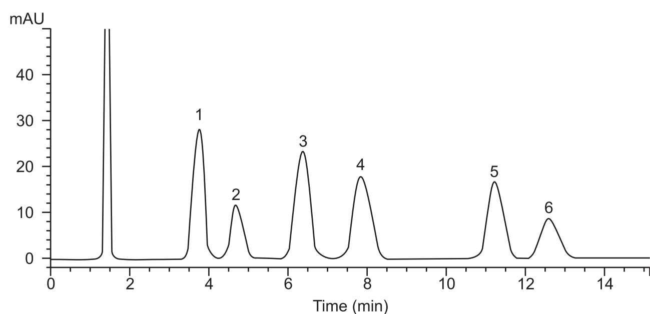

FIGURE 1. Chromatographic separation of flavonols, flavones and the internal standard – morin in standard solution (1 – myricetin, 2 – IS morin, 3 – quercetin, 4 – luteolin, 5 –kaempferol, 6 – apigenin).

myricetin and quercetin and 2 mg/kg f.w. for kaempferol, luteolin and apigenin. The standard calibration curves of flavonols and flavones were linear with coefficients of correlation $R^2>0.999$ within the concentration range of 0.25–25 μg/mL. The analytical recovery was determined by analysis of spiked samples and was more than 80%. The repeatability of the method was measured by analysis of six parallel samples within one day (n=6), by using a laboratory control sample for flavonols and flavones. The relative standard deviation was as follows – myricetin: RSD % = 4.5 %; quercetin: RSD % = 2.8 %; kaempferol: RSD % = 1.5 %; luteolin: RSD % = 3.1 %; and apigenin: RSD % = 1.7 %

Applying the validated analytical HPLC method our results are shown in Table 1. The results are presented as average value in mg/kg fresh food. In order to assess the biological diversity the standard deviation of the mean (±SD) and the minimum and the maximum values found are also presented. First of all we have to point out that flavonol myricetin was not found in any of the food samples analysed in this study, therefore data for myricetin were not included in the table of results.

Malvaceae family

The results of the analysis of three samples of *Abelmoschus esculentus* revealed that okra was excessively rich in quercetin (164.6–235.0 mg/kg) and only dill contained greater amounts. The evidence for flavonols content in okra in the third edition of USDA Database for Flavonoid Content of Selected Food quoted two publications and showed a mean value almost identical to our finding – 209.9 mg/kg but with greater dispersion of the values – in the range from 111.0 to 332.2 mg/kg [Sakakibara *et al*., 2003; Huang *et al.*, 2007]. Our national cuisine uses okra for the preparation of different dishes both in summer and in winter and this vegetable is a specific source of flavonols in Bulgarian diet.

Umbiliferae family

Carrots, celery and parsley do not contain flavonols, but dill is very rich in quercetin (mean value 403 mg/kg) and has a significantly lower content of kaempferol (17.9 mg/kg). The presented data support the results obtained by Justesen & Knuthsen [2001], showing that dill is very rich in quercetin and its availability even in small amounts in various dishes can affect the total flavonols dietary intake.

The representatives of this plant family are the richest source of flavones. The determined level of luteolin in the analyzed carrots was from 3.1 to 14.3 mg/kg. The obtained data differ from those listed in the USDA database (n=7, mean value – 1.1 mg/kg; minimum – 0.0 mg/kg; maximum – 8.0 mg/kg). This difference should be explained, although the explanation could sound scientifically speculative to some extent. As the characteristics of the applied analytical method ensure the accuracy of the obtained results, our explanation would rather focus on the responsibility of biological variability for the established difference. The conditions of cultivation in the various geographic regions affect the spectrum and composition of bioactive ingredients.

Celery and parsley are the main sources of flavones in our diet. Celery leaves are very rich in luteolin and apigenin; their amount in the root is lower, but still significant (see Table 1). Our results show that parsley contains only apigenin but in such high amounts that only one gram of this green leaf herb

TABLE 1. Flavonols and flavones content in plant foods from *Malvaceae* and *Umbiliferae* family.

Plant food	Flavonols						
	Quercetin				Kaempferol		
	mg/kg fresh weight						
	n	Average value ±SD	Min	Max	Average value ±SD	Min	Max
Okra	3	200.3±35.2	164.6	235.0	n.d.	–	–
Dill	3	403.0±35.9	367.4	439.2	17.9±2.1	15.8	19.7
Plant food	Flavones						
	Luteolin				Apigenin		
	mg/kg fresh weight						
	n	Average value ±SD	Min	Max	Average value ±SD	Min	Max
Carrots	5	8.8±4.7	3.1	14.3	n.d.	–	–
Celery, root	5	16.9±4.7	11.7	21.7	29.5±7.1	20.1	38.3
Celery, leaves	3	228.9±36.1	187.2	250.4	152.4±25.4	124.2	173.3
Parsley	3	n.d.	–	–	747.9±101.8	639.9	842.0

(-) – non detected

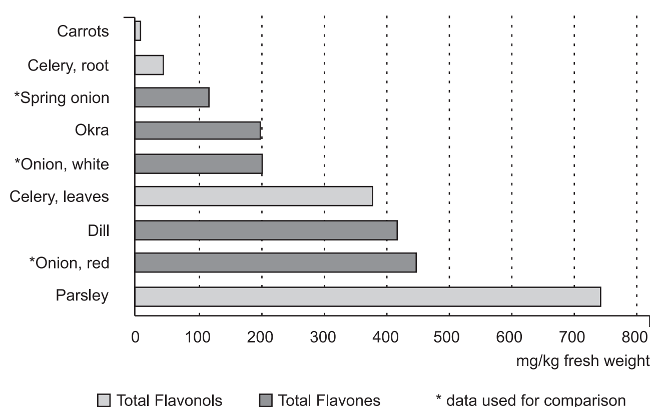

FIGURE 2. Total flavonols and total flavones in some Bulgarian foods. *Data used for comparison after: Tsanova-Savova [2011].

provides about 0.7 mg of apigenin (see Table 1). The values, communicated by other researchers on flavones content in celery and parsley are similar [Crozier *et al.,* 1997; Justesen & Knuthsen, 2001; Lugassi & Hovari, 2000; Huber *et al.,* 2009].

In order to demonstrate more expressively the assessment for flavonols and flavones content in plant foods, we took the opportunity to make a comparative assessment of the content of those flavonoids with the richest vegetable source – onion – that has been analysed by us in previous studies [Tsanova-Savova, 2011]. Figure 2 presents the results for total flavonols content (sum of quercetin and kaempferol) and flavones (sum of luteolin and apigenin) in the studied products, compared to three types of onion – red, yellow and spring onion.

Figure 2 shows the highest amount of flavones in parsley (747.9 mg/kg) exceeding substantially the level of total flavonols and flavones in all other studied vegetables. With about 300 mg/kg less emerges the group of celery leaves (479.3 mg/kg flavones), red onions (452.5 mg/kg flavonols) and dill (420.9 mg/kg flavonols). The celery roots have the lowest content of total flavones (46.4 mg/kg).

CONCLUSION

The modern scientific network demands much more food composition data as well as data corresponding to the contemporary requirements for representativeness and accuracy. The data presented in this paper can be used for biological characterisation of various plant species, can be implemented successfully in practice for compilation of preventive, antioxidant diets to be administered in case of various modern diseases. Those data are important also for the assessment of Bulgarian dietary traditions.

ACKNOWLEDGEMENTS

We gratefully acknowledge the support of Medical University, Sofia, Medical College "Yordanka Filaretova".

REFERENCES

1. Bhagwat S., Haytowitz D.B., Holden J.M., USDA Database for the Flavonoid Content for Selected Foods. Release 3. Nutrient Data Laboratory, Beltsville Human Nutrition Research Center, Agricultural Research Service, U.S. Department of Agriculture, 2011, Web site: [http://www.ars.usda.gov/nutrientdata].
2. Crozier A., Lean M.E.J., McDonald M.S., Black C., Quantitative analysis of the flavonoid content of commercial tomatoes, onions, lettuce, and celery. J. Agric. Food Chem., 1997, 45, 590–595.
3. Gao X., Cassidy A., Schwarzschild M.A., Rimm E.B., Ascherio A., *Habitual intake of dietary flavonoids and risk of Parkinson disease*. *Neurology*, 2012, 78, 1138–1145.
4. Garcia-Closas R., Gonzalez C.A., Agudo A., Riboli E., Intake of specific carotenoids and flavonoids and the risk of gastric cancer in Spain. Cancer Causes Control, 1999, 10, 71–75.
5. Geleijnse J.M., Launer L.G., Hofman A., Pols H.A.P., Witteman J.C.M., Tea flavonoids may protect against atherosclerosis – The Rotterdam study. Arch. Intern. Med., 1999, 159, 2170–2174.
6. Harborne J.B., Flavonoid pigments of *Lathyrus odoratus*. Nature, 1960, 187, 240–241.
7. Haytowitz D.B., Lemar L.E., Pehrsson P.R., USDA's Nutrient Databank System – A tool for handling data from diverse sources. J. Food Comp. Anal., 2009, 22, 433–441.
8. Hertog M.G.L., Feskens E.J.M., Hollman P.C.H., Katan M.B., Kromhout D., Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. Lancet, 1993, 342, 1007–1011.
9. Hertog M.G.L., Hollman P.C.H., Venema D.P., Optimization of quantitative HPLC determination of potentially anticarcinogenic flavonoids in vegetables and fruits. J. Agric. Food Chem., 1992a, 40, 1591–1598.
10. Hertog M.G.L., Hollman P.C.H., Katan M.B., Content of potentially anticarcinogenic flavonoids of 28 vegetables and 9 fruits commonly consumed in the Netherlands. J. Agric. Food Chem., 1992b, 40, 2379–2383.
11. Huang Z., Wang B., Eaves D.H., Shikany J.M., Pace R.D., Phenolic compound profile of selected vegetables frequently consumed by African Americans in the southeast United States. Food Chem., 2007, 103, 1395–1402.
12. Huber L.S., Hoffmann-Ribani R., Rodriguez-Amaya D.B., Quantitative variation in Brazilian vegetable sources of flavonols and flavones. Food Chem., 2009, 113, 1278–1282.
13. Justesen U., Knuthsen P., Composition of flavonoids in fresh herbs and calculation of flavonoid intake by use of herbs in traditional Danish dishes. Food Chem., 2001, 73, 245–250.
14. Knekt P., Kumpulainen J., Jarvinen R., Rissanen H., Heliovaara M., Reunanen A., Hakulinen T, Aromaa A., Flavonoid intake and risk of chronic diseases. Am. J. Clin. Nutr., 2002, 76, 560–568.
15. Knekt P., Jarvinen R., Seppanen R., Heliovaara M., Teppo L., Pukkala E., Arommaa A., Dietary flavonoids and the risk of lung cancer and other malignant neoplasms. Am. J. Epidemiol., 1997, 146, 223–230.
16. Kozics K., Valovicova Z., Slamenova D., Structure of flavonoids influences the degree inhibition of Benzo(a)pyrene – induced DNA damage and micronuclei in HepG2 cells. Neoplasma, 2011, 58, 516–24.
17. Kuhnau J., The flavonoids. A class of semi-essential food components: their role in human nutrition. World Rev. Nutr. Diet., 1976, 24, 117–191.

18. Le Marchand L., Murphy S.P., Hankin J.H., Wilkens L.R., Kolonel L.N., Intake of flavonoids and lung cancer. J. Natl. Cancer Inst., 2000, 92, 154–160.
19. Lugasi A., Hovari J., Flavonoid aglycons in foods of plant origin I. Vegetables. Acta Aliment. Hung., 2000, 29, 345–352.
20. Mulvihill E.E., Huff M.W., Antiatherogenic properties of flavonoids: implications for cardiovascular health. Can. J. Cardiol., 2010, Suppl A, 17A-21A.
21. Rice-Evans C.A., Miller N.J., Paganga G., Structure-antioxidant activity relationships of flavonoids and phenolic acids. Review article. Free Rad. Biol. Med., 1996, 20, 933–956.
22. Rossi L., Mazzitelli S., Arciello M., Capo C.R., Rotilo G., Benefits from dietary polyphenols for brain aging and Alzheimer's disease. Neurochem. Res., 2008, 33, 2390–2400.
23. Sakakibara H., Honda Y., Nakagawa S., Ashida H., Kanazawa K., Simultaneous determination of all polyphenols in vegetables, fruits, and teas. J. Agr. Food Chem., 2003, 51, 571–581.
24. Sampson L., Rimm E., Hollman P.C.H., de Vries J.H., Katan M.B., Flavonol and flavone intakes in US health professionals. J. Am. Diet Assoc., 2002, 102, 1414–1420.
25. *Tsanova-Savova S., Biologically active composition and health impact of Allium cepa. Acta Medica Bulgarica*, 2011, 1, 99–104.
26. Von Elbe J.H., Schwartz J.S., Food Chemistry (ed. O.R.Fennema). 1996, Marcel Dekker, Inc. New York, pp. 651–718.

Submitted: 28 November 2012. Revised: 1 March 2013. Accepted: 21 March 2013. Published on-line: 31 July 2013.