

## Polyphenol content and antioxidant activity of phytoestrogen containing food and dietary supplements: DPPH free radical scavenging activity by HPLC

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### ABSTRACT

Soy, red clover, chaste tree, hop and flax have all been found to contain a wide range of phytoestrogenic compounds, and a large number of dietary supplements contain their extracts as principal ingredients. This study is aimed to evaluate the total polyphenolic content and antioxidant activity of phytoestrogen-containing food and formulated dietary supplements. The HPLC-DPPH method was applied for DPPH free radical scavenging activity testing of various phytoestrogen-containing samples. Polyphenol content and antioxidant activity in dietary supplements were higher than in functional food samples; multiple-botanical-source preparations showed higher polyphenol content and antioxidant activity than the mono-botanical counterparts. Furthermore, the correlation between polyphenol content and antioxidant activity was strongly statistically significant, so it might be concluded that antioxidant activity is proportional to the content of these secondary metabolites. The most striking batch-to-batch deviations were represented by one chaste berry-based product (RSD 41.3 %) and one red clover derived product (RSD 57.9 %). The results of this study contribute to a better understanding of the phenolic profile and antioxidant properties of phytoestrogen containing food and dietary supplements.

**Keywords:** functional food, dietary supplements, phytoestrogens, polyphenol content, antioxidant activity, HPLC

### INTRODUCTION

Phytoestrogens are phytochemicals of phenolic origin found in a variety of plants, that are structurally similar to estrogen (1). Mimicking the conformational structure of estradiol they act as agonists or antagonists for estrogen receptors inducing estrogen-

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-responsive gene products, but may also exert metabolic effects not related to estrogen receptors. The most common source of phytoestrogen exposure to humans is soybean-derived foods. Consumed in many traditional Asian countries for millennia, soya has only been a common part of the Western diet in recent years. Although there is an increased interest in soybean-derived food among the population of Western countries, dietary supplements containing soy, chaste tree, red clover, flax and hop remain the main source of phytoestrogens for women in these countries. In addition to positive effects for menopausal symptoms, these compounds can quench reactive free radicals, prevent the oxidation of other molecules and may, therefore, have health-promoting effects in the prevention of degenerative diseases (2).

The trend in the implementation of high-performance liquid chromatography (HPLC) technique in 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay offers the high-throughput capability of the method essential to support routine analysis (3–6). According to our findings, limited research is performed on the antioxidant activity of phytoestrogen containing dietary supplements. DPPH• assay was used for the evaluation of soy- (7) and red clover-containing (7, 8) dietary supplements. The main drawback of these studies is the limited number and types of samples tested. Furthermore, no prior studies have examined the antioxidant activity of complex multiple-botanical-source dietary supplements.

Recently, the research of our scientific group was focused on the evaluation of active (9, 10) and toxic (9, 11) ingredients in phytoestrogen-containing dietary supplements. This study is a continuation of our previous work but focuses on the antioxidative activity of phytoestrogen-containing dietary supplements.

In consideration of the beneficial nutritional, physiological and health-promoting effects of phytoestrogens, in this study the macronutritive composition, the total polyphenol content (TPC), the antioxidant activity of soy, chaste tree, red clover, flax and hop as functional food ingredients or dietary food supplements is being evaluated.

## EXPERIMENTAL

### *Samples*

Four commercially available food samples of soybeans (F1 and F2) and flaxseed (F3 and F4) were collected from a local health-food store, while twenty phytoestrogen-containing dietary supplements (DS1-DS20) were obtained from a public pharmacy in Zagreb, Croatia. A list of the products, together with ingredients declared by manufacturers is given in the Supplementary materials, Table SI.

Briefly, fifteen dietary supplements were single-botanical-source preparations containing the following herbal extracts: soy (DS1-DS4), chaste berry (DS5-DS9), red clover (DS10 and DS11), hop (DS12-DS14), and flax (DS15). Five samples were classified as multiple-botanical-source dietary supplements containing several bioactive ingredients: soy and chaste berry (DS16 and DS17), soy and red clover (DS18), soy and flax (DS19), and red clover and hop (DS20). The dietary supplements were formulated as liquid extracts (2 samples), tablets (5 samples) or capsules (13 samples). Liquid samples were pure herbal extracts in ethanol, while other products contained additional ingredients, such as vitamins and minerals. Two different batches of each dietary supplement product were analysed to investigate batch-to-batch variability.

### *Chemicals and reagents*

Standards of gallic acid and TROLOX (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were provided from Sigma-Aldrich (Germany). As a source of free radical, DPPH (2,2-diphenyl-1-picrylhydrazyl, 95 %) was purchased from Sigma-Aldrich, while Folin-Ciocalteu's phenol reagent was obtained from Fluka (Switzerland). Ethanol, methanol and acetonitrile (98–100 %) HPLC grade were delivered by Merck (Germany), while hydrochloric, sulphuric, and boric acids were obtained from Kemika (Croatia). Kjeltabs Cu/3.5 (3.5 g potassium sulfate and 0.4 g cupric sulfate pentahydrate), which was used as a catalyst, was provided by Foss Tecator (Sweden). Ammonium sulphate, anhydrous sodium carbonate, sodium hydroxide, indicators bromocresol green and methyl red, acetone and petroleum ether (40–70 °C) were supplied by Kemika. Other reagents used in this work were of analytical reagent grade or higher. Ultra-pure water from a WaterPro water system Labconco (USA) with a resistivity of 18.2 MΩ cm (25 °C) was used in all experiments.

### *Nutritional composition of food samples*

Nutritional composition of food samples was evaluated by known procedures: total nitrogen concentration (Kjeldahl method, Kjeltec TM 2300, Foss Tecator, Sweden), total fat content (Soxhlet method, Foss Soxtec, model 2055, Foss, Denmark), ash content (muffle furnace L3/11/C6, Nabertherm®, Germany), moisture (oven UM300, Memmert GmbH, Germany), total dietary fiber content (Fibertec dietary fiber system, Foss) (12). Total carbohydrates and total energy value were calculated according to the Food and Agriculture Organization of the United Nations (FAO) recommendation (13).

### *Analysis of total polyphenol content and antioxidant activity*

*Sample preparation.* – The food samples, soybeans and flaxseeds, were crushed to a fine powder using Knifetec 1095 sample mill (Foss).

Ten tablets of dietary supplement samples were weighed, and the average mass of one tablet was determined. Afterwards, all tablets were finely ground and used in further investigations. Likewise, the content of ten capsules was pooled, and the average mass of one capsule was calculated.

*Extraction procedures.* – A sample portion of  $25 \pm 2$  mg was accurately weighed in a screw-capped 15-mL centrifuge tube. After adding 10 mL of methanol/ultra-pure water (80:20, V/V), the samples were sonicated for 15 min at room temperature in Elmasonic X-TRAH ultrasonic bath (Elma, Germany). The supernatant obtained after centrifugation (Hermle Z 306, Germany) at 3000xg for 10 min at 25 °C was filtered through a 0.45-µm Chromafil membrane filter (Macherey-Nagel, Germany) before UV-Vis and HPLC analysis.

*Preparation of stock and working solutions.* – The standard gallic acid solution (2 mg mL<sup>-1</sup>) was prepared by dissolving in methanol/ultra-pure water (80:20, V/V), while stock solution of TROLOX was prepared in methanol at a concentration of 1 mmol L<sup>-1</sup>. All stock solutions were stored in a refrigerator at 4 °C in 25-mL amber-glass volumetric flasks, and working solutions were prepared daily just prior to measurement. The DPPH solution was freshly prepared by dissolving 25 mg of DPPH in methanol using a 25-mL amber-glass volumetric flask class A.

*Determination of total polyphenol content (TPC).* – TPC in phytoestrogen-containing samples was assayed by the procedure using the Folin-Ciocalteu reagent (14). Briefly, 0.20 mL of methanolic extract was pipetted into a 5-mL volumetric flask and diluted with ultra-pure water to 2.5 mL. Following this, 0.25 mL of Folin-Ciocalteu reagent was added, and after 3 min 0.5 mL of 10 % sodium carbonate solution as well. Then, the mixture was well shaken using a vortex mixer (ZX3, Velp scientifica, Italy) and made up to volume with ultra-pure water. The solution was allowed to stand in the dark at room temperature for 2 hours before reading at 740 nm using a UV-Visible spectrophotometer (model Lambda 25, Perkin-Elmer, USA). The blank was prepared with methanol (80:20, V/V). Gallic acid was used as a standard and the calibration curve of gallic acid was prepared in the range from 0.4 to 6.0  $\mu\text{g mL}^{-1}$  at 5 concentration levels ( $R = 0.9999$ ). The results were expressed as gallic acid equivalents (GAE) ( $\text{mg GAE g}^{-1}$  sample).

*Determination of antioxidant activity.* – The antioxidative activity of the phytoestrogen-containing sample was determined using the HPLC-DPPH assay method (15). Briefly, 400  $\mu\text{L}$  of DPPH solution was added to a 1-mL aliquot of the sample extract. Following this, the mixture was shaken well on a vortex mixer in an Eppendorf tube and placed in the dark, at room temperature. The reaction time was 30 min. Afterwards, the solution was filtered through a Minisart RC4 0.20- $\mu\text{m}$  injection filter (Sartorius, Germany) and placed in an amber-glass HPLC vial.

Chromatographic analyses were performed on a Dionex chromatographic system (USA), equipped with a P680 pumping system, an ASI 100 automated sample injector, a TCC-100 thermostated column compartment, a UVD170S detector and Chromeleon 6.8 software (Dionex). The chromatographic conditions were a modification of those reported by Chandrasekar *et al.* (15) and used for DPPH assay. XBridge C18 column (4.6 x 150 mm, particle size 3.5  $\mu\text{m}$ ) from Waters (USA) was used as the stationary phase, while the mobile phase consisted of methanol:ultra-pure water (80:20, V/V). Isocratic elution was carried out at a flow rate of 1  $\text{mL min}^{-1}$ , and the column was thermostatically controlled to maintain a temperature of  $25.0 \pm 0.1$  °C. Finally, 20  $\mu\text{L}$  of the sample was injected into the HPLC system for analysis. During each run, absorbance was recorded at 517 nm, and the total run time was 5 min. Sample preparation and analysis was performed in triplicate.

The ability of a sample to scavenge the 'stable' free DPPH radical was determined from the difference in the peak area of the initial solution of the radical itself and the solution of the radical after reaction with the sample. TROLOX was used as a standard antioxidant and the calibration interval was in the range from 0.05 to 0.30  $\text{mmol L}^{-1}$  at 6 concentration levels. The results were expressed as TROLOX equivalent antioxidant capacity (TEAC) determined from a standard calibration curve.

### *Preliminary model validation*

To ensure the high reliability of the analytical procedure the HPLC-DPPH method was validated according to the ICH guidelines (16). The following parameters were assessed: selectivity, linearity, accuracy and precision. The linearity was examined by analysing the reaction mixture of DPPH radical and TROLOX standard in the concentration range from 0.05 to 0.3  $\text{mmol L}^{-1}$ . The accuracy of the method was investigated by a recovery study. At each concentration level (low, medium and high), three samples of

DPPH solution after the reaction with different TROLOX standard solutions were analysed. The percentage recovery and the RSD values were calculated for each replicate sample. The method precision includes repeatability and intermediate precision (inter-day precision). They were tested by repeated injections of the DPPH and standard solution mixture ( $n = 3$ ) over three consecutive days.

### *Data analysis*

Results are expressed on a dry mass (dm) basis for all types of analyses, except for the moisture content, based on 3 independent analyses.

The variables with a normal distribution are described by the arithmetic mean and standard deviation (SD), and those not showing a normal distribution are represented by the median and interquartile range. The Pearson product-moment correlation coefficient and Spearman rank correlation coefficient were determined to examine potential relationships between the concentrations of different compounds.  $p < 0.05$  was considered statistically significant and  $p < 0.01$  was considered highly significant. The statistical package STATISTICA v. 12.1 from StatSoft® (Tulsa, OK, USA) was used for data analysis.

## RESULTS AND DISCUSSION

### *Macronutritive composition of phytoestrogen containing food samples*

As it can be seen from Fig. 1a, the protein content of the investigated samples of soybeans and flaxseeds varied significantly, ranging from 209.4 g kg<sup>-1</sup> dm (SD = 3.08,  $n = 3$ ) in flaxseeds up to 383.2 g kg<sup>-1</sup> dm (SD = 1.48,  $n = 3$ ) in soybeans. The results obtained are in accordance with the data reported for various soy samples (350–420 g kg<sup>-1</sup>) (17–20) and flax varieties (178–246 g kg<sup>-1</sup>) (21). Flaxseeds, as a rich source of healthy fat, contained significantly higher fat content (458.9 ± 5.6 g kg<sup>-1</sup> dm) as compared to soybeans (195.6 g ± 23.3 kg<sup>-1</sup> dm). The obtained results are in accordance with those for flaxseeds (328.4–465.6 g kg<sup>-1</sup>) and soybeans (151–217 g kg<sup>-1</sup>) previously published (17, 21, 22). Soybeans, as a component of the daily diet, contained a higher amount of carbohydrates (326.4 g kg<sup>-1</sup> dm) compared to flaxseeds (233.4 g kg<sup>-1</sup> dm). At the same time, it has been observed that the mean value of dietary fiber was similar for both types of functional food samples. These results are close to those reporting carbohydrates (297–229 g kg<sup>-1</sup>) and crude fiber (57–81 g kg<sup>-1</sup>) contents of flaxseed varieties (21) and content of crude fiber (71 g kg<sup>-1</sup>) for soybeans (19). The moisture content in the studied soya bean was higher (114.3 g kg<sup>-1</sup>) than flax samples (67.1 g kg<sup>-1</sup>) and these results are in conformity with the data reported for soya (20) and flax (21, 23, 24). The total energy value of flax samples (average: 6040 kcal kg<sup>-1</sup> dm) was significantly higher in comparison to soybean samples (average: 4623 kcal kg<sup>-1</sup> dm) (Fig. 1b).

### *The total polyphenol content (TPC) of oral phytoestrogen-containing samples*

The obtained results for TPC (Table SII, Supplementary material), expressed as gallic acid equivalents, in food samples were in a rather narrow range of values, from 4.52 (F2 – soy) to 7.72 (F3 – flax) mg GAE g<sup>-1</sup> dm (Table I). The average content of TPC was higher in both flax samples (average 6.72 mg GAE g<sup>-1</sup>) than in two soybean samples (average 5.64

mg GAE g<sup>-1</sup>). From a nutritional standpoint, it is important to establish TPC per portion since nutritional recommendations are usually expressed in terms of portion. Hence, the amount of polyphenols taken by consuming recommended quantities (40–90 g of soybean

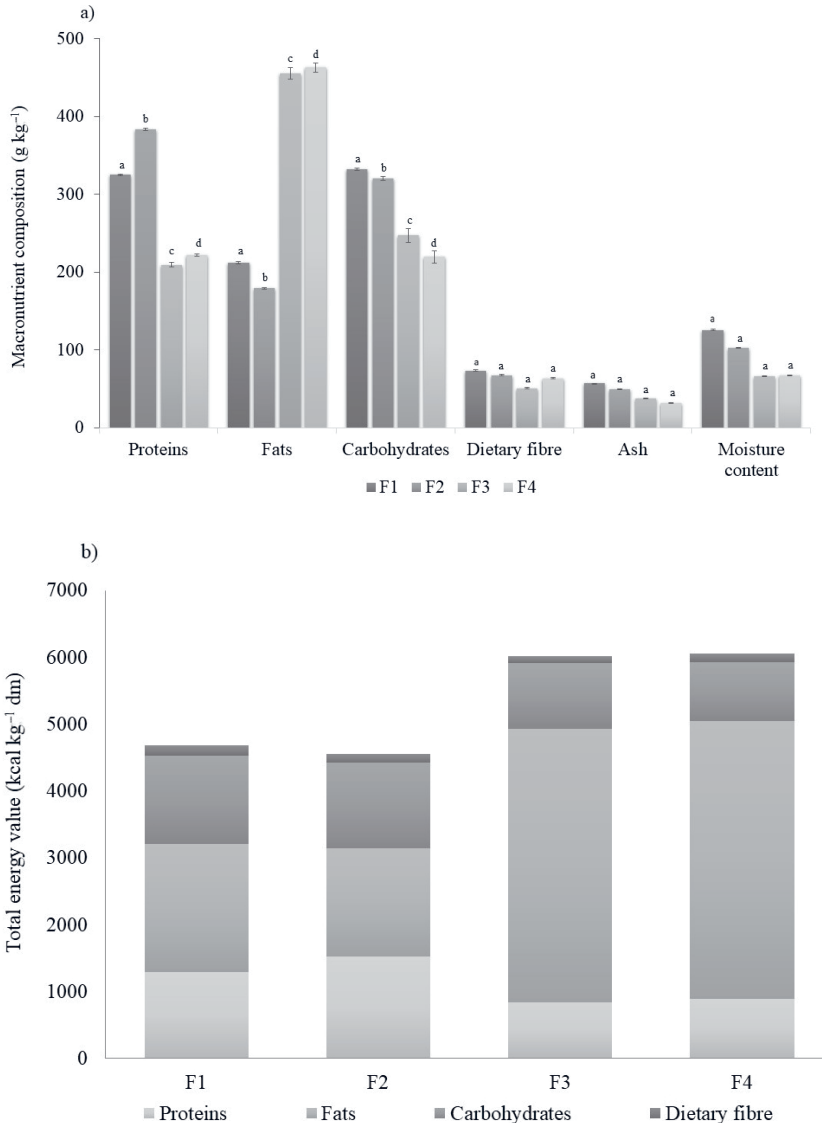


Fig. 1. Macronutrient composition of food samples (soybeans F1, F2 and flax seeds F3, F4): a) average value  $\pm$  SD (g kg<sup>-1</sup> dm; moisture content excepted,  $n = 3$ ) and b) their contribution to the total energy value. Statistically significant differences marked by different letters within each nutritional parameter group:  $p < 0.05$ .

Table 1. Total polyphenol content and antioxidant activity of phytoestrogen containing samples

Sample	Total phenolic content			DPPH <sup>a</sup>	
	mg GAE g <sup>-1</sup> sample <sup>b</sup>	Total amount of phenolics per dosage form unit (mg) <sup>cd</sup>	Total amount of phenolics per recommended daily serving (mg) <sup>de</sup>	mmol TEAC mL <sup>-1</sup> extract	mmol TEAC g <sup>-1</sup> sample
All samples					
Mean value	15.74			0.235	92.51
Median	13.43			0.185	72.23
Range of values	2.34–38.21			0.046–0.537	20.37–208.5
Interquartile range	7.60–24.52			0.112–0.364	44.22–143.4
RSD (%) <sup>f</sup>	0.1–2.7			0.9–7.0	
Food samples					
Mean value	6.18		284.1	0.093	40.01
Median	6.24		278.2	0.092	40.34
Range of values	4.52–7.72		57.24–608.0	0.046–0.141	20.37–58.99
Interquartile range	5.42–6.99		154.9–391.1	0.077–0.108	34.41–45.94
RSD (%) <sup>f</sup>	0.15–0.9			1.8–4.1	
Dietary supplements					
Mean value	17.65	12.18	49.47	0.263	103.01
Median	15.93	9.26	37.96	0.216	84.09
Range of values	2.34–38.21	1.87–51.95	6.28–154.0	0.054–0.537	21.30–208.5
Interquartile range	8.95–25.67	5.8–13.17	28.89–61.48	0.130–0.385	51.30–153.3
RSD (%) <sup>f</sup>	0.1–2.7			0.9–7.0	
Batch-to-batch variability RSD (%) <sup>g</sup>	0.1–57.9				

<sup>a</sup> DPPH – antioxidant activity measured by HPLC-DPPH scavenging assay.

<sup>b</sup> Expressed in mg g<sup>-1</sup> of dry matter.

<sup>c</sup> Dosage form unit: 1 tablet or capsule or 1 mL liquid extract.

<sup>d</sup> Based on GAE results.

<sup>e</sup> Recommended daily servings: 1 to 6 tablets or capsules, 30 drops for liquid extract, 40–90 g for soya or 10–50 g for flaxseed.

<sup>f</sup> RSD (%) range obtained by analysis of each sample in triplicate.

<sup>g</sup> Batch-to-batch variability is given for dietary supplement products.

and 10–50 g of flaxseed) is given in Table I. Despite the fact that TPC is somewhat higher in flax than soy, the daily intake of polyphenols through the ingestion of flax (from 57.24 to 386.85 mg day<sup>-1</sup>) still stays lower compared to soya (from 180.8 up to 608.0 mg), expressed as GAE.

Due to variations in the extraction process and differences in expressing results, it was challenging to compare the obtained content of total polyphenols in investigated food products with the data available in the literature. Our results were compared with the results of other scientific groups that have used methanol/water solutions as extraction solvent. It was found that TPC in our soya samples was similar to the values obtained for Bulgarian samples (2.18–5.06 mg GAE g<sup>-1</sup>) (25). On the other hand, the analyzed samples had higher polyphenol content than Croatian soya samples investigated by Mujić *et al.* (17) (0.87–2.16 mg GAE g<sup>-1</sup>) and Josipović *et al.* (26) (2.12–3.23 mg GAE g<sup>-1</sup>). Regarding the TPC in the currently tested flaxseed samples, higher values were obtained than for varieties from the major planting areas in China (1.10–2.47 mg GAE g<sup>-1</sup>) (22), Canada (2.09 mg GAE g<sup>-1</sup>) (27) and the United States (1.54 mg GAE g<sup>-1</sup>) (28).

Unlike food samples, the dietary supplement samples showed large differences with respect to TPC, and more than 15-fold difference was recorded between chaste berry sample (DS8: 2.34 mg GAE g<sup>-1</sup> sample) and soy sample (DS2: 38.21 mg GAE g<sup>-1</sup> sample). The obtained values imply significant polyphenol content in the latter sample. As was expected, higher TPC values were obtained for dietary supplements than for food samples (DS: 17.65 ± 10.28 mg GAE g<sup>-1</sup>, F: 6.18 ± 1.37 mg GAE g<sup>-1</sup>).

Overall, multiple-botanical-source products (23.70 ± 9.09 mg GAE g<sup>-1</sup>) have higher polyphenol content than single-botanical-source preparations (15.63 ± 10.11 mg GAE g<sup>-1</sup>), though this difference was not statistically significant. Similar findings regarding TPC in single- and multiple-botanical-source products was reported by Romani *et al.* (7).

TPC in solid single-botanical-source preparations ranged from the flax-based product (DS15: 7.24 mg GAE g<sup>-1</sup>) to soy-based product (DS2: 38.21 mg GAE g<sup>-1</sup>). Generally, the highest value was obtained for soy-based products (23.18 ± 11.58 mg GAE g<sup>-1</sup>) followed by red clover (18.69 ± 7.87 mg GAE g<sup>-1</sup>), chaste berry (12.82 ± 11.33 mg GAE g<sup>-1</sup>) and hop (11.02 ± 4.14 mg GAE g<sup>-1</sup>) products. Liquid single-botanical-source preparations showed somewhat lower values than solid preparations (< 4.18 mg GAE g<sup>-1</sup> for DS7), regardless of the botanical source.

In the studied soybean single-botanical-source preparations, TPC was higher than in samples investigated by Romani *et al.* (7) (12.5 and 15.1 mg GAE g<sup>-1</sup>) except for sample DS3 (11.14 mg GAE g<sup>-1</sup>). The highest value of TPC found in the red clover-based dietary supplement (DS11: 24.26 mg GAE g<sup>-1</sup>) was comparable to the one found in the red clover dietary supplement (29.6 mg GAE g<sup>-1</sup>) reported by Romani *et al.* (7), while same cannot be noted for sample DS10 (13.12 mg GAE g<sup>-1</sup>). On the other hand, Kroyer *et al.* (8) reported markedly higher TPC in one red clover single-botanical-source preparation (113.9 ± 3.3 mg g<sup>-1</sup>) compared to those found in our samples. This difference probably stems from the fact that the authors analysed fortified commercially available products, labelled as highly isoflavone-concentrated.

Limited data are available on TPC in multiple-botanical-source preparations. Analysis shows that our values for soybean multiple-botanical-source preparations [from 16.12 (DS17) to 36.42 (DS16) mg GAE g<sup>-1</sup>] are in accordance with those obtained previously by Romani *et al.* (7) (37.1 and 33.4 mg GAE g<sup>-1</sup> sample).



According to our findings, published investigations on TPC in dietary supplements are focused on polyphenol content by sample mass (7, 8). This information is useful to evaluate the quality of botanical material used in the production of dietary supplements. Our previous studies, however, have shown that the active substance content per formulation unit or daily intake can usefully contribute to the patient's choice of an appropriate dietary supplement product (10, 29). For this purpose, the TPC administered by dosage form or recommended daily dose is emphasized. Analysed samples demonstrated a wide range of dosage form unit masses, from 0.143 (DS10, capsule) to 1.85 g (DS9, tablet). The reasoning that follows is based on GAE results obtained: considering the average mass of individual tablet or capsule, polyphenol administration following single solid dosage form lies in the wide range of values from 1.87 mg (DS10) to 51.95 mg (DS9) (Table I). According to products' labels, there is a large diversity among the serving sizes of each product (up to 6 solid dosage form units). Hence, the daily administration of polyphenols that patients would ingest if they follow the daily serving recommendation by the manufacturer varies greatly: from 1.87 (DS10) to 103.9 (DS9) mg g<sup>-1</sup> per day, for the adult population.

#### *Antioxidant activity of oral phytoestrogen containing samples*

The DPPH radical is an organic stable free radical with an absorption band at 517 nm because of its spare electron delocalization over the whole molecule. This chromogen radical, on interaction with antioxidants, accepts an electron or hydrogen atom to become a DPPH-H non-radical stable molecule (3–6). It loses this absorption, resulting in a visually noticeable discoloration from deep purple to pale yellow solution. A representative chromatogram of the DPPH solution (DS 19) before and after the sample reaction is presented in Fig. 2.

The DPPH peak ( $t_R$  4.2 min, relative standard deviation 0.1 %,  $n = 6$ ,  $N = 9723$ ,  $k' = 2.5$ ) was well separated (resolution factor was higher than 3.7 in all test solutions) without interferences (peak purity factor was higher than 999) with acceptable tailing (1.07).

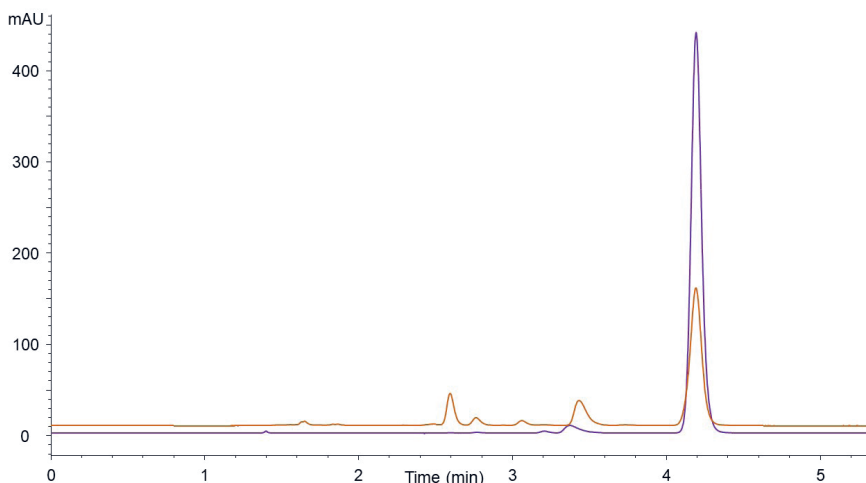


Fig. 2. The chromatogram of the DPPH solution before (purple) and after the sample (DS19, vegetal jelly, flaxseed extract + soybean extract) reaction (orange) recorded at 517 nm.

The high correlation coefficient ( $R = -0.9995$ ) of the linear regression ( $y = -129.89x + 49.367$ ) indicates the appropriate linearity of the method. The accuracy of the method was investigated by recovery study:  $97.5 \pm 2.1$  % for  $0.05 \text{ mmol L}^{-1}$ ,  $103.9 \pm 2.7$  % for  $0.15 \text{ mmol L}^{-1}$  and  $99.1 \pm 1.4$  % for  $0.30 \text{ mmol L}^{-1}$  standard solution) were within validation protocol acceptance limit of  $\pm 5.0$  % for recovery and RSD. The results show that the method is precise within the validation protocol acceptance limit (not more than 5.0 %) with RSD values from 3.6 to 4.8 % for repeatability and from 4.1 to 4.7 % for intermediate precision.

Table I shows the antioxidant activity of oral phytoestrogen-containing samples; the results for the DPPH-HPLC assay are presented as TROLOX equivalents. It is worth noting that the DPPH values obtained for food samples are in a rather narrow range ( $20.37\text{--}58.99 \text{ mmol TEAC g}^{-1}$ ) indicating conformity with the previous results (Supplementary materials, Table SII) (24, 30–33).

All the investigated dietary supplements exhibited some antioxidant properties, however, there was a large diversity among them (from 21.30 for DS8 – chaste berry to  $208.5 \text{ mmol TEAC g}^{-1}$  sample for DS2 – soy). Furthermore, a statistically significant difference ( $p < 0.05$ ) was found between the DPPH values for food (mean value  $40.01 \text{ mmol TEAC g}^{-1}$  sample,  $SD = 15.8$ ,  $n = 4$ ) and dietary supplement samples (mean value  $103.01 \text{ mmol TEAC g}^{-1}$  sample,  $SD = 58.42$ ,  $n = 20$ ) indicating that investigated dietary supplements are a more concentrated source of antioxidants. It is noteworthy that DPPH values, in general, were higher in multiple-botanical-source dietary supplements (average  $0.365 \text{ mmol TEAC mL}^{-1}/143.49 \text{ mmol TEAC g}^{-1}$ ) than in single-botanical-source preparation (average  $0.229 \text{ mmol TEAC mL}^{-1}/89.52 \text{ mmol L}^{-1} \text{ TEAC g}^{-1}$ ), still not significantly different.

Literature survey reveals that there is a general lack of research on the antioxidant activity of phytoestrogen-containing dietary supplements. In addition, DPPH values

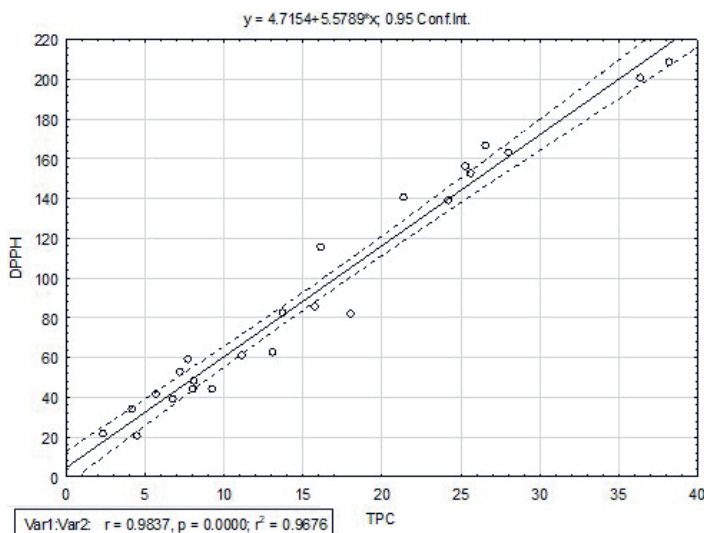


Fig. 3. Correlation between total polyphenol content and antioxidant activity estimated by HPLC-DPPH method in phytoestrogen-containing food samples and dietary supplements.

obtained in different studies was rather difficult to compare due to the differences in the sample preparation (different standard antioxidant: ascorbic acid, TROLOX, butylated hydroxyanisole, *etc.*) and the expression of the results (*e.g.*,  $EC_{50}$  value) (8). Only two research groups have evaluated the antioxidant activity of these products (7, 8).

Researchers have noted a correlation between DPPH assay and polyphenol content in phytoestrogen-rich food and dietary supplement samples (7, 17, 34). In contrast to these studies, a weak positive correlation of DPPH data with polyphenol content was also published, which questions the nature of polyphenols that have been extracted (35). The statistical analysis of our results has demonstrated a significant correlation between the TPC and antioxidant activity estimated by the HPLC-DPPH method (Fig. 3). Pearson correlation coefficient indicated a correlation for all investigated samples ( $R = 0.9837$ ,  $p < 0.0001$ ), food samples ( $R = 0.9295$ ,  $p < 0.01$ ) and formulated dietary supplements ( $R = 0.9821$ ,  $p < 0.01$ ).

Our results for food samples agree in general with the results of earlier studies of soybean (17, 35). The literature data show that the antioxidant activity of soybeans was correlated with TPC in Indian and Taiwan black soybean ( $R = 0.916$ ) (34) and Croatian soybean cultivars ( $R = 0.896$ ) (17). In both studies, antioxidant activity was evaluated by the UV-Vis method. Spectrophotometric assay of antioxidant activity *via* DPPH determination is not specific; food constituents and excipients in dietary supplements could co-absorb with DPPH radicals and affect the results. However, the HPLC-DPPH method is more selective than the commonly used spectrophotometric method.

According to the results of this study, a considerable amount of polyphenols can be found in phytoestrogen-containing food and formulated dietary supplements. Also, the extent of the antioxidant activity of these samples is in conformity with TPC.

### *Batch-to-batch variability*

Since our previous study on the dietary supplements (10) demonstrated batch-to-batch variability regarding the individual phytoestrogen content, our research is extended to variability in the TPC. The batch-to-batch quality consistency of selected products was evaluated using two different batches of each dietary supplement, and variability was found among various brands [RSD<sub>TPC</sub> values were between low 0.4 (DS17) and high 57.9 % (DS11)]. The variation in TPC of up to 10 % was found in 7 investigated products (35.0 % of all samples), while two products exhibited a dramatic batch-to-batch variation. The most striking deviations were represented by one chaste berry-based formulated product [RSD value 41.3 % (DS5)] and one red clover [RSD value 57.9 % (DS11)] derived product.

## CONCLUSIONS

The obtained data show that soybean and flaxseed are very good sources of nutritional constituents. The validated chromatographic method was suitable for determining the antioxidant activity of the investigated samples.

Results of this study contribute to a better understanding of the phenolic content and antioxidant properties of phytoestrogen-containing dietary supplements. Polyphenol content and antioxidant activity in formulated DS products were higher than in functional

food samples. Also, the results indicate that multiple-botanical-source preparations have higher polyphenol content and antioxidant activity than mono-botanical ones.

The correlation between polyphenol content and the antioxidant activity is strongly statistically significant both in food and formulated dietary supplements.

Supplementary materials available upon request.

*Conflicts of interest.* – The authors declare no conflict of interest.

*Authors contributions.* – Conceptualization, D.A.K.; methodology, D.A.K. and I.K.; analysis I.B., D.A.K. and I.K.; investigation, I.B., D.A.K. and I.K.; writing, original draft preparation, J.K., D.A.K., I.K. and A.M.; writing, review and editing, J.K., M.-L.J., E.B., D.A.K. and A.M. All authors have read and agreed to the published version of the manuscript.

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