# ANTIOXIDANT ACTIVITY IN TWO SPECIES COMMON BEANS (PHASEOLUS VULGARIS L.) FROM VILLAGE OF SMILYAN, BULGARIA

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**Abstract**

*Smilyan beans are called bean seeds, one of the few Bulgarian foods protected by a trademark patent for its cultivation in the area of the upper valley of Arda River. Smilyan beans have a unique taste and are famous both in Bulgaria and abroad. Common beans (Phaseolus vulgaris L.) from two different cultivars from village of Smilyan were sieve analyzed and assessed for antioxidant activities.*

**Keywords:** *Phaseolus vulgaris L., common bean, TPC, antioxidant activity, DPPH, ABTS, CUPRAC*

## 1. INTRODUCTION

*Phaseolus vulgaris L.*, a recently introduced species in Europe, is one of the most important protein sources for people until a few decades ago and after a period of decline its use is now being reevaluated of dietary reasons.

Smilyan is located in the central parts of Rhodope Mountains. Its climate belongs to the mountainous climate region, i.e. to the middle mountainous part of South Bulgaria climate area of the Continental Mediterranean climate region of Bulgaria. The relief is mountainous and brown forest soils are predominant. The average annual air temperature is about 8ºC and the average annual amplitude is hardly 19.2ºC. The spring is late, cool and humid. Summer is late, cool and very humid. The maximum temperatures are 25-29ºC and very rare above 30ºC. The autumn is short, cool and excessively humid. All these weather conditions affect the conditions of beans growing in Smilyan region [1].

Consummation of dry beans has positive impacts including maintenance and even improvement of human health and wellness. A group of international authors have investigated the consummation of legumes including dry bean was the most critical factor when compared to other food classes in determining longevity [2]. Many other reports have shown that diets comprised of pulses can significantly lower LDL-cholesterol levels [3], reduce the risk of prostate cancer [4], and inhibit mammary cancer development even at relatively low bean intake levels [5]. A lot of the nutritional benefits from consuming beans have been largely accredited to its dietary fiber content [4].

The common beans (Phaseolus vulgaris L.) are widely consumed throughout the world. They play a very important function in human nutrition, being a source of proteins, minerals, and certain vitamins. In last years, bioactive effects, associated with the fibers, polyphenols, and other beans components related to human health have gained attention [6, 7]. Beans contain substantial amount of phenolic acids and flavonoids; some cultivars (red, black, and blue-violet colored beans) show also anthocyanins, such as delphinidin and cyanidin, that overall attribute them a very strong antioxidant and antiradical activities [8]. Polyphenols are essentially present in the seed coats and in minor amount in cotyledons.

The presence of different phytochemicals including polyphenols, lead to the physiological effects of dry bean consumption, which possess both anticarcinogenic and antioxidant properties. It's believed that antioxidants scavenge free radicals and reactive oxygen species can be very important in inhibiting

oxidative mechanisms that lead to degenerative diseases. Bean extracts are known to possess antioxidant activity. A freeze-dried navy bean hull extract shows strong antioxidant activity [9]. Mung bean hulls have antioxidant activity, which has reducing power, scavenge DPPH radicals, and inhibit lipid peroxidation and nonlipid oxidative damage [10, 11].

There is a lack of information in the literature regarding the antioxidant activity of the common beans species *Phaseolus vulgaris L.*, cultivated in village of Smilyan, Bulgaria. In relation with this the aim of our study is to investigate the antioxidant activity of the two types beans after sieve analysis have been applied.

## 2. MATERIALS AND METHODS

### *2.1. Bean Materials*

The different bean seeds (*Phaseolus vulgaris L.*) were grown in village of Smilyan in the Rhodope Mountains region (Figure 1). The samples were harvested in 2017 year. The raw material was stored at -20 °C in sealed plastic bags in absence of air prior to performing the experiments.




**Fig. 1.** Different type common beans: S-6-dark bean; S-15- speckled bean.

### *2.2. Milling and sieve analysis of samples of black and speckled beans*

The raw materials used for milling are dried in advance beans with moisture content less than 10%. The samples were ground by means of a "Schule" laboratory pin mill - Germany at a peripheral speed of 64 m/s and they were passes through the mill once. The sieve analysis of the final ground product was carried out using a LP-200 laboratory plan sifter with a single sieve box with five sieves, frame size 200 x 200 mm and mesh sizes: 1000; 800; 670; 560; 450; 355; 280; 200; 180; and 150 μm. The sifting time was 3 min and the plan sifter rotation speed was 180 $min^{-1}$. In order to assist the sifting process two rubber balls were placed in each frame [12, 13]. The average sample collection and the sieve analysis of the ground product have been carried out in accordance with BDS 754:1980 "Mill products. Rules for testing and methods for testing". The distribution density has been determined in accordance with DIN 66 141

*2.3. Chemicals and reagents*

Chromatographic grade methanol was used for HPLC analyses (VWR, Austria). Water for HPLC was prepared with Millipore purifier (Millipore, USA). Ammonium acetate ($NH_4Ac$), cupper(II) chloride, gallic acid, cyaniding, 6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid (Trolox,) and reagents 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), neocuproine (2,9-dimethyl-1,10-phenanthroline), Folin–Ciocalteu reagent, phosphate buffered saline, pH 7.4, (PBS), were purchased from Sigma-Aldrich.

*2.4. Preparation of extracts*

The extraction of the components of the polyphenol complex was carried out according to the procedure described by Zhang with slight modifications [14]. The milled beans material was weighed with 0.0001 g precision and 5g were used for analysis. The initial milled material was extracted with 70% methanol containing 1% hydrochloric acid for 12 hours in dark at room temperature. All samples were filtered under vacuum. The extraction was repeated three times, as the second and third extractions were performed via ultrasonic for 30 minutes. The supernatants were combined, centrifuged (5000 rpm for 10 min) and evaporated under vacuum to dryness with temperature of the water bath 40°C. The dry extracts were weighed and dissolved in methanol containing 0.1% hydrochloric acid. The volume of all samples was adjusted to 3 ml and passed through a membrane filter with pore size of 0.45 μm prior for analysis.

*2.5. Determine of total phenolic content (TPC)*

The total phenolic content in crude extracts was determined with colorimetric method using Folin-Ciocalteau's reagent [15] with slight modifications [16]. Calibration curve was achieved using as standard ethanolic solution of gallic acid at concentrations between 50 and 1000 μg/ml. Briefly, 50 μl of extract or gallic acid standard was mixed with 3 ml distilled water, 250 μl of 0.2 M Folin-Ciocalteu's reagent and 1.7 ml of 7.5 % sodium carbonate ($Na_2CO_3$) solution. The tested samples were incubated for 2h in dark at room temperature. The absorbance of the samples was measured at 765nm with a spectrophotometer (Camspec M508, England), using a blank sample. The total phenolic content was expressed as mg gallic acid equivalent per grams of dry weight (mg GAE/g DW) based on the calibration curve.

*2.6. Free radical scavenging assay*

The DPPH• method was assessed according to the method of Blois [17] with some modifications [18]. Daily a solution of DPPH• reagent was prepared with concentration 0.12 mM. Two milliliters of the DPPH• solution was placed in a vessel and 2 mL of the extract was added in various concentrations (2.5, 5, 10, 20, 50, 100, 200 and 300 μg/mL extract). The mixtures were placed in the dark for 30min at room temperature. The absorbance of the samples was measured at 517 nm using a spectrophotometer. All measurements were done under dim light. The percentage RSA of the samples was evaluated by comparing with a control and calculated using the following formula:

$$\text{RSA, \%} = \left[\frac{A_0 - A_b}{A_0}\right] \times 100$$

where $A_0$ is the absorbance of the control blank and $A_b$ is the absorbance of the samples. $IC_{50}$ (μg/mL) is defined as the concentration of an extract that causes 50% loss of the colour. The higher the radical scavenging potential is, the lower is the value of $IC_{50}$. The mean $IC_{50}$ was calculated on the basis of three repetitions and by means of interpolation of graphical dependence of concentration and degree of inhibition of the DPPH radical. The DPPH• assay was calibrated using standard cyanidin ($IC_{50}$ 0.25 μg/ml) and was determined for every set of samples.

*2.7. Trolox Equivalent Antioxidant Capacity (TEAC)*

*2.7.1. ABTS assay*

The Trolox Equivalent Antioxidant Capacity (TEAC) was determined by using the colorimetric method reported from Re et al. [19]. For this assay, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) cation radical (ABTS•+) solution was prepared by dissolving 7 mM of ABTS in 2.45 mM $K_2S_2O_8$. This mixture was shaken for 12 - 16h at ambient temperature in the dark until obtaining a stable oxidative state. For the study of the extracts, the ABTS•+ stock solution was diluted with PBS until absorbance became 0.70 ± 0.02 at 734 nm. Sample analysis was performed as follows: 2 ml of ABTS solution and 20µl of sample or standard were mixed. Absorbance of sample was measured at 734nm with a spectrophotometer Camspec M508, England after samples incubation at 25°C for 5 min. The calibration curve was plotted by using 6-hydroxy-2,5,7,8- -tetramethylchromane-2-carboxylic acid (Trolox) as a standard. The results were expressed as mmol Trolox equivalents per g of dry weight (mM TE/g DW).

*2.7.2. Cupric ion reducing antioxidant capacity (CUPRAC) assay*

The bean extracts were investigated by cupric ion reducing antioxidant capacity (CUPRAC) method described by Apak et al [20]. In test tubes were mixed 1 ml $CuCl_2$ solution (10 mM), 1 ml neocuproine alcoholic solution (7.5 mM) and 1M $NH_4Ac$ buffer solution (pH = 7), 0.2 ml tested extract or trolox and 0.9 ml water (final volume, 4.1 ml). Absorbance against a blank sample was measured at 450 nm with a spectrophotometer Camspec M508, England after 30min in dark at room temperature. Calibration curve was achieved using 6-hydroxy-2,5,7,8- -tetramethylchromane-2-carboxylic acid (Trolox) as a standard ethanolic solution at concentration ranges between 0.045 and 1.5 mM. The trolox equivalent antioxidant capacity was plotted as mmol Trolox equivalents per g of dry weight (mM TE/g DW).

*2.7.3. DPPH assay*

Antioxidant activity was measured according to Brand-Williams [21] procedure. In the test tubes were mixed 100 µl of extract or Trolox and 2.9mL of 0.12mM DPPH (2,2-diphenyl- 1-picrylhydrazyl) reagent, which was prepared with 4,8mg DPPH dissolved in 100 mL $CH_3OH$). The mixtures were shaken and then incubated for 30 min at room temperature. The absorbance was recorded at 517 nm with a spectrophotometer Camspec M508, England. To quantify the antioxidant activity a standard Trolox curve (6-hydroxy-2,5,7,8-tetramethylchroman-2- carboxylic acid) is used with concentration from 0.045 to 1.5mmol Trolox. The results were expressed as mM TE/g dry weight.

*2.8. Statistical Analysis*

All the analyses were made in triplicate. Statistical differences between samples were tested using ANOVA. Dates were expressed as mean ± SD. The level of significance was set at $p<0.05$.

## 3. RESULTS AND DISCUSSION

*3.1. Sieve analysis of dark and speckled beans samples.*

The obtained results were processed and a distribution density curve of the obtained fractions is shown (Fig. 2). The distribution density of the fractions in both samples of ground beans in the range of 150 and 1000 µm is characterized by significant changes, i.e. the distribution of the fraction sizes is uneven.

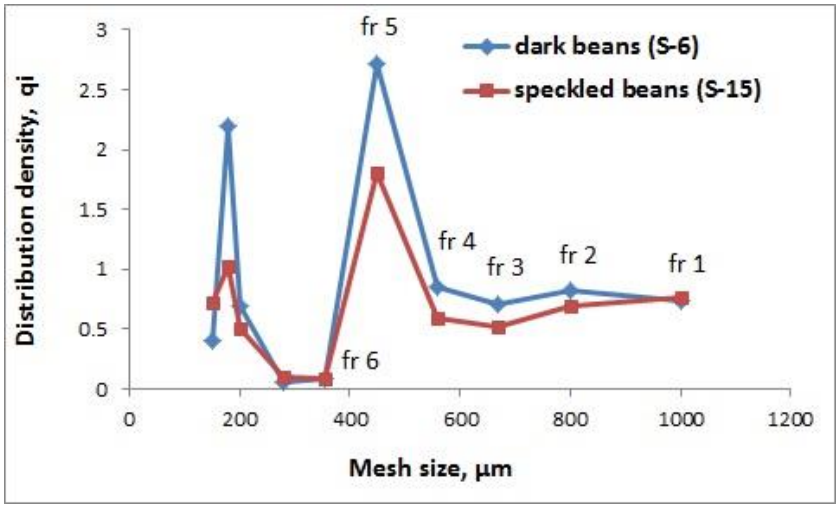



**Fig. 2.** Distribution density of the fractions of both types beans.

Two characteristic peaks are shown in the values of this indicator – at 180 µm (fractions with particle size similar to the particle size of flours from cereals) and at 450 µm (similar to the size of semolina fractions from cereals). The course of the curves across the entire range of sizes in both samples is very close to overlap. Based on the observations it can be assumed that the milling process of the dark and speckled beans on impact at a peripheral speed of 64 m/s results in the formation of a conglomerate of particles obtained from cotyledons and husks with significant differences in size and quantity and this is probably due to the difference in the strength of the internal grain structure. The sieve analysis allows the study of the distribution of the total phenolic content in the all 6 fractions (Fig. 2). The experiments were directed to establish a correlation between distribution density, TPC and antioxidant activity.

*3.2. Analysis of total phenolic content (TPC)*

The TPC of the examined fractions of both samples (S-6, S-15) are shown in Tables 1 and 2. The values of TPC are in the range from 1.04-1.84 mg GAE/g DW for fractions of S-6 and 2.2-3.7 mg GAE/g DW for fractions of S-15. It's obvious that fractions 3 and 4 from sample S-15, as well as fractions 4, 5 and 6 of sample S-6, are characterized by a high content of total phenols. The results were statistically processed at $p > 0.05$. It is known from the literature that the TPC of the colored bean types is higher than the white beans. Our received data shows the opposite case as the total phenolic content is lower in the dark bean fractions instead in the speckled beans (Fig. 3). We have established that the low distribution density fractions show high phenolic content. These results of TPC correlate very well with the antioxidant activity.

| **S-6** | **fr 1** | **fr 2** | **fr 3** | **fr 4** | **fr 5** | **fr 6** |
|---|---|---|---|---|---|---|
| **TPC, mg GAE/g DW** | 1,04 ± 0,05 | 1,18 ± 0,04 | 1,29 ± 0,07 | 1,81 ± 0,08 | 1,55 ± 0,06 | 1,84 ± 0,10 |
| **$_{DPPH}IC_{50}$ , µg/mL** | 54,71 ± 0,58 | 43,07 ± 0,92 | 41,82 ± 1,44 | 44,40 ± 0,51 | 44,62 ± 2,61 | 47,90 ± 1,23 |
| **$TEAC_{DPPH,}$ mM TE/g DW** | 4,82 ± 0,54 | 5,74 ± 0,54 | 5,86 ± 0,16 | 7,24 ± 0,16 | 7,93 ± 0,27 | 6,51 ± 0,33 |
| **$TEAC_{CUPRAC,}$ mM TE/g DW** | 4,56 ± 0,10 | 4,13 ± 0,15 | 4,63 ± 0,20 | 7,84 ± 0,50 | 7,05 ± 0,31 | 6,26 ± 0,45 |
| **$TEAC_{ABTS,}$ mM TE/g DW** | 3,87 ± 0,27 | 3,80 ± 0,22 | 4,08 ± 0,31 | 5,18 ± 0,24 | 4,56 ± 0,49 | 4,42 ± 0,33 |

**Table 1.** Results for TPC, $IC_{50}$ and TEAC for all dark bean (S-6) fractions.

| S-15 | fr 1 | fr 2 | fr 3 | fr 4 | fr 5 | fr 6 |
|---|---|---|---|---|---|---|
| **TPC, mg GAE/g DW** | 2,20 ± 0,04 | 2,75 ± 0,36 | 3,71 ± 0,11 | 3,42 ± 0,18 | 2,56 ± 0,25 | 2,25 ± 0,23 |
| **$_{DPPH}IC_{50}$ , µg/mL** | 35,14 ± 1,14 | 34,23 ± 0,42 | 33,58 ± 0,59 | 36,63 ± 0,64 | 34,84 ± 0,23 | 41,54 ± 0,62 |
| **$TEAC_{DPPH,}$ mM TE/g DW** | 9,36 ± 0,78 | 10,36 ± 0,55 | 11,85 ± 0,22 | 11,41 ± 0,22 | 10,02 ± 0,41 | 10,38 ± 0,19 |
| **$TEAC_{CUPRAC,}$ mM TE/g DW** | 11,23 ± 0,39 | 14,57 ± 1,13 | 18,41 ± 1,02 | 17,02 ± 1,16 | 13,59 ± 0,53 | 12,38 ± 0,64 |
| **$TEAC_{ABTS,}$ mM TE/g DW** | 10,57 ± 0,69 | 11,98 ± 1,01 | 14,62 ± 1,02 | 13,99 ± 0,82 | 11,95 ± 1,06 | 12,16 ± 0,43 |

**Table 2.** Results for TPC, $IC_{50}$ and TEAC for all speckled bean (S-15) fractions.

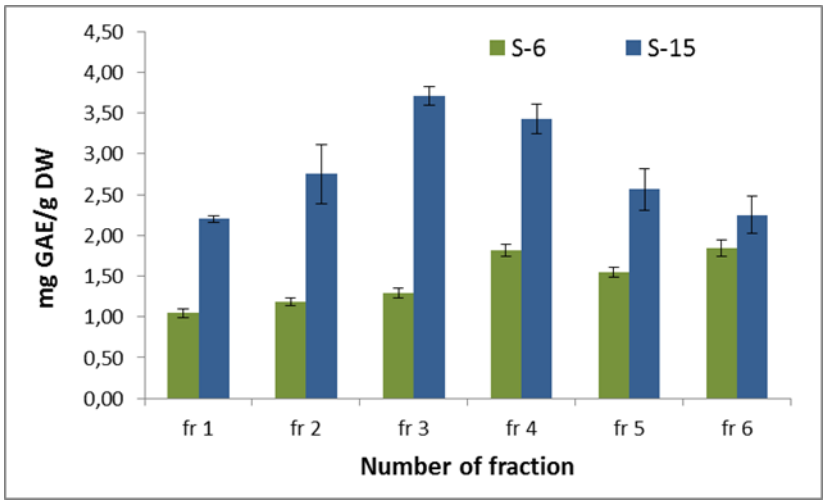



**Fig. 3.** Total phenolic content (TPC) of the dark and speckled beans examined fractions.

*3.3. Antioxidant activity*

The antioxidant activity of all methanol extracts varies significantly. The antioxidant activity of the various extracts was evaluated by various methods, such as the ability to deactivate free radicals as well the ability to reduce copper (II) ions using ABTS, CUPRAC and DPPH analysis, respectively. The results of the antioxidant activity study are presented in Tables 1 and 2.

The TEAC results are determined by ABTS analysis are presented in Table 1 and 2 and shown at Fig. 4. The analysis is based on the ability of the antioxidants to decolorize the stable blue/green form of ABTS•+. The TEAC grade of the dark and speckled bean fractions varies widely 3,8-5,8 mM TE/g DW for S-6 and 10,57-14,62 mM TE/g DW for S-15 (Fig. 4).

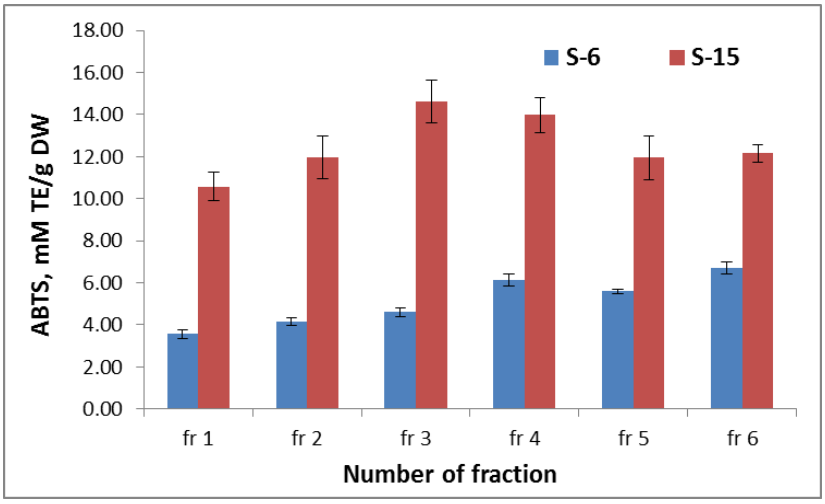



**Fig. 4.** ABTS assay of the dark and speckled beans examined fractions.

The results for antioxidant activity show that the speckled bean (S-15) fractions are characterized by the highest TEAC. From the literature is known that the values of TEAC for the white beans are in the range 27-43 μM TE/g DW [22], for the red beans between 0.19 and 1.49 mM TE/g DW [23,24], and for the black and brown bean is in the range 2.2-5.2 μM TE/g DW [25]. In the sieve analysis the fractions were enriched with total phenols. This can be seen from the antioxidant activity, which increases in the order of 100 to 4000 times. We also found that the distribution density of fractions 3 and 4 (S-15) were low and exhibited the highest trolox equivalent antioxidant capacity (TEAC). This dependence is well expressed for fractions 4 and 6 of S-6.

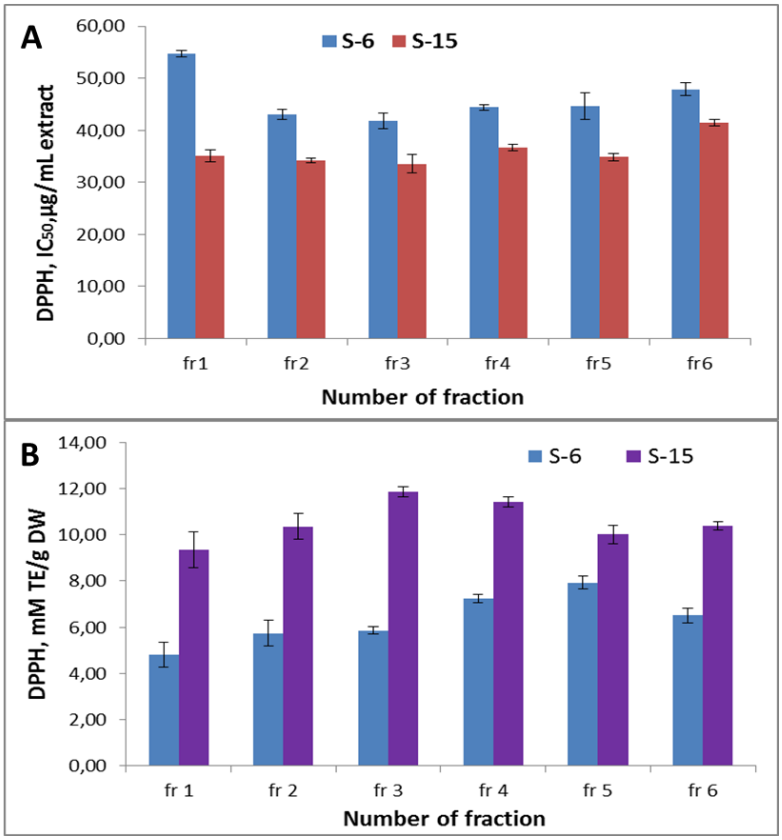



**Fig. 5.** DPPH assay of the dark and speckled beans examined fractions: A-$IC_{50}$; B-TEAC.

The antioxidant activity of the extracts was investigated by DPPH analysis, including the $IC_{50}$ and TEAC assay. Research has shown that extracts have high antioxidant properties. For inhibition of 50% of free DPPH radical, several μg (Table 1, 2) are required. All speckled beans fractions show higher antioxidant activity compared to the dark beans with an $IC_{50}$ of 33.58-35.14 μg/ml (Fig. 5a). The TEAC values for the speckled beans fractions vary in the range of 9.36-11.85 mM TE/g DW (Fig. 5b).

It was also interesting the performing of CUPRAC method analysis of the methanol extracts of all examined fractions of the dark and speckled beans, as the results shows that the values of the antioxidant activity vary widely (Table 1,2 and Fig. 6). High antioxidant properties were observed in all examined speckled beans fractions in the range of 11.23-18.41 mM TE/gDW, as the values are 2 to 4 times higher than those of the dark bean results (Table 1, 2 and Fig. 6). The resulting high values for the speckled bean fractions obtained by the CUPRAC method correlate very well with both the low distribution density and the high content of total phenols.

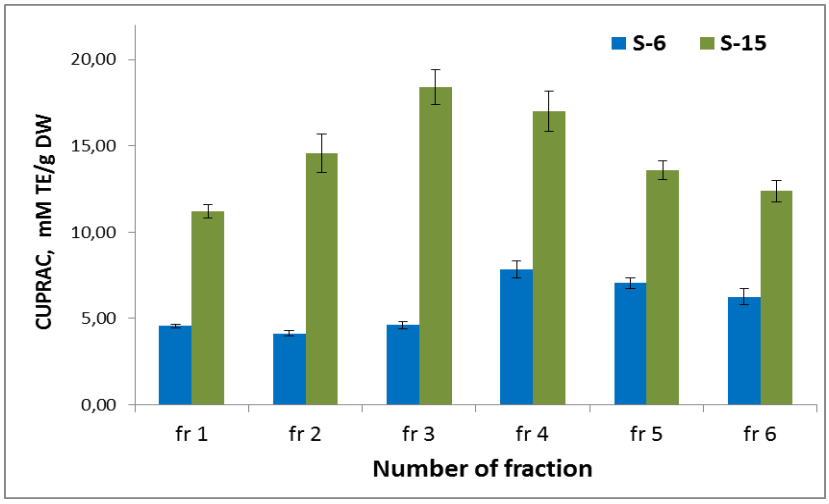



**Fig. 6.** CUPRAC assay of the dark and speckled beans examined fractions.

| S-6 | mg GAE/g DW | $TEAC_{CUPRAC}$ | $TEAC_{ABTS}$ | $TEAC_{DPPH}$ |
|---|---|---|---|---|
| mg GAE/g DW | 1 | | | |
| $TEAC_{CUPRAC}$ | 0.7328 | 1 | | |
| $TEAC_{ABTS}$ | 0.7262 | 0.9328 | 1 | |
| $TEAC_{DPPH}$ | 0.5614 | 0.7431 | 0.6215 | 1 |

| S-15 | mg GAE/g DW | $TEAC_{CUPRAC}$ | $TEAC_{ABTS}$ | $TEAC_{DPPH}$ |
|---|---|---|---|---|
| mg GAE/g DW | 1 | | | |
| $TEAC_{CUPRAC}$ | 0.9827 | 1 | | |
| $TEAC_{ABTS}$ | 0.8810 | 0.9119 | 1 | |
| $TEAC_{DPPH}$ | 0.8725 | 0.9077 | 0.9864 | 1 |

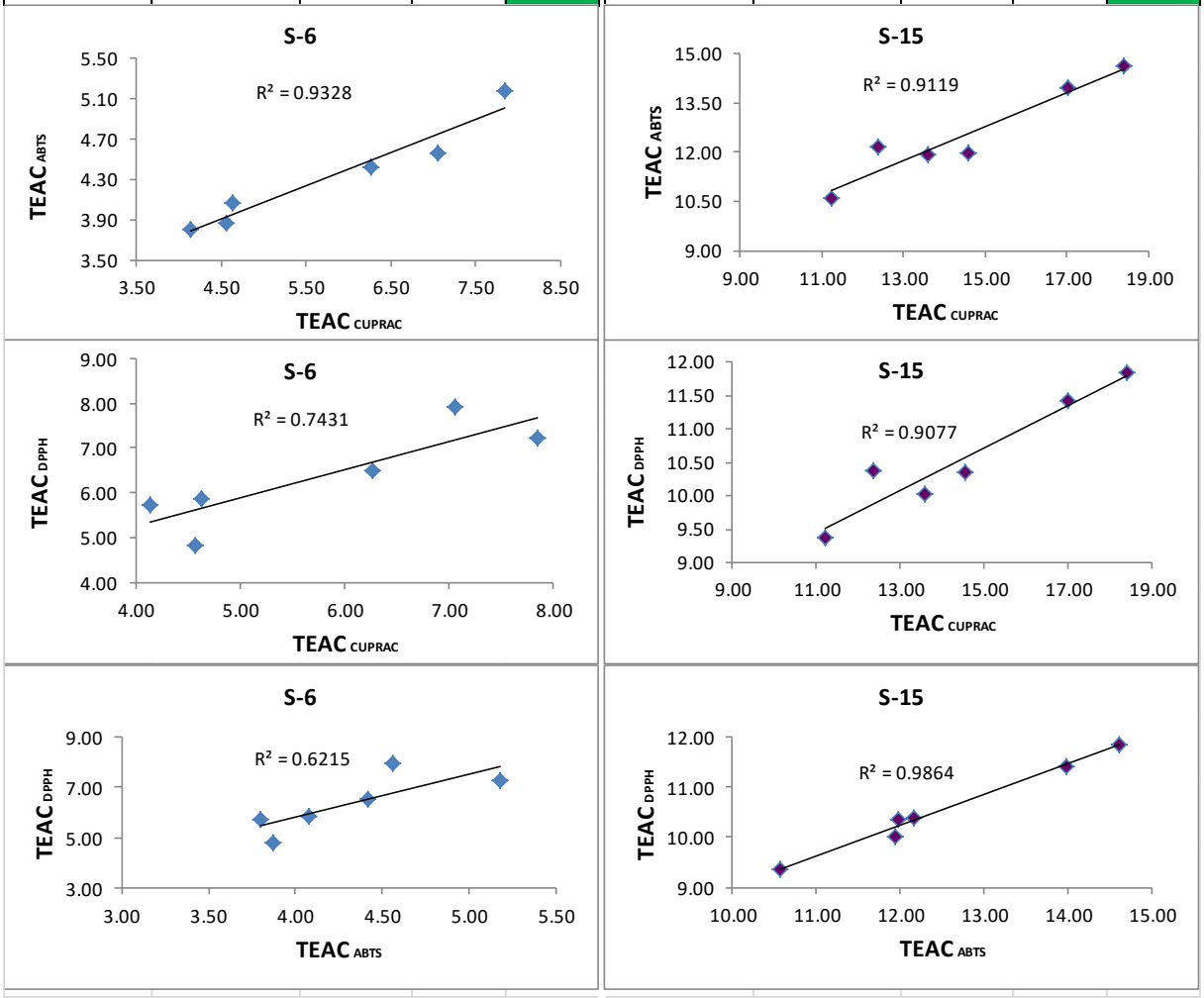



**Fig. 7.** Correlations between TPC/TEAC and TEAC/TEAC of the dark and speckled beans examined fractions.

The results of all analyzed fractions allowed us to establish a correlation between the total phenolic content (mg GAE/g) and trolox equivalent antioxidant capacity (mM TE/g), expressed as $R^2$. Figure 7 shows the correlation between the total phenolic content (TPC) and the different TEAC methods, as well as between the antioxidant activity methods. The obtained values ($P > 0.05$) indicate that $R^2$ between the total phenolic content (TPC) (mg GAE/g) and trolox equivalent antioxidant capacity (TEAC) (mM TE/g) are in the range of 0.5614 to 0.9827. From these results it can be concluded that the best correlation is found in the speckled beans unlike the dark beans. The obtained values for $R^2$ are 0.9827 for CUPRAC relative to Folin, 0.8810 for ABTS versus Folin and 0.8725 for DPPH versus Folin. From the presented data, it was found that the CUPRAC values best correlate with the total phenolic content. The CUPRAC analysis is the most recent method of measuring the total amount of antioxidants relative to the total phenolic content and was determined by the Folin reagent [20]. It was observed a very good correlation between different TEAC methods as well (Fig. 7), as this dependence is being best expressed for the fractions of the speckled bean (S-15).

## 4. CONCLUSIONS

The results obtained from the analysis shows that speckled bean fractions are characterized by the highest total phenolic content. This correlates with the high TEAC (ABTS, CUPRAK, DPPH) and low values of $IC_{50}$ to DPPH. The sieve analysis have been made gave the opportunity the distribution of total phenolic content to be established in the different fractions depending on the distribution density. The results of this study showed that the speckled bean fraction 3 and 4 had the highest antioxidant activity. These results are also confirmed in the DPPH, ABTS and CUPRAC analyzes. Analyzes have shown that speckled beans exhibit a higher antioxidant activity than dark beans. However the fractions 3 and 4 have low distribution densities, they exhibit the highest antioxidant activity. This tendency is also observed in the dark beans, but with few exceptions. The enrichment of phenols in these fractions is very important because high phenolic content and high antioxidant activity are directly related to the antitumor activity [26]. This is why the sieve analysis was carried out; it is related to the separation of flour and meal and enrichment of the fractions (1-6) with biologically active phenolic compounds.

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