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Determination of Total Content of Phenolic Compounds and Their Antioxidant Activity in Vegetables—Evaluation of Spectrophotometric Methods

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This research studies in detail the contents of phenolic compounds determined by the Folin−Ciocalteu reagent and the antioxidant activities determined by the TEAC (Trolox equivalent antioxidant capacity), DPPH (using diphenyl-*p*-picrylhydrazyl radical), and FRAP (ferric reducing antioxidant power) methods, and their correlations for used standards with these methods (catechine, gallic acid, caffeic acid, ferulic acid, Trolox, ascorbic acid, and ferrous sulfate) and extracts from several species of commonly consumed vegetables were studied in detail. The comparison of absolute values of absorption coefficients for used standards and for individual methods allows one to choose optimal common standards for methods to be compared. The procedures applied for the same sets of the extracts using identical calibration procedures and common standards allowed better comparison of the results obtained by the TEAC, DPPH, and FRAP methods. The values of content of phenolic substances and total antioxidant activity of the sets of samples correlate very well for all used methods. The very high values of antioxidant activity were found in intensely colored vegetables (red cabbage, red onion, etc.), and the values were very low in watery vegetables such as potato, marrow, and cucumber.

KEYWORDS: Vegetables; phenolic compounds; antioxidant activity; spectrophotometry; unified standardization

INTRODUCTION

Phenolic antioxidants, a specific group of secondary metabolites, play the very important role of protecting organisms against harmful effects of oxygen radicals and other highly reactive oxygen species. Their formation in human organisms is closely connected with the development of a wide range of degenerative and nondegenerative diseases, mainly arteriosclerosis and other associated complications, cancer, indispositions, and last but not least with the accelerated aging of organisms (*1*−*4*).

Some preventive and defensive systems against the attack of the reactive substances exist in the human organism; however, they cannot eliminate harmful activities of such substances completely, particularly when their production is increased in some metabolic, physiologic, pathologic, and other situations. An adequate intake of natural antioxidants in food is therefore of great importance for protection of macromolecules against oxidative damage (*4*, *5*) in cells (mainly unsaturated fatty acids in lipids, cholesterol, different functional polypeptides and proteins, and nucleic acids).

Plants are rich in phenolic compounds of different origins and functions. Most of them belong to the principal, biologically highly active components (antiviral, anticancerogenic, etc.) of foodstuffs of plant origin (*2*, *6*−*8*). Antioxidant activity plays, in many cases, a basic role in their pharmacological effects; thus, it can be considered the most important (*9*). The basic plant constituents of food should be a sufficient source of phenolic compounds for humans. However, there is a limited amount of information on the content of phenolic compounds in common foodstuffs of plant origin and their antioxidant activities.

The content of phenolic compounds in plant materials can be determined by several separation methods [high-performance liquid chromatography (HPLC), gas chromatography (GC), capillary zone electrophoresis (CZE), etc.] as a set of individual substances or by a specific chemical reaction as a group of chemically similar reactive compounds. The chromatographic determination is highly precise and accurate with high informative value, but it is highly problematic to identify all of the phenolic compounds in a specific plant material at acceptable times and costs of analysis. The determination of antioxidant activity by HPLC is experimentally more complicated, and at present, procedures are mostly presented to show methodological principles.

Generally, it is necessary to analyze several samples since the contents of phenolic substances can vary in different varieties and cultivars. In addition, contents of phenolic substances are influenced by a large number of external factors such as agrotechnical processes, climatic conditions and ripeness during

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harvest, postharvest manipulations, and time of consummation (*10*). At the present time, sufficient information for the evaluation of content of total phenolic compounds and total antioxidant activity as a criterion of nutritional value of plant foodstuffs can be easily obtained from spectrophotometric measurements employing specific analytical reagents.

The determination of antioxidant activity of plant extracts is still an unresolved problem. Approximately 20 analytical methods such as applying different reagents, reaction mixture composition, standards, analytical evaluations, and others are used. The exact comparison of the results and their general interpretation are practically impossible due to the variability of experimental conditions and differences in physicochemical properties of oxidizable substrates. Furthermore, the antioxidant activity of the substances in foodstuffs and other biological systems depends on the applied test system (method) and substrate that should be protected by the antioxidative substance.

A huge number of factors, including colloidal properties of substrate, experimental conditions, reaction medium, oxidation state, and antioxidant localization in different phases, can influence the activity. The applied methods can be divided into two basic groups: (i) the test with lipophilic substrates and (ii) the test with hydrophilic substrates. Furthermore, the composition of the system, type of oxidizable substrate, method used, and the way of quantification of antioxidant activity are of great importance during the tests (*11*).

Each determination should be done at different experimental conditions of the oxidation reaction using at least two or more methods for quantification of different products of oxidative reaction, and finally, the general trends of values for individual samples should be compared. In addition, a unified standardization of the antioxidant activity test is highly recommended (*11*). Hydrolyzed extracts contain more active antioxidants since it is well-known that the glycosides of phenolic substances are weaker antioxidants than the corresponding aglycones (*12*, *13*).

The main purpose of the present study was to determine the total content of phenolic compounds in selected species of commonly consumed vegetables and to determine and compare the antioxidant activity of plant extracts applying three commonly used spectrophotometric methods: TEAC (Trolox equivalent antioxidant capacity), FRAP (ferric reducing/antioxidant power), and DPPH (with 2,2-diphenyl-1-picrylhydrazyl assay). The analytical procedures were modified to allow semimicroscale measurements at the same experimental conditions using a large set of extracts with acceptable reproducibility. The selection of a suitable, generally applicable standard for all methods allowed us to obtain a set of simply comparable results.

MATERIALS AND METHODS

Instruments. A spectrophotometer HELIOS β (declared reliability of measuring to 2.0 AU) controlled with program VISION 32 Software (Spectronic Unicam, Cambridge, United Kingdom), an ultrasonic bath HD 2070 (model M8 72, Bandelion Sonoplus, Germany), a high-speed mill Grindomix (Retsch, Germany), and a CHRIST ALPHA 1-2 B lyophiliser (Braun Biotech International, Germany) were used for sample preparation. An HP 1100 chromatographic system (Hewlett-Packard, Waldbronn, Germany) with a mass-selective HP MSD detector (G1946A, Hewlett-Packard, Palo Alto, CA) was used for ascorbic acid determination.

Chemicals. Caffeic acid, ferulic acid, ascorbic acid (purity ≥ 99.0% each), 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•, ≈90.0%), and 2,2′-azino-bis-(3-ethylbenzothiazolin-6-sulfonate) diammonium salts (ABTS, ≈98.0%) were purchased from Sigma-Aldrich Chemical Co. (United States); gallic acid monohydrate (≥98.0%), Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid, a hydrophilic derivative of tocopherol, purum, ≥99%, for HPLC), Folin−Ciocalteu reagent (FC reagent), and 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ, puriss, ≥99.0%) were from Fluka Chemie (Buchs, Switzerland). Methanol and acetonitrile of gradient grade were from Merck (Darmstadt, Germany). Other chemicals of p.a. purity were from Pliva-Lachema (Brno, Czech Republic). All reagents and standard solutions were prepared using Milli Q deionized water (Millipore, Bedford, MA).

Table 1. Comparison of Our Contents of Phenolic Compounds Determined by FC Method and Dry Matter (DM) with Published Results of Kähkönen et al. (*31*) and Vinson et al. (*16*)

		content of phenolic compounds							
		total[a]		free[b]		total[b]		DM	
		x	$x \pm s_x$	x		x		%	
n	vegetable	S[c]	*31*	S[c]	*16*	S[c]	*16*	S[c]	*16*
1	carrots (garden)	9.9	0.6 ± 0.0	5.4	4.7	20.3	15.3	19.8	
2	carrots (Delvita stores)	14.5		13.9		29.9		12.5	10.6
3	parsley	10.9		6.3		22.5		36.1	
4	celery	10.6		7.6	7.0	21.7	13.6	13.1	8.8
5	red beet	20.7	4.3 ± 0.2	20.7	45.2	42.6	53.4	22.0	15.4
6	red radish	15.0	0.9 ± 0.1	28.7		30.9		3.9	
7	onion yellow	18.2	2.5 ± 0.1	12.4	4.7	37.5	22.9	16.2	10.5
8	onion red	21.2	3.0 ± 0.9	25.5	11.4	43.6	41.0	13.6	9.8
9	garlic	18.9		10.5	5.2	38.9	34.3	35.2	37.6
10	leek	13.7		11.3		28.3		13.2	
11	potato, var. Laura (red)	4.5	4.3 ± 0.2	5.8	3.5	9.3	5.9	24.6	
12	potato, var. Korela	6.8	2.5 ± 0.1	8.1		13.9		19.1	20.3
13	potato, var. Katka	5.4		7.4		11.2		23.9	
14	lettuce	26.8		20.3	8.4	55.2	16.9	3.4	4.7
15	spinach paste	15.5		20.4	13.4	32.0	27.6	7.2	6.2
16	cabbage white	12.1		10.5	9.5	25.0	19.2	11.6	9.4
17	cabbage red	36.3		129.0		74.7		13.6	
18	cabbage green	13.5		11.1		27.9		18.9	
19	cabbage Chinese	11.0		5.3		22.6		5.5	
20	cauliflower	14.0		10.8	11.7	28.7	20.9	11.2	8.6
21	kohlrabi green	10.0		7.9		20.5		13.3	
22	broccoli	18.1		18.2	17.5	37.3	40.6	10.1	8.9
23	tomato	10.7	2.0 ± 0.1	9.4	9.5	22.0	18.9	8.3	6.9
24	pepper green	15.1		18.4	16.4	31.2	26.1	8.1	5.7
25	pepper red	19.3		23.1		39.7		11.1	
26	pepper red (Spain)	16.5		25.6		34.0		11.0	
27	pepper red (Spain)	12.6		32.6		25.9		12.5	
28	cucumber	10.6	3.8 ± 0.1	7.7	4.4	21.9	15.5	5.0	2.6
29	marrow	7.7		6.6	5.6	15.9	17.6	5.6	5.1
30	little bean, frozen	10.6	1.6 ± 0.1	3.9		21.8		25.6	
31	little bean green, frozen	10.5		6.2	8.0	21.6	17.8	11.0	8.4
32	maize delicates, frozen	12.7		7.8	13.9	26.2	19.1	33.5	25.7

[a] GE, gallic acid equivalent in mg/g DM. [b] CE, catechin equivalent in μmol/g DM. [c] Our results.

Sample Preparation. Samples (26 kinds of vegetables commonly consumed in the Czech Republic; see **Table 1**) were purchased at a local market (Delvita stores). Edible parts of the vegetables (20−50 g) were cut into small peaces (<0.5 cm) and lyophilized at −52 °C for 48−72 h in a vacuum. The dry matter was determined gravimetrically (±1 mg) by the difference before and after lyophilization. Lyophilizates were homogenized in a laboratory ultramixer to tiny particles and sieved through a plastic sieve (<0.5 mm). The powder was stored in plastic bottles under nitrogen at −20 °C until analyses. Lyophilization of the native sample was assumed to be the most cautious method for isolation of phenolic compounds, and it was preferred as the less cautious homogenization of fresh samples (*14*). It minimized aerial and enzymatic oxidation of phenolic compounds that are more intense at a higher content of water and at laboratory temperature.

Extraction and Hydrolysis. A modification of procedure for extraction of phenolic compounds published by Vinson et al. (*15*, *16*) was employed. Briefly, the homogenized powder (2 × 500 mg; ±0.2 mg) was weighed and equally divided into two plastic bottles with screw cups. An aqueous methanol (1:1, v/v, 10 mL) was added into one bottle to extract free phenolic compounds while the same amount of acidified aqueous methanol (1:1, v/v, 2.4 mol/L HCl, 10 mL) was added into the second bottle to extract all phenolic compounds (free and conjugated usually with saccharide moiety). The suspensions were sonicated under nitrogen atmosphere for 2 min at 90% power for better destruction of cell walls and then incubated at 82−83 °C (bp of the extraction agents) for 150 min with vortexing every 30 min for 20 s. After incubation, methanol (10 mL) was added into the cooled samples, the mixture was vigorously agitated, and the suspension was centrifuged at 6000*g* for 10 min. Acidic extracts were neutralized to pH 7 since results of some methods were pH-dependent. Supernatants were stored at −20 °C under nitrogen in plastic vials with screw caps. Extracts were stable for at least several days or several weeks at 4 and −20 °C, respectively.

Determination of Phenolic Compounds in Plant Extracts. The Folin−Ciocalteu method (FCM), based on the reduction of a phosphowolframate−phosphomolybdate complex by phenolics to blue reaction products, was used to determine phenolic compounds (*16*−*18*). The total volume of reaction mixture was miniaturized to 1 mL. An absorbance was measured twice (four or six times in some cases) for each sample at 760 nm against blank (100 μL water) using 2 mmol/L gallic and ferulic acids and (+)-catechine as standards. Five points calibration was linear to a concentration of 0.2 mmol/L in the reaction mixture and an absorbance range up to 3.0 AU. Highly reproducible results for standards ($R^2 > 0.997$) and samples were obtained except for some samples with high concentrations of phenolics, partly due to dilution and dosing of small volumes (5 μL) of extracts.

Ascorbic acid and main mono- and disaccharides (glucose, fructose, and saccharose) present in vegetables and fruits can interfere. Corresponding corrections for ascorbic acid interferences are experimentally complicated due to its instability and due to fast subsequent reactions. In addition, most of the published methods for ascorbic acid determination are unsuitable for plant extracts. Thus, the HPLC/MS method was applied. Interferences of saccharides were less evident than those of ascorbic acid (see parameters of calibration curves), and their influence for the samples with higher contents of saccharides could be estimated on the basis of the mean contents of phenolics and saccharides (*18*).

Determination of Antioxidant Activity/Capacity of Plant Extracts. Three methods, TEAC, FRAP, and DPPH$^\bullet$, based on reaction with electron-donating or hydrogen radicals (H$^\bullet$) producing compounds/antioxidants according to the reaction $R^\bullet + \text{Aox-H} \rightarrow \text{RH} + \text{Aox}^\bullet$, were used. Electron transfer and hydrogen atom transfer reactions can be difficult to distinguish. Hydrogen atom transfer reactions can be the result of proton-coupled electron transfer. Despite the similar redox mechanisms of the methods, reagents and products are different. Also, performance and interpretation differ considerably in publications. Thus, reciprocal comparison of results of individual methods and results among publications for the same method is often problematic.

Trolox and ferrous sulfate are usually used for calibration of the TEAC and FRAP methods, respectively, while the DPPH$^\bullet$ method is usually interpreted on the basis of antioxidant amount needed for the decrease of the initial DPPH$^\bullet$ concentration to 50% (EC_{50}); thus, they are the initial DPPH$^\bullet$ concentration dependent. A more objective comparison of the results could be possible by applying the same interpretation procedure with the same common standard and unified standardization procedure.

TEAC Method. The TEAC (or total antioxidant activity) method (*19*−*22*) was modified. Briefly, the total volume of the original analytical procedure (*23*, *24*) was reduced to 1 mL. A (1:1, v/v) mixture of ABTS (7 mmol/L) and potassium persulfate (4.95 mmol/L) was left to stand for 12 h at laboratory temperature in the dark to form radical cation ABTS$^{\bullet+}$. The solution was stable for at least 1 week at 4 °C in the dark.

A working solution was diluted to absorbance values between 1.0 and 1.5 AU at 734 nm with phosphate buffer solution (constant initial absorbance values must be used for standard and samples). Standards or plant extracts (from 5 to 25 μL according to reaction intensity) were mixed with the working solution (975 μL) and diluted up to 1000 μL with deionized water. A decrease of absorbance was measured at 734 nm after 20 min. Aqueous phosphate buffer solution (1 mL, without ABTS$^{\bullet+}$ solution) and Trolox (1.0−2.0 mmol/L) were used as a control and main calibrating standard, respectively.

FRAP Method. A FRAP method (*25*−*27*) was modified to a semimicroscale with a total volume of 1 mL. Briefly, a portion of an aqueous 10 mM solution of TPTZ reagent in 40 mmol/L HCl was mixed with the same volume of 20 mmol/L $FeCl_3 \cdot 6H_2O$ and 10 times higher volume of acetate buffer of pH 3.6 (3.1 g sodium acetate and 16 mL acetic acid per liter). The mixture was incubated at 37 °C for several minutes. A portion (900 μL) of the Fe^{3+}−TPTZ mixture and 20−50 μL of the sample (or standard or water for blank) were diluted up to 1000 μL with deionized water and incubated for at least 4 min (better 10−30 min) (*27*), and the absorbance was measured at 593 nm. Fresh 1 mmol/L working solutions of $FeSO_4$ were used for calibration (dilution of 20 mmol/L stock solution). The antioxidant capacity was calculated from the linear calibration curves (*25*, *28*). The total antioxidant activity further increased seriously (*26*, *27*) between 4 and 30 min (more then 40−50% in most cases).

DPPH$^\bullet$ Test/Method. An original procedure (*29*) was modified (to 1 mL total volume) with calibration using standard solution. The working solution was prepared by dilution of methanolic DPPH$^\bullet$ (98 mg/L, absorbance ≅ 1.9) to absorbance at ≅ 1.5 AU (exactly the same for sample and standard) to gain the sufficient reaction capacity for higher contents of antioxidants in extracts. A portion (950 μL) of the working solution and 5−50 μL of the sample in water or (1:1, v/v) methanolic extract of phenolics (or standard) were diluted up to 1000 μL with aqueous methanol (1:1, v/v) and incubated for at least 20 min (better 30 min to reach plateau) (*30*), and the absorbance was measured at 515 nm against methanol and methanol with a DPPH$^\bullet$ blank.

RESULTS AND DISCUSSION

As can be seen from the **Figure 1A**−**D**, the rate of the reactions of individual standards and samples differs substantially, and the reaction kinetics of samples can be expressed by exponential curves reaching the plateau after 30−60 min, where about 95% yield is obtained. This reaction time corresponds to the optimum incubation period. The best time for absorbance measurements was near 30 min for all four methods. Further prolongation of the reaction time led to some other disadvantages in some cases, i.e., adsorption of color products on the test tube or cuvette walls or evaporation of methanol and thus the increase of absorbance values (concentration of products—this phenomenon can be prevented by using stoppered cuvettes).

Choice of a Suitable Common Standard for the Tested Methods. Strictly linear calibration curves were obtained for all of the methods using different calibration standards (gallic, ferulic, and caffeic acids, catechine, Trolox, and ferrous sulfate) and main interferents (ascorbic acid, glucose, fructose, and saccharose) in concentrations up to 50, 100, or 200 μmol/L of individual compounds in dependence of their antioxidant activity, up to 100 mmol/L of saccharides, and up to absorbance values of 2.0−3.0 AU as long as the reagent was not completely depleted. The linearity of calibration curves allowed quantification of phenolics using any of the above-mentioned standards.

Different calibration standards and different ways of expression of concentrations (dry weight, DW; fresh weight, FW; in molar or mass units) have been applied to express the results in the literature. These facts complicate the comparison of the results from one source to another one.

For example, the content of phenolics (free and total) has been expressed mainly as gallic (GE) or ferulic acids (FE) or catechine (CE) equivalents in molar units or using a mean value of molecular weight (of phenolics, 290.0; of catechine, 298.3; and of gallic acid, 170.1) using DW or FW of analyzed samples.

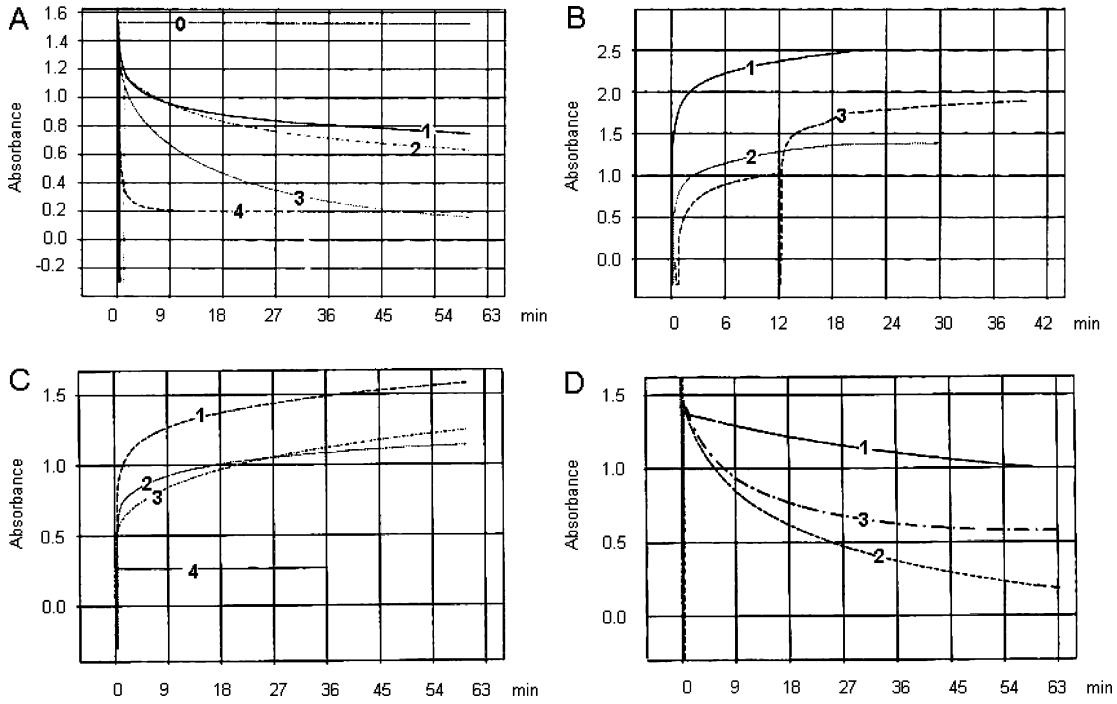

Figure 1. Rate of the reactions of the DPPH (**A**), FCM (**B**), FRAP (**C**), and TEAC (**D**) methods. **A**: 0, DPPH alone; 1, extract of plumb (free phenolics); 2, extract of peach (25 μL); 3, extract of peach (50 μL); and 4, extract of plumb (total phenolics). **B**: 1, ferulic acid; 2, extract of orange; and 3, gallic acid (two injections). **C**: 1, extract of peach; 2, extract of red pepper; 3, extract of green pepper; and 4, reagent alone. **D**: 1, reagent alone; 2, extract of green beans; and 3, extract of carrot.

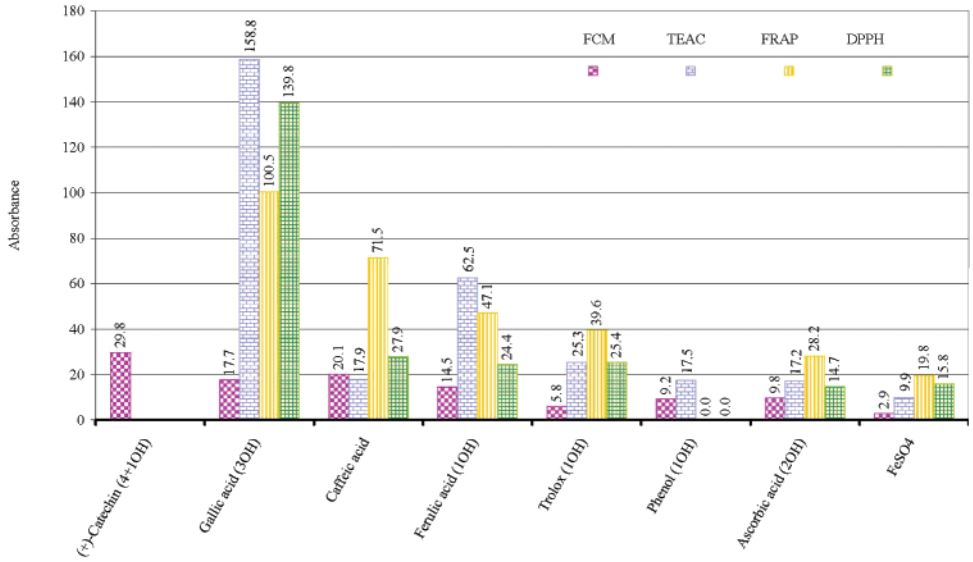

Figure 2. Comparison of the absolute values of mmol absorption coefficients for used standards, phenol, and ascorbic acid determined by the FCM, TEAC, FRAP, and DPPH methods.

A recalculation of determined values in different publications and their comparison is possible if the values of DW (or FW, usually given), parameters of calibration equations (mostly not given), and type of calibration standards are presented. The dry matter values of the samples of vegetables varied in the range of 4−36% in our experiments and were in very good agreement with those in the literature (*16*), with few exceptions (**Table 1**).

Some interpretations of values determined by individual method can be deduced from the comparison of the absolute values of molar absorptivities of samples (**Figure 2** and **Table 2**). Two of three hydroxyl groups of gallic acid and three of four free hydroxyl groups (five −OH groups are present in total) of (+)-catechine react with FC reagent while one reacting −OH group is free in the case of ferulic acid (*18*). The final absorbance values are usually proportional to the number of reacting phenolic hydroxyl groups and also depend on a structure of the molecule, i.e., reactivity of individual phenolic hydroxyl (**Figure 2**).

For example, two of three reacting hydroxyl groups of gallic acid are less reactive in the FC method then two reacting hydroxyl groups of caffeic acid. The absorbance value for caffeic acid (two reacting OH) is approximately twice and for (+)-catechine (three reacting OH) three times higher than that for phenol (one reacting OH). A higher value was found for ferulic acid (one reacting OH) than for phenol (substituents of the

Table 2. Parameters of Calibration Equations $A = a \times c + b$ for the FC, FRAP, TEAC, and DPPH Methods

	coefficients of calibration equation[a] $A = a \times c + b$			
compound	FCM[b]	FRAP[b]	TEAC	DPPH
gallic acid	0.0177	0.1005	–0.1588 + 1.4875	–0.1398 + 1.4511
caffeic acid	0.0201	0.0715	–0.0179 + 1.4668	–0.0279 + 1.3195
ferulic acid	0.0145	0.0471	–0.0624 + 1.0204	–0.0244 + 1.4363
catechin	0.0298	N	N	N
Trolox	0.0058	0.0396	–0.0235 + 1.0921	–0.0217 + 0.9278
Trolox[c]	N	0.0388	–0.0261 + 1.4325	–0.0254 + 1.4215
phenol	0.0101	NR	–0.0174 + 1.3440	NR
$FeSO_4$	0.0028	0.0198	–0.0099 + 1.3771	–0.0158 + 1.4763
ascorbic acid	0.0128	0.0282	–0.0172 + 0.9906	–0.0147 + 1.3080
glucose	0.0006	NR	–0.0002 + 1.4266	NR
fructose	0.0022	0.0004	–0.0006 + 1.3563	NR
saccharose	0.0005	NR	–0.0011 + 1.4848	NR

[a] A, absorbance; c, concentration (μmol/L, for saccharides in mmol/L); N, not repeated; NR, no reaction. [b] $b = 0$. [c] Separate experiment.

benzene ring decrease the electron density on the hydroxyl group). The value is lower for gallic acid (two reacting OH groups) than the doubled value for phenol (the liberation of the second electron/hydrogen radical is more difficult).

The absolute values of molar absorption coefficients of FRAP methods were proportionally increasing with the number of reacting phenolic hydroxyl groups, and their reactivity corresponded to the influence of the molecular structure on the electron density of the particular phenolic group. A significant decrease of the overall reactivity of phenolic hydroxyls of caffeic acid was observed for the TEAC method. The reactivity of the hydroxyl group of Trolox and ferulic acid was nearly the same for TEAC and DPPH methods, and the reactivity of the second hydroxyl group of caffeic acid was reduced while the reactivity of all three hydroxyl groups of gallic acid was increased (**Figure 2** and **Table 2**).

Measured values by particular methods are therefore determined by selected standards. If the standard used for calibration is highly reactive and gives a high absorbance, then the measured values of samples will be low. A standard with different reactivity by particular method will give very different values for sets of samples measured by a particular method. According to the results in **Figure 2**, Trolox seems to be the best standard for all three methods, since nearly the same results were obtained for the TEAC and DPPH methods and about 50% higher values were obtained for the FRAP method. Slightly less suitable reaction properties were observed for the other standards. On the other hand, for the FCM method, caffeic acid (eventually gallic acid or catechine) seems to be the very suitable standard while Trolox is not suitable due to relatively low reactivity and low values of absorbance values.

Determination of Phenolic Compounds in Plant Extracts. The contents of phenolic compounds (free and total) determined by the FC method for different analyzed vegetables are presented in **Figure 3** as the corrected values for ascorbic acid interferences. Conjugated phenolic compounds prevailed in the vegetables, and their contents varied between 33.7 and 82.2% with the exception of one sort of pepper (24.9%) and radish (7.1%). Contents higher (lower) than 50% were found for 20 (10) species of vegetables, respectively, with a maximum value for little beans at 82.2%. The extreme value of 7.1% for radish might be seriously influenced by the presence of sulfur-containing compounds and may be an artifact.

Our results are mostly similar to those for similar sets of vegetables and fruits (**Table 1**) published by Vinson et al. (*16*). Higher deviations among the results in some cases can be explained by the fact that the content of phenolic compounds in some species of vegetables can be influenced by several internal and external factors. The contents of conjugated phenolics ranged from 23 to 87% for most of the vegetables. Significantly higher (lower) values of free phenolics were found for seven (four) types of vegetables while very similar results were obtained for another seven species from the 18 analyzed. Slightly or significantly higher contents of total phenolics were found for 13 samples, and lower contents were found for five samples.

The content of free and total phenolic compounds for 30 samples (two out-layers) correlates highly and significantly ($r = 0.8092$). The published values from different sources can seriously differ as can be seen from the values for our red beet, which was measured to be five times higher than that of Kähkönen (*31*), but our value is still about 20% lower than that of Vinson et al. (*16*). The total content of phenolic compounds in three varieties of pepper ranged between 810 and 1430 CE mg/kg (CE = catechine equivalent) and was similar to the data of Karakaya (*32*). Colored varieties of vegetables (red onion, red cabbage, and red pepper) are especially rich in phenolic compounds. Red onion contains a higher quantity of both colored and colorless phenolics than the yellow variety. This is in correspondence with the findings of HPLC analyses of the main flavonole of onion, quercetine (*33*). In addition, they also contain anthocyanins, the strongly absorbing phenolic colorants that represent their red color.

The contents of phenolic compounds decrease in the order red cabbage, garlic, red beet, delicates maize, parsley, red onion, yellow onion, green pea, white cabbage, pepper, and broccoli while the lowest values were found for marrow, cucumber, Chinese cabbage, and radish (**Figure 3**).

Determination of Antioxidant Activity/Capacity of Extracts. *Determination by TEAC Method.* The TEAC method is one of the most often used methods for the determination of total antioxidant capacity (*19*−*22*). It is based on a neutralization of radical cation formed by a single-electron oxidation of a synthetic ABTS chromophore to a strongly absorbing $ABTS^{\bullet+}$ radical (700−750 nm) according to the reaction $ABTS - e^- \rightarrow ABTS^{\bullet+}$. The radical is prepared by an oxidation reaction of ABTS with oxidized methmyoglobine by enzymatic peroxidase reaction with H_2O_2 (original method) or (*11*) with potassium persulfate (higher sensitivity).

The radical reacts quickly with electron/hydrogen donors to form colorless ABTS. The reaction is pH-independent. A decrease of the $ABTS^{\bullet+}$ concentration is linearly dependent on the antioxidant concentration, including Trolox as a calibrating standard (*23*, *34*, *35*). The results correlated very well with biological redox properties of phenolics (*36*, *37*).

The highest, however, the less reproducible values of antioxidant activity were obtained in comparison with the other tested methods. The $ABTS^{\bullet}$ reagent was very unstable, and it was slowly degraded at the given experimental conditions. The measurable continuous decrease of absorbance was observed during the incubation period (c. 30% per hour; **Figure 1D**). While the reaction with Trolox (standard) was very fast (reaction time about 1 min) and was constant for at least 30 min (not presented in figures), the reactions with plant extracts were slow with exponential form of the time dependence. The relatively very long time was needed to reach a plateau (even more than 1 h in most cases) although most of the reaction product was formed in the first 10 min.

The continuous decrease of sample absorbance could be affected by spontaneous degradation of $ABTS^{\bullet}$ reagent (they

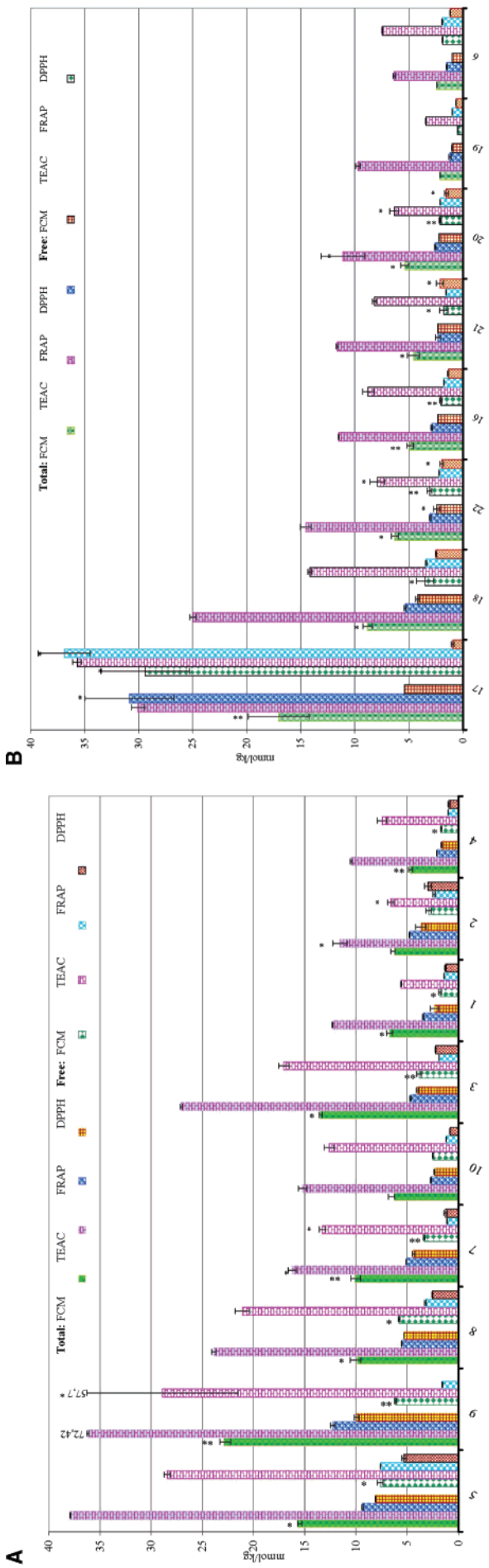

Figure 3. Content of total and free phenolic compounds determined by the FC method (GE) and antioxidant activity determined by the TEAC, FRAP, and DPPH methods (TE) in extracts of vegetables in mmol/kg fresh mass (the numbers correspond to those in **Table 1**). $x \pm$ absolute mean deviation, $n = 2$, $*n = 4$, $**n = 6$), and TEAC × 2 for no. 9.

closely correlate). The different rate of the reactions of ABTS• with Trolox and plant extracts could not eliminate the decrease in absorbance values due to degradation of ABTS• reagent. A steady linear decrease of absorbance after 7 min in the presence of some individual compounds (i.e., ascorbic acid) or an exponential decrease for some other compound (i.e., quercetin) was published (*34*). A measured spontaneous exponential decrease of ABTS• reagent absorbance, an exponential decrease of absorbance at reaction of ABTS• with extracts (**Figure 1D**), and the decrease in absorbance (0.538 AU) for 20 μmol/L Trolox are in agreement with the published data (*24*, *38*).

From the above-mentioned facts, it could be deduced that the determined values for samples should be corrected (decreased) by the values of absorbance decrease obtained for the pure ABTS• reagent. The values without this correction are overestimated.

The antioxidant activity values determined by TEAC method for individual species of vegetables decreased in the order red beet > garlic > freezed maize delicates > red cabbage > parsley > white cabbage > red onion > green pea > potato > yellow onion > leeks > broccoli > spinach > pepper, etc., while the lowest values in increasing order are for cucumber < marrow < kidney bean < cabbage < lettuce < tomato < Chinese cabbage, etc. (**Figure 3**).

Determination by FRAP Method. A FRAP method is based on the ability of antioxidant to reduce (electron transfer) Fe^{3+} to Fe^{2+} ions in the presence of TPTZ forming an intense blue Fe^{2+}−TPTZ complex with an absorption maximum at 593 nm. The reaction is pH-dependent (optimum pH 3.6). The absorbance decrease is proportional to the antioxidant (reductant) content (*25*). Compounds that are active in Fe^{3+} reduction also stimulate the formation of OH• (prooxidation activity).

An absorbance exponentially increased due to the formation of absorbing product of the reaction of antioxidants with Fe^{2+}−TPTZ complex. The reaction with extracts was slow, and the constant and quantitative yield of the reaction plateau was not reached even after 1 h (**Figure 1C**). The results of our observation are in agreement with some published data (*27*). Ferrous sulfate is usually used as a standard; however, the application of more widely used Trolox (TEAC and DPPH) can be advantageous due to linear dependence of absorbance vs antioxidant concentration and its, approximately, twice higher absorbance values as compared to $FeSO_4$. The reaction with Trolox reached the maximum in 1 or 2 min, and the absorbance is constant up to 30 min.

The results of the FRAP method correlated very well with the antioxidant activity measured by electron spin resonance spectroscopy (ESR), i.e., the ability to donate electron or hydrogen atom to the synthetic free radical (*28*) of potassium nitrosodisulfonate (radical of Fremy's salt). A correlation between results of FRAP method and antioxidant activity is questionable (*39*). Carotenoids probably are not active in the FRAP method (*26*, *40*).

The antioxidant activity of phenolic substances in the method increased in agreement with the number of hydroxyl groups. The antioxidant activity was higher for phenolic substances than for ascorbic acid where only one hydroxyl group reacted at the given experimental conditions. The reactivity decreased in the order gallic acid (three OH) > caffeic acid (two OH) > ferulic acid (one OH) > Trolox (one OH) > ascorbic acid (two OH) > $FeSO_4$ in agreement with the parameters of calibration curves (**Table 2**). Of all the used methods, the obtained absorptivity values for standards correlated the best with the number of phenolic hydroxyl groups (**Figure 2**).

The determined values in extracts of vegetables were usually slightly higher than those obtained by DPPH method; however, they were several times lower than those of the TEAC method. They correlated highly significantly with them (**Figure 3**) with the exception of red cabbage.

Determination by DPPH• Method. The DPPH• test/method is one of the oldest and the most frequently used methods for total antioxidant potential/capacity of food extracts (*41*, *42*). It is based on the ability of antioxidant to give hydrogen radical to synthetic long-lived nitrogen radical compounds DPPH• having a radical localized on the N-atom: DPPH• + Aox → DPPH, i.e., $=N - N^{\bullet -} + H^{\bullet} \rightarrow = N - NH^{-}$.

A blue-violet color changes gradually to green and yellow (absorption maximum at 405 nm), and a decrease in absorbance at 515 nm is monitored during the reaction in neutral medium. The reaction is pH-dependent. Published results are based on the very different interpretation; therefore, comparison is very problematic both in rank of the method and among others methods. Two versions of DPPH• method of evaluation (*39*) have been used, (i) dynamic and (ii) static.

The rate of DPPH• destruction after addition of a sample containing phenolic compounds is measured in dynamic version. The reaction rate characterizes the reactivity, and it can be calculated as a slope of the reaction kinetics at the very beginning of the reaction ($t \sim 0$). Usually, applied graphical interpretations are of limited accuracy and precision. In addition, the starting reaction rate and the total antioxidant activity are not completely two independent values.

The amount of DPPH• inactivated by the sample is measured graphically or numerically [as a % of consumed or unconsumed DPPH•, % of discoloration $100 \times (1 - A_{sample}/A_{control})$ etc.] in a static version. The H-donor potential, in the form IC_{50} (*29*), expressed percentage decrease of initial concentration (EC_{50}), i.e., amount of antioxidant needed for decrease of DPPH• concentration by 50%. Direct comparison of the results in rank of the method and among other methods is again highly problematic (*39*) since they depend on the initial concentration of DPPH• radical.

Radical scavenging efficiency (RSE, the ratio of initial reaction rate of DPPH• radical decomposition to EC_{50}) was recommended for characterization of radical inhibition ability (*43*). Antiradical efficiency (AE), expressed in two forms, $AE = 1000 \times 1/IC_{50}$ or $AE = 1/IC_{50} \cdot t_{50}$, where t_{50} is the time needed for 50% transformation of DPPH• radical, were also recommended for presentation of results (*29*, *44*, *45*). It is clearly evident that the parameters are hardly reproducible, since the values depend on the initial DPPH• concentration.

The percentage of DPPH• inhibition (I %), $I\% = (A_{blank} - A_{sample}/A_{blank}) \times 100$, i.e., ratio of nonconsumed DPPH• in the presence of antioxidants to initial DPPH• in %, where A_{sample} and A_{blank} are absorbance values of the reaction mixture with and without sample, respectively (*44*, *46*). Other modifications (in combination with HPLC, TLC, sequential injection analysis, etc.) of the method were also recommended in the literature. Unfortunately, new more or less serious advantages and disadvantages appeared in most of them, but the main principle of the basic reaction remains unchanged.

The amount of inactivated DPPH• is proportional to the concentration of added flavonoids (*47*); thus, the classical calibration procedure based on Trolox as a standard can be used for quantification (*48*) and 1 mmol/L of Trolox corresponds to antioxidant activity of 1 mmol/L phenolic compounds.

In our experiment, the yield of the reaction with Trolox was finished during 2 min, while with plant extracts the yield

Table 3. Determined Millimolar Absorptivity Coefficients and Ratio of Absolute Values of Molar Absorptivity Coefficients Related to Ferulic Acid (after / Symbol) of All Used Methods for Applied Standards

method	gallic	caffeic	ferulic	phenol	Trolox	ascorbic	$FeSO_4$
FCM	0.177/1.22	0.201/1.39	0.145	0.092/0.63	0.058/0.40	0.098/0.68	0.028/0.20
TEAC	−1.588/2.54	−0.179/0.29	−0.625	−0.175/0.28	−0.253/0.40	−0.172/0.28	−0.099/0.16
FRAP	1.005/2.13	0.715/1.52	0.471	0	0.396/0.84	0.282/0.60	0.198/0.42
DPPH	−1.398/5.70	−0.279/1.14	−0.244	0	−0.254/1.04	−0.147/0.60	−0.158/0.65

exponentially decreased and reached the plateau after 45−60 min (**Figure 1A**). The reaction rate was very fast in the beginning (90% decrease in first 20−30 min while the rest of 10% in the next 30 min). The published values of antioxidant activity increased with the incubation period, i.e., the values 2.2 and 3.5 were determined in the 2nd and 15th min, respectively (*49*).

The very good stability of the DPPH• radical (no measurable decrease of absorbance values was observed for DPPH• reagent) was the main advantage of the method over the TEAC method. The very long reaction times for obtaining plateau and thus the very long incubation times needed for measurements were the main disadvantage of the method (similar to the other ones) as can be seen from the kinetic data (**Figure 1**). The very short incubation times used in most of the published papers were probably the main source of the very low reproducibility of the results. Classical procedures for evaluation of the results on the basis of a suitable standard can be used due to the reaction kinetic and the linear relationship between absorbance and antioxidant concentration. Exactly the same initial concentrations must be used for the standard and the sample solutions. Trolox was found as the most suitable standard (see above) since the quantitative yields of the reaction were obtained in 1 min and the values of decreased absorbance were similar to those of caffeic and ferulic acids.

Hydrogen-donor capacities of polyphenols for DPPH• were proportional to the number of hydroxyl groups (*50*). The amount of inactivated DPPH• was proportional to the concentration of added flavonoids (*47*). The values for catechins in tea were practically equal to the number of active OH groups present in catechol and pyrogallol parts of molecules. The reactivity of DPPH• with flavonoids not containing OH groups in the B ring and with the aromatic acids containing a single OH group is negligible (*39*), but the validity of the findings probably might be limited.

Ferulic acid (one OH group) was also active, and the value was practically equal to the value for caffeic acid (two OH groups). Five times higher values were obtained for gallic acid (three OH groups) when we compared the reactivity of the acids tested in our experiments (**Figure 2**). The explanation for the lowest determined values of extracts by these methods can be due to the fact that DPPH• is a long-lived little reactive radical reacting only with very reactive phenolic and other antioxidants.

The results were comparable for the TEAC and DPPH methods despite the fact that the reactivity of some compounds seriously differs. For example, the orders of decrease in antioxidant activity determined by the TEAC (with ABTS) and the DPPH• methods were practically equal for seven phenolic compounds (*30*); however, for some other compounds, they differed substantially (i.e., for rutin and Trolox).

Table 4. Correlation of the Results of Different Methods

	total			free		
methods	n^a	r^b	F^c	n^a	r^b	F^c
FCM vs TEAC	32	++	++	31/32	++	++
FCM vs FRAP	31	++	++	30/32	++	−
FCM vs DPPH	32	++	++	30/32	+±	+
TEAC vs FRAP	31	++	++	31/32	++	++
TEAC vs DPPH	31	++	++	31/32	+±	++
FRAP vs DPPH	31	++	−?	31/32	+±	−

[a] Number of samples. [b] Regression coefficient. [c] Paired test; ++, highly significant correlation; +, significant correlation; −, nonsignificant correlation. Left part determined by least-squares method, right half by *t*-test.

The determined values in the plant extracts were very similar, and they correlated very well with those determined by the FRAP method (**Figure 3**). Because of the same basic principles of the applied methods for the determination of phenolic compounds and the antioxidant activity (redox properties), one can expect a high correlation of determined values among all methods. Correlations (**Table 4**) were determined by two methods—linear least-squares method (Excel) and *t*-test counted by statistical system Unistat 4.53e. Indeed, the correlation of determined values of the some extracts among all used methods (FCM−TEAC, FCM−FRAP, FCM−DPPH, TEAC−FRAP, TEAC−DPPH, and FRAP−DPPH) for extracts of free and total phenolics was highly significant (for 30−32 values, 1−2 extreme values deleted for some methods).

A consensus on correlation is not general since some literature data on the contents of phenolics and their antioxidant activity are very contradictory. Some authors observed close or very close correlations (*31*) while the others did not find any or nonsignificant direct correlation. The discrepancies could be influenced by the estimation and interpretation of the results of individual methods, the differences in evaluation of interferences of other substances (like ascorbic acid, saccharides, and eventually carotenoids).

Ascorbic acid and other endiols react positively not only with the FC reagent but also with reagents of the TEAC, DPPH, and FRAP methods (values of ascorbic acid are equivalent to ca. two-thirds values of Trolox applied as a standard in the methods). The corrections on the content of ascorbic acid are not critical and suitable for determination of overall antioxidant activity and for eventual comparison of the values determined by different methods, since the ascorbic acid participates in the total antioxidant capacity.

However, a correction on ascorbic acid content could be applied for correlation of the total content of phenolic compounds and the total antioxidant capacity. The negligible increase of the values (<5% and mostly <1%) was observed for the TEAC, FRAP, and DPPH methods. Interference of

TEAC: gallic ac. > quercetin > epicatechin > catechin > ascorbic ac. > rutin > chlorogenic ac.> Trolox
(3 OH) (1+4 OH) (1+4 OH) (1+4 OH) (2 OH) (4 OH) (3+2 OH) (1 OH)

DPPH: gallic ac. > quercetin > epicatechin > catechin > ascorbic ac.≥ Trolox > rutin > chlorogenic ac.

saccharides is negligible for all of the methods except for the TEAC method (**Table 2**).

In conclusion, a detailed study was performed of the contents of phenolic compounds determined by the Folin−Ciocalteu reagent and the antioxidant activities determined by the TEAC, DPPH, and FRAP methods and their correlations for extracts from several species of commonly consumed vegetables. The FC method for the determination of phenolic compounds is, such as the methods of antioxidant activity determination, based on redox properties of the compounds; thus, the values could partially express the antioxidant activity. This confirms a highly significant correlation between the values of FC method and the values of individual methods for antioxidant activity.

The three used methods for the determination of antioxidant activity applied for the same sets of the extracts using identical calibration procedures and common standard allowed the better comparison of the results obtained by the TEAC, DPPH, and FRAP methods.

The absolute values of individual methods for determination of antioxidant activity differed proportionally. The highest values were obtained using the TEAC method (mean 31 of extracts total/free 15.3/10.1) than for the FRAP method (3.46/2.08), while the lowest ones were obtained with the DPPH method (2.90/1.75).

The simultaneous determination of ascorbic acid and the correction of absorbance values according to its contribution play the key role in correct determination of phenolic compounds, mainly in vegetables rich in ascorbic acid. The determined contents of phenolic compounds could be corrected for the ascorbic acid content to eliminate or minimize the misinterpretation of the ratio of real values when comparing the content of phenolics and the antioxidant activity. The interference of saccharides could be more significant in samples rich in saccharides, but it is mostly negligible in vegetables.

The content of phenolic substances and total antioxidant activity correlate very well for most of the samples. There is no significant difference in the values determined by the individual methods, since the absorbance values for the methods correspond to the absorbing substances with different molar absorptivities. More important is the common trend, i.e., the constancy of the ratio between compared values for the wider set of samples. The higher deviations of the values determined by the different methods for the same sample from the common trend could be caused by higher content of specific phenolic compounds or other compounds of different reactivity (for example, in garlic). The highest antioxidant activities were obtained for the TEAC method, substantially lower for FRAP, and the lowest for the DPPH method.

The content of phenolic compounds and the antioxidant activity are partly dependent on the color of the variety of the vegetables and total sunlight irradiation (intensity × time) during vegetative period and with the water content. The very high values of antioxidant activity were found in intensely colored vegetables (red cabbage, red onion, etc.), and very low values were found in watery vegetables such as potato, marrow, and cucumber.

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