Flavonols and Flavones in Some Bulgarian Plant Foods

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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 Umbelliferae 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 Umbelliferae 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-pyrene 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 flavonoid 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 flavonoids: 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.

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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 Umbelliferae 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 Umbelliferae family: carrot (Daucus carota, root), celery (Apium graveolens, root), celery leaves (Apium graveolens, leaves), parsley (Petroselium sativum, leaves), and dill (Anethum graveolens, leaves) were the focus of this 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 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 was 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: Rhdonye 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 0.5 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 filtered 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:

\[
SD = \sqrt{\frac{\sum (x_i - \bar{x})^2}{n-1}},
\]

where: \(x_i\) is the individual result of each food sample analysed, \(\bar{x}\) 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
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 analysed 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 | Flavones |
|------------|-----------|----------|
|            | Quercetin | Kaempferol |
|            | Average value ±SD | Min | Max | Average value ±SD | Min | Max |
| Okra       | 200.3±35.2 | 164.6 | 235.0 | n.d. | – | – |
| Dill       | 403.0±35.9 | 367.4 | 439.2 | 17.9±2.1 | 15.8 | 19.7 |
| Carrots    | 8.8±4.7 | 3.1 | 14.3 | n.d. | – | – |
| Celery, root | 16.9±4.7 | 11.7 | 21.7 | 29.5±7.1 | 20.1 | 38.3 |
| Celery, leaves | 228.9±36.1 | 187.2 | 250.4 | 152.4±25.4 | 124.2 | 173.3 |
| Parsley    | n.d. | – | – | 747.9±101.8 | 639.9 | 842.0 |

(-) – non detected
The temporary requirements for representativeness and composition data as well as data corresponding to the diversity, so diseases. Those data are important also for the study of oxidant diets to be successfully in mg/kg flavonols and flavones, red onions (452.5 mg/kg flavonols). The group of flavonoids with the highest content of flavones in vegetables and fruits, J. Agric. Food Chem., 1992a, 40, 1591–1598.

In order to demonstrate more expressively the assessment for flavonoids 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 flavonoids 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 flavonoids and flavones in all other studied vegetables. With about 300 mg/kg less emerges the group of celery leaves (479.3 mg/kg flavonols), red onions (452.5 mg/kg flavonols) and dill (420.9 mg/kg flavonols). The celery roots have the lowest content of total flavonoids (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.

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