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Use of Cyclic Voltammetry, Photochemiluminescence, and Spectrophotometric Methods for the Measurement of the Antioxidant Capacity of Buckwheat Sprouts

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This paper describes the use of cyclic voltammetry, photochemiluminescence (PCL), and spectrophotometric methods (TEAC and FCR reducing capacity) for the measurement of the antioxidant capacity of dark- and light-grown buckwheat sprouts. Moreover, the flavonoid profile of studied material is provided. Methanol extracts (80%) from ungerminated buckwheat grain and 6 and 8 DAS (days after seeding) sprouts were used. The 8 day germination period was sufficient to obtain good quality sprouts with completely removed pericarps. The ungerminated buckwheat grain contained only rutin, whereas in sprouts produced in dark or light, a high level of isoorientin, orientin, vitexin, rutin, and isovitexin was found. The flavonoid content in sprouts produced under light was almost 2 times higher than those of sprouts produced in the dark. The antioxidant capacity of light-grown sprouts was higher than that of dark-grown ones. The results from voltammetric experiments obtained for buckwheat seeds and 6 and 8 DAS sprouts harvested under dark or light conditions highly correlated with those obtained by PCL antioxidant capacity of water-soluble substances (PCL ACW) ($r = 0.99$), PCL antioxidant capacity of lipid-soluble substances ($r = 0.99$), TEAC ($r = 0.99$), and FCR reducing capacity ($r = 0.99$). The use of cyclic voltammetry, PCL ACW, and TEAC was fully applicable for the evaluation of the antioxidant capacity of buckwheat sprouts.

KEYWORDS: Buckwheat sprouts; light conditions; antioxidant capacity; cyclic voltammetry; chemiluminescence; spectrophotometric methods; flavonoid profile

INTRODUCTION

One of the major challenges faced by the food industry is the need to develop products which can contribute to the customer's desire for a health-protective diet. In the past few years, new foods containing bioactive compounds with antioxidative properties have been extensively sought. As a result of this searching, the level of interest in buckwheat (*Fagopyrum esculentum*) and its products has grown (*1*). Buckwheat has been used both as a food and as a traditional medicine (*2, 3*). Buckwheat is abundant in antioxidants, and it is cited as an origin plant of rutin (*4, 5*). Moreover, Holasova et al. (*6*) suggested buckwheat as source of antioxidant activity in functional foods. More recently, Kim et al. (*7*) recommended the buckwheat sprouts as a new vegetable for the first time. These sprouts not only had a soft and slightly crispy texture and attractive fragrance but also had abundant nutrients. It was reported that buckwheat sprouts provided larger amounts of monosaccharides, unsaturated fatty acids, free amino acids, including lysine, γ-amino-*n*-butyric acid (GABA), and sulfur-containing amino acids, rutin (quercetin 3-*O*-rutinoside), quercitrin (quercetin 3-*O*-rhamnoside), and water-soluble vitamins, B_1, B_6, and C (*8*).

In numerous papers dealing with measurement of antioxidant capacity, very rarely, a single method is applied. Moreover, the number of methods for measuring antioxidant capacity of foods, nutraceuticals, and other dietary supplements has increased considerably. Because of the complexity of the composition of foods, the investigation of each single antioxidant compound is costly and inefficient; moreover, possible synergistic interactions among the antioxidant compounds in a food mixture are not taken into account (*9*). A cyclic voltammogram (CV tracing) provides information describing the integrated antioxidant capacity without the specific determination of the contribution of each individual component. It is based on the analysis of the anodic current (AC) waveform which is a function of the

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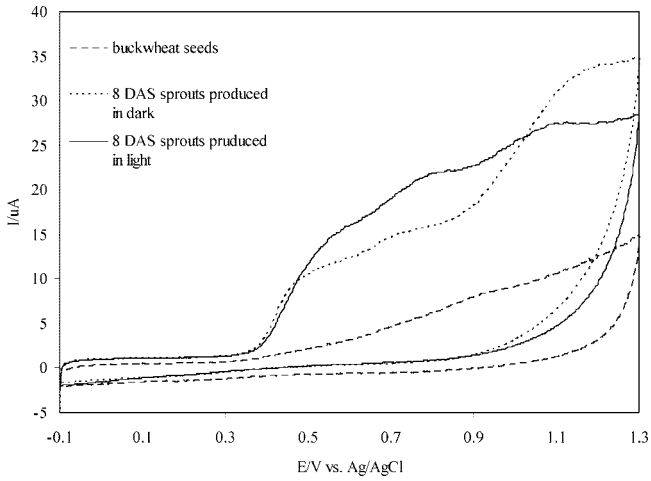

Figure 1. Cyclic voltammograms of analyzed extracts that originated from whole buckwheat seeds and 8 DAS buckwheat sprouts produced under dark and light conditions. Operative conditions: concentration of each extract, 20 mg/mL of 80% methanol; sample preparation, 80% methanol extract mixed with 0.2 M sodium acetate/acetic buffer (pH 4.5) at a 1:1 (v/v) ratio; scan rate, 100 mV s^{-1}.

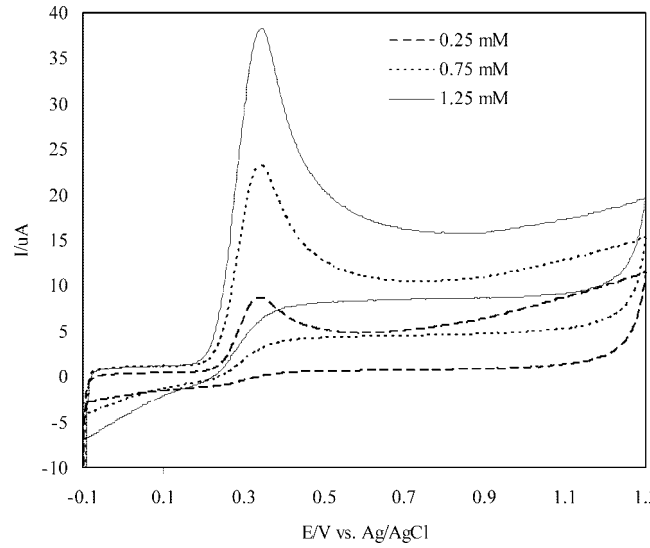

Figure 2. Selected cyclic voltammograms at Trolox concentrations within the range of 0.05–2.5 mM. Operative conditions: 80% methanol solution of the standard mixed with 0.2 M sodium acetate/acetic buffer (pH 4.5) at a 1:1 (v/v) ratio; scan rate, 100 mV s^{-1}.

reductive potential of a given compound in the sample and/or a mixture of components. The total antioxidant capacity of the sample is a function combining two sets of parameters. The first parameter is the biological oxidation potential, whereas the second parameter is the intensity of the anodic AC current (Ia), reflecting the concentration of the components. Recently, it has been proposed that the area under the AC wave (*S*; related to the total charge) is a parameter that better reflects the antioxidant capacity of the sample (*10*). For example, when cyclic voltammetry (CV) is used, it is often compared to either some spectrophotometric method or HPLC methods (*11*). In the PCL (photochemiluminescence) assay, the photochemical generation of free radicals is combined with the sensitive detection by using chemiluminescence. The PCL is based on the photoinduced autoxidation inhibition of luminol by antioxidants, mediated by the radical anion superoxide ($O_2^{\bullet-}$) and is suitable for measuring the radical scavenging properties of single antioxidants as well as more complex systems in the nanomolar range (*12*). The TEAC assay was first reported by Miller et al. (*13*) and is based on the radical scavenging ability of antioxidants on the long-life radical cation $ABTS^{\bullet+}$. In this assay, ABTS is oxidized by peroxyl radicals or other oxidants to its radical cation $ABTS^{\bullet+}$, which is intensely colored and reacts rapidly with antioxidants. Antioxidant capacity, termed Trolox, is measured as the ability of test compounds or investigated food extracts to weaken the color reacting directly with the $ABTS^{\bullet+}$ radical.

To the best of our knowledge, voltammetric, chemiluminescent, and spectrophotometric methods have never been simultaneously applied in studying the antioxidant capacity of buckwheat sprouts. Therefore, the principal aims of the work were therefore (1) to compare the antioxidant capacity of ready-to-eat buckwheat sprouts obtained under light or dark conditions and (2) to compare widely differing voltammetry, chemiluminescence, and spectrophotometry methods for their suitability in measuring the antioxidant capacity when simultaneously applied to the same food samples.

MATERIALS AND METHODS

Reagents. Rutin (quercetin 3-rutinoside), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma Chemical Co. (St. Louis, MO). ACW (antioxidant capacity of water-soluble substances) and ACL (antioxidant capacity of lipid-soluble substances) kits (model no. 400.801) for the PCL assay were from Analytik Jena AG (Jena, Germany). Orientin (3′,4′,5,7-tetrahydroxyflavone 8-glucoside), homoorientin (3′,4′,5,7-tetrahydroxyflavone 6-glucoside), vitexin (4′,5,7-trihydroxyflavone 8-glucoside), and isovitexin (4′,5,7-trihydroxyflavone 6-glucoside) standards (HPLC-grade) were obtained from Extrasynthese Company Inc. (Lyon, France). All other reagents of reagent-grade quality were from POCh (Gliwice, Poland). Water was purified with a Mili-Q system (Millipore, Bedford, MA).

Seed Germination. Buckwheat (*Fogopyrum esculentum*, variety Luba) was used for the germination. Whole buckwheat seeds (25 g) were soaked in 125 mL of distilled water at room temperature and shaken every 30 min. After 12 h, the water was drained off and the seeds were transferred to an incubator (Cliambic Cabinet, model Economic Deluxe EC00-065, Snijders Scientific B.V.). Sprouting was carried out in triplicate at 25 °C and 95% humidity, with or without exposure to light for 24 h. Buckwheat sprouts were harvested 6 and 8 days after seeding (DAS) and then lyophilized.

Preparation of 80% Methanol Extracts. Approximately 100 mg of sprouts and ungerminated whole buckwheat seeds were lyophilized and ground using a laboratory mill. Then, the material was extracted with 1 mL of 80% methanol via a 30 s sonication. Next, the mixture was vortexed for 30 s, again sonicated and vortexed, and centrifuged for 5 min (5000*g* at 4 °C). That step was repeated five times, and the supernatant was collected in a 5 mL flask. Finally, all extracts were kept at −80 °C prior to further determination of the antioxidant capacity and phytochemical profile.

Voltammetric Experiments. Cyclic voltammetric experiments were performed in 80% methanol extracts mixed with 0.2 M sodium acetate/acetic buffer (pH 4.5) at a 1:1 (v/v) ratio by following the method of Cosio et al. (*14*). The sodium acetate/acetic buffer acted as a supporting electrolyte for the voltamperometric measurement. The measurements were carried out using a conventional three-electrode system: (a) a 3 mm diameter glassy carbon working electrode (BAS MF-2012), (b) a Ag/AgCl electrode as a reference electrode, and (c) a platinum electrode as a counter electrode. The voltammetric experiments were performed at room temperature using a voltammetric apparatus cell, to which analyzed buckwheat extract previously mixed with the supporting electrolyte had been introduced. Exactly 100 μL of the extract and 100 μL of buffer were used in this respect. To avoid a reduction in sensitivity, the working electrode was carefully polished with 0.05 μm alumina paste (Polishing alumina, BAS) and ultrasonically rinsed in deionized water at the end of each cycle. After being washed with

methanol, the electrode was then ready for further tests. The cyclic voltammograms were recorded by scanning the potential from −100 to +1300 mV. Cyclic voltammograms were recorded with a potentiostat/galvanostat KSP system at a scanning rate of 100 mV s^{-1}. For the purpose of testing, the total charge below the anodic wave curve of the voltammogram was calculated. This method is actually based on the correlation between the total charge below the anodic wave of cyclic voltammograms and the antioxidant capacity of the sample and reference substance. The 80% methanol solution of Trolox within the concentration range of 0.05–2.50 mM was used, and the results are expressed as micromoles of Trolox per gram of dry matter. The total charge under the anodic wave of the background signal (solvent + supporting electrode) was subtracted from the total charge under the anodic wave obtained for each standard and sample measured.

Determination of Antioxidative Capacities of Water-Soluble (ACW) and Lipid-Soluble (ACL) Compounds by the Photochemiluminescence (PCL) Method. The photochemiluminescence (PCL) assay, based on the methodology of Popov and Lewin (*15*), was used to measure the antioxidant activity of buckwheat extracts with a Photochem apparatus (Analytik Jena, Leipzig, Germany) against superoxide anion radicals generated from luminol, a photosensitizer, when exposed to UV light. The antioxidant activity of 80% methanol extracts of sprouts was measured using both ACW and ACL kits provided by the manufacturer designed to measure the antioxidant activity of hydrophilic and lipophillic compounds, respectively. For ACW studies, the luminal reagent and Trolox work solution were prepared on the day during which they were needed according to the ACW protocol. The presence of Trolox (or any other antioxidants from the extracts) retarded luminescence for a period; hence, a lag time was noted before a signal was measured. The duration of the lag, which is calculated by the computer software from the first derivative of the detector signal at its turning point and intersection with the *x*-axis, was plotted against the concentration of Trolox added to the assay medium. The concentration of the added extract solution was such that the generated luminescence fell within the limits of the standard curve. Therefore, the lag time (seconds) for the ACW assay was used as the radical scavenging activity and the antioxidant capacity calculated by comparison with a Trolox standard curve and then expressed as micromoles of Trolox per gram of dry matter of sprouts. In ACL studies, the kinetic light emission curve, which exhibits no lag phase, was monitored for 180 s and expressed as micromoles of Trolox per gram of dry matter. The areas under the curves were calculated using the PCLsoft control and analysis software. As greater concentrations of Trolox working solutions were added to the assay medium, a marked reduction in the magnitude of the PCL signal and hence the area calculated from the integral was observed. This inhibition was used as a parameter for quantification and related to the decrease in the integral of PCL intensities caused by varying concentrations of Trolox. The observed inhibition of the signal was plotted against the concentration of Trolox added to the assay medium. The concentration of the added extract solution was such that the generated luminescence during the 180 s sampling interval fell within the limits of the standard curve. The extracts for ACW and ACL measurements were centrifuged (5 min at 16000*g*) prior to analysis. The antioxidant assay was carried out in triplicate for each sample, and 20 μL of the diluted extract (1:40, v/v) in HPLC-grade water (ACW) or HPLC-grade methanol (ACL) was sufficient to correspond to the standard curve.

Trolox Equivalent Antioxidant Capacity (TEAC) Assay. Trolox equivalent antioxidant capacity was determined following a procedure described by Re et al. (*16*) with a minor modification described below. For measurements, the $ABTS^{\bullet+}$ solution was diluted with 80% methanol to an absorbance of 0.70 ± 0.02 at 734 nm. For the photometric assay, 1.48 mL of the $ABTS^{\bullet+}$ solution and 20 μL of the extracts or Trolox standards were mixed, and then the absorbance was measured immediately after 6 min at 734 nm at 30 °C using a spectrophotometer (UV-160 1PC, Shimadzu, Kyoto, Japan). Appropriate solvent blanks were run in each assay. The Trolox equivalent antioxidant capacity of 80% methanol extracts was calculated, using the Trolox standard curve, on the basis of the percent inhibition of absorbance at 734 nm. The 80% methanol solution of Trolox within the concentration range of 0.1–2.0 mM was used for construction of the calibration curve. Additionally, a Trolox equivalent antioxidant activity of rutin was determined in parallel with the Trolox standard curve.

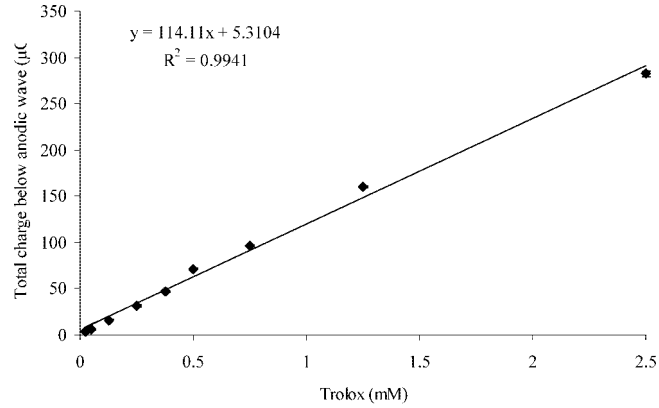

Figure 3. Dependency of the total charge under the anodic wave as a function of increasing concentration of Trolox (0.05–2.5 mM).

FCR Reducing Capacity Assay. Assessment of the FCR reducing capacity via application of Folin-Ciocalteu reagent (FCR) was carried out according to the method described by Shahidi and Naczk (*17*). Briefly, 0.25 mL of the 80% methanol extract was mixed with 0.25 mL of FCR previously diluted with distilled water (1:1, v/v), 0.5 mL of saturated sodium carbonate (Na_2CO_3), and 4 mL of water. The mixture was incubated at room temperature for 25 min and centrifuged at 2000*g* for 10 min. The supernatant absorbance was measured at 725 nm using a spectrophotometer (UV-160 1PC, Shimadzu). The data were calculated as milligrams of rutin equivalents on a dry matter basis.

Flavonoid Profile. The extracts prepared for the flavonoid analyses were diluted in 80% methanol (1:100, v/v), and then they were submitted to HPLC analysis (Shimadzu), consisting of two pumps (LC-10 AD_{VP}), an UV detector (SPDM-$10A_{VP}$) set at 330 nm, an autosampler set to 5 μL injection (SIL-10 AD_{VP}), a column oven (CTO-10 AS_{VP}), and a system controller (SCL-10 A_{VP}). All chromatographic determinations were performed at 35 °C with a flow rate of 1 mL/min on a C18(2) Luna 5 μm column, 4.6 mm × 250 mm (Phenomenex, Torrance, CA). The flavonoids were eluted in a gradient system composed of 4% aqueous formic acid (solvent A) and acetonitrile containing 4% formic acid (solvent B). Gradients were as follows: 13% B at gradient time (t_G) 0, 13% B at t_G = 7 min, 20% B at t_G = 20 min, 40% B at t_G = 25 min, 80% B at t_G = 30 min, 13% B at t_G = 35 min, and 13% B at t_G = 45 min. Rutin, orientin, homoorientin, vitexin, and isovitexin stock solutions were prepared in methanol at concentrations of 500, 517, 477, 509, and 574 μM, respectively, taking into account the purity of the standards. For quantitative analysis, calibration standards were prepared in duplicate at five concentrations within the range of 0.1–40 μM for each compound.

Statistical Analysis. The results are given as the means and the standard deviation of three independent experiments. Statistical analysis was performed using Student's *t* test, and a $P < 0.05$ significance level was used.

RESULTS AND DISCUSSION

Buckwheat Sprouts. Sprouts produced in the dark had a light-yellow cotyledon, bright-white hypocotyls, and brown roots, whereas those produced under light exhibited a dark green cotyledon, yellow-red hypocotyls, and bright-white roots. The buckwheat sprouts produced in the dark looked like soybean sprouts. After germinating for 8 days, the obtained sprouts did not contain their pericarps which were completely removed. The duration of the germination period is very important in terms of the quality of buckwheat sprouts because in the sprouts that included pericarps, they not only are difficult to remove but also become an obstacle in using them as fresh vegetables. The 8 day germination time applied in this work was in accordance with the report of Kim et al. (*8*), who introduced the mass

Table 1. Antioxidant Capacity of Buckwheat Sprouts Measured by the Cyclic Voltammetric Method[a]

	dark-grown		light-grown	
days after seeding (DAS)	total charge below anodic wave (μC)	antioxidant capacity (μmol of Trolox/g of dry matter)	total charge below anodic wave (μC)	antioxidant capacity (μmol of Trolox/g of dry matter)
0	23.41 ± 1.20 a	7.69 ± 0.51 a	23.41 ± 1.20 a	7.69 ± 0.51 a
6	122.90 ± 0.85 b	50.91 ± 0.37 b	129.60 ± 2.11 b	52.62 ± 0.89 b
8	120.70 ± 0.49 b	49.62 ± 0.21 b	134.30 ± 1.53 b	55.46 ± 0.66 b

[a] Data expressed as means ± the standard deviation ($n = 3$). Means in a column followed by the same letter are not significantly different ($p \leq 0.05$).

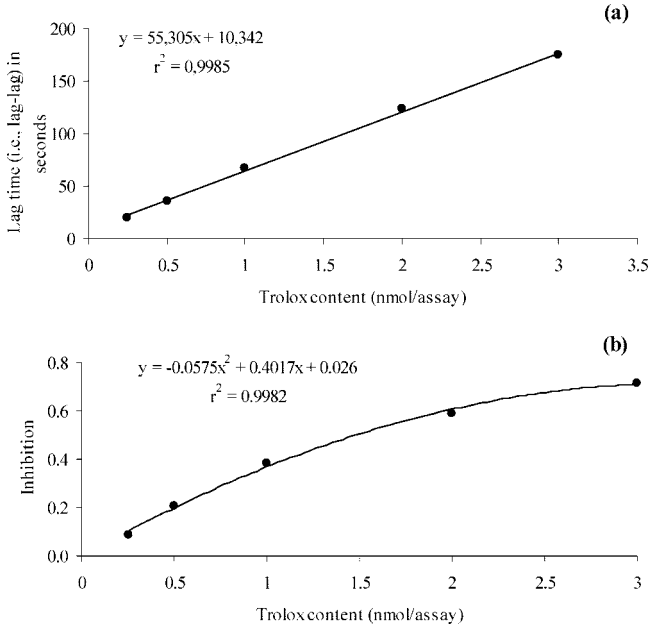

Figure 4. Calibration curve of Trolox in the calculation of Trolox equivalents for ACW (**a**) and ACL (**b**) measurements.

production system to produce buckwheat sprouts, and the same period of germination was sufficient to obtain good quality sprouts with almost completely removed pericarps. The dry matter contents of 6 and 8 DAS sprouts produced in the dark were 25.9 and 17.5%, respectively, whereas the values of those produced under light were 13.6 and 10.3%, respectively.

Antioxidant Capacity of Sprouts Derived from Voltammetric Experiments. Cyclic voltammograms of analyzed 80% methanol extract of buckwheat sprouts were recorded as shown in **Figure 1** (buckwheat seeds and 8 DAS buckwheat sprouts produced under dark or light conditions). The observed anodic wave was broadened due to the response of several antioxidants with different oxidation potentials, including mainly flavonoids, phenolic acids, and water-soluble vitamins like B_1, B_6, and C (*6*, *8*, *18*). In contrast, voltammograms obtained for the standard solutions of Trolox (0.05–2.50 mM) showed well resolved peaks and a shoulder in the potential region up to 1.1 V. A typical CV tracing of different Trolox concentrations is shown in **Figure 2**. The total charge under the anodic current (AC) waveform, provided by CV computer software, was used to calculate the antioxidant capacity of the sample, based on the function AC versus a set of Trolox solutions (**Figure 3**) as suggested by Chevion et al. (*10*) and Martinez et al. (*11*). The results showed that sprouts produced in the dark or under light for up to 8 days had antioxidant capacity almost 7 times higher than that of ungerminated seeds (**Table 1**). There was no statistical difference between the antioxidant capacity of buckwheat sprouts collected at 6 and 8 DAS under dark or light conditions. This finding indicates that a period of germination of up to 8 days is sufficient in terms of obtaining buckwheat sprouts with established antioxidative capacity. Moreover, 8 DAS sprouts obtained under light conditions showed 12% higher antioxidant capacity compared to those harvested in the dark (**Table 1**). It can be suggested that ready-to-eat buckwheat sprouts are a very rich source of 80% methanol-soluble compounds, having the ability to be oxidized on a glassy carbon electrode. Recently, cyclic voltammetry (CV) was shown to be an efficient instrumental tool for evaluating the integrated antioxidant capacity of low-molecular weight antioxidants (LMWAs) in human plasma, animal tissues, edible plants, wines, different types of tea and coffee (*10*, *19*–*22*), and pseudo-cereal-based products (*23*). Therefore, it can be suggested that the CV assay is an efficient tool for describing the antioxidant capacity of plant material provided by LMWAs.

Antioxidative Capacities of Water-Soluble (ACW) and Lipid-Soluble (ACL) Compounds. The antioxidant capacity of buckwheat sprouts was evaluated by using the Photochem device and the ACW and ACL kits supplied by Analytik Jena AG. The Photochem device is the first system than can quantitate the antioxidant capacity of water- and lipid-soluble substances. It combines the very fast photochemical excitation of radical generation with the highly sensitive luminometric detection. Because of the high sensitivity of the PCL of luminol, only nanomolar concentrations of nonenzymatic antioxidant substances are required to observe PCL (i.e., lag – lag_0). The principles of the assay are as follows: a standard volume of photosensitizer substance (i.e., luminol) is added to the assay medium; the photosensitizer is optically excited to produce the superoxide anion radical ($O_2^{\bullet -}$); the sample containing antioxidative compounds scavenges a part of the $O_2^{\bullet -}$; the remaining radicals are quantified by comparison against an ascorbic acid or Trolox standard (i.e., ACW or ACL, respectively) or against only Trolox. A typical calibration curve of Trolox for ACW and ACL calculation is shown in **Figure 4**. In this study, the 80% methanol extracts were taken for ACW and ACL measurements with this system, and the results are expressed as Trolox equivalents. The same system was considered by Oomah et al. (*24*) when different genotypes of lupin seeds were evaluated for their antioxidant capacity by ACW and ACL measurements. More recently, this method was applied in assessing the integral antioxidant capacity of baobab products (*12*).

Germination for up to 8 days increased significantly the ACW of sprouts by an average of 47 times (dark conditions) and 94 times (light conditions), while the ACL increased approximately 5 times for sprouts produced under dark or light conditions (**Table 2**). However, ACL values of sprouts planted in dark or light were higher by average an 125 or 30%, respectively, when compared to the respective average ACW values. It was found that the ACL of 8 DAS buckwheat sprouts produced under dark or light conditions was higher by only 7 or 6%, respectively, when compared to sprouts harvested after 6 DAS. No differences were found between the ACW of 6 and 8 DAS sprouts produced

Table 2. Antioxidative Capacity of Water-Soluble (ACW) and Lipid-Soluble (ACL) Compounds of Buckwheat Sprouts[a]

days after seeding (DAS)	ACW (μmol of Trolox/g of dry matter)		ACL (μmol of Trolox/g of dry matter)	
	dark-grown	light-grown	dark-grown	light-grown
0	2.52 ± 0.10 a	2.52 ± 0.10 a	59.51 ± 1.37 a	59.51 ± 1.37 a
6	116.23 ± 2.45 b	233.76 ± 2.09 b	251.78 ± 12.72 b	300.72 ± 4.58 b
8	123.10 ± 4.26 b	239.70 ± 2.26 b	288.73 ± 13.50 b	318.16 ± 0.28 c

[a] Data expressed as means ± the standard deviation ($n = 3$). Means in a column followed by the same letter are not significantly different ($p \leq 0.05$).

Table 3. Trolox Equivalent Antioxidant Capacity (TEAC) and FCR Reducing Capacity of Buckwheat Sprouts[a]

days after seeding (DAS)	dark-grown		light-grown	
	TEAC (μmol of Trolox/g of dry matter)	FCR reducing capacity (mg of rutin equiv/g of dry matter)	TEAC (μmol of Trolox/g of dry matter)	FCR reducing capacity (mg of rutin equiv/g of dry matter)
0	41.55 ± 0.78 a	10.99 ± 0.60 a (43.2 ± 2.4)[b]	41.55 ± 0.78 a	10.99 ± 0.60 a (43.2 ± 2.4)[b]
6	123.48 ± 5.59 b	60.04 ± 1.84 b (236.0 ± 7.2)[b]	212.84 ± 2.07 b	91.38 ± 1.70 b (359.2 ± 6.7)[b]
8	137.52 ± 1.63 c	70.52 ± 2.64 c (277.2 ± 10.4)[b]	218.36 ± 1.45 b	92.17 ± 1.62 b (363.3 ± 6.4)[b]

[a] Data expressed as means ± the standard deviation ($n = 3$). Means in a column followed by the same letter are not significantly different ($p \leq 0.05$). [b] Values in parentheses show the FCR reducing capacity converted into antioxidant capacity expressed as micromoles of Trolox per gram of dry matter when a TEAC of rutin of 2.4 mM was used.

under dark or light conditions. This finding supports our previous conclusion based on the voltammetric experiments that germination of buckwheat is completed after 8 days from the point of view of antioxidant capacity. Moreover, 8 DAS sprouts obtained under light conditions exhibited ACW and ACL higher by approximately 94 and 19%, respectively, when compared to those harvested in the dark. These findings clearly indicate that light conditions are an important factor affecting the ACL values of ready-to-eat sprouts. On the other hand, it can be suggested that both sprouts are a very rich source of 80% methanol-soluble compounds having antioxidant activity against superoxide anion radicals. The scavenging and dismutation of active oxygen radicals by enzymes such as superoxide dismutase and catalase and by low molecular-weight antioxidant compounds such as ascorbic acid and glutathione have been the subject of extensive studies from a pharmacological point of view. It is well-known that $O_2^{\bullet -}$ is one of the most important free radicals in the body which has been implicated in the initiation of oxidation reactions associated with aging, and it plays an important role in the formation of other reactive oxygen species (*25*, *26*). Therefore, the data provided by the PCL method indicate the importance of antioxidants that originate from germinated buckwheat in the further potential application in nutrition as well as in pharmacology. Moreover, the Photochem device was fully applicable for the evaluation of the antioxidant capacity originating from 80% methanol-soluble compounds of buckwheat sprouts. The ACW and ACL results provided for buckwheat seeds and 6 and 8 DAS sprouts produced under dark or light conditions were correlated with those from voltammetric experiments ($r = 0.99$).

Trolox Equivalent Antioxidant Capacity (TEAC) and FCR Reducing Capacity. Recently, it was proposed that following procedures and applications of three assays can be considered for standardization of antioxidant capacity measurements: the oxygen radical absorbance capacity (ORAC) assay, the Folin-Ciocalteu method, and possibly the Trolox equivalent antioxidant capacity (TEAC) assay (*9*). In this study, two of these spectrophotometric assays were used for the evaluation of antioxidant capacity of buckwheat sprouts. Therefore, the 80% methanol extracts of the ungerminated buckwheat seeds and 6–8 DAS sprouts were examined for their free radical scavenging activity against $ABTS^{\bullet +}$ cation radical (TEAC) and for their reducing capacity by the means of Folin-Ciocalteu reagent (FCR) application.

The sprouts produced under dark or light conditions for up to the 8 days exhibited TEAC values almost 3 or 5 times higher, respectively, than the TEAC values of ungerminated whole buckwheat (**Table 3**). It was found that TEAC of 8 DAS buckwheat sprouts produced under dark and light conditions was higher by 11% and only 3%, respectively, when compared to spouts harvested after 6 days. This finding supports our previous conclusion based on voltammetric experiments and PCL data that germination of buckwheat is completed after 8 days from the point of view of antioxidant capacity. Moreover, sprouts obtained under light conditions showed TEAC higher by approximately 66% when compared to those harvested in the dark (**Table 3**). These findings clearly indicate the impact of light conditions on TEAC values of sprouts. On the other hand, it can be suggested that sprouts are a very rich source of 80% methanol-soluble compounds having antioxidant activity against ABTS cation radicals.

The TEAC values of buckwheat sprouts produced under dark or light conditions were almost 2 or 3 times higher, respectively, than the TEAC values of ready-to-eat cruciferous sprouts (*27*, *28*). It was also found that buckwheat sprouts produced in the dark had a TEAC almost 15 times higher than the TEAC of sprouts obtained from four cultivars of lentil originating from Spain (*29*). The TEAC of rutin determined in this study was equal to 2.4 mM Trolox. This value was identical as reported Rice-Evans et al. (*30*), and it was used to express FCR reducing capacity as antioxidant capacity (micromoles of Trolox per gram of dry matter) to further compare data obtained from different assays. In this study, the provided TEAC values of buckwheat sprouts produced under dark or light conditions were exactly the same as those described by ACW, but they were almost 2 times lower than ACL data provided by the PCL assay. It indicates that the photochemiluminescence (PCL ACW) assay is a more accurate method for the determination of antioxidative properties of sprouts. Moreover, TEAC data for buckwheat seeds and 6 and 8 DAS sprouts harvested under dark or light conditions were correlated with those from voltammetric experiments ($r = 0.99$).

In this study, the reducing capacity of the buckwheat sprouts was measured by the means of Folin-Ciocalteu reagent (FCR) application. The Folin-Ciocalteu reagent (FCR) actually mea-

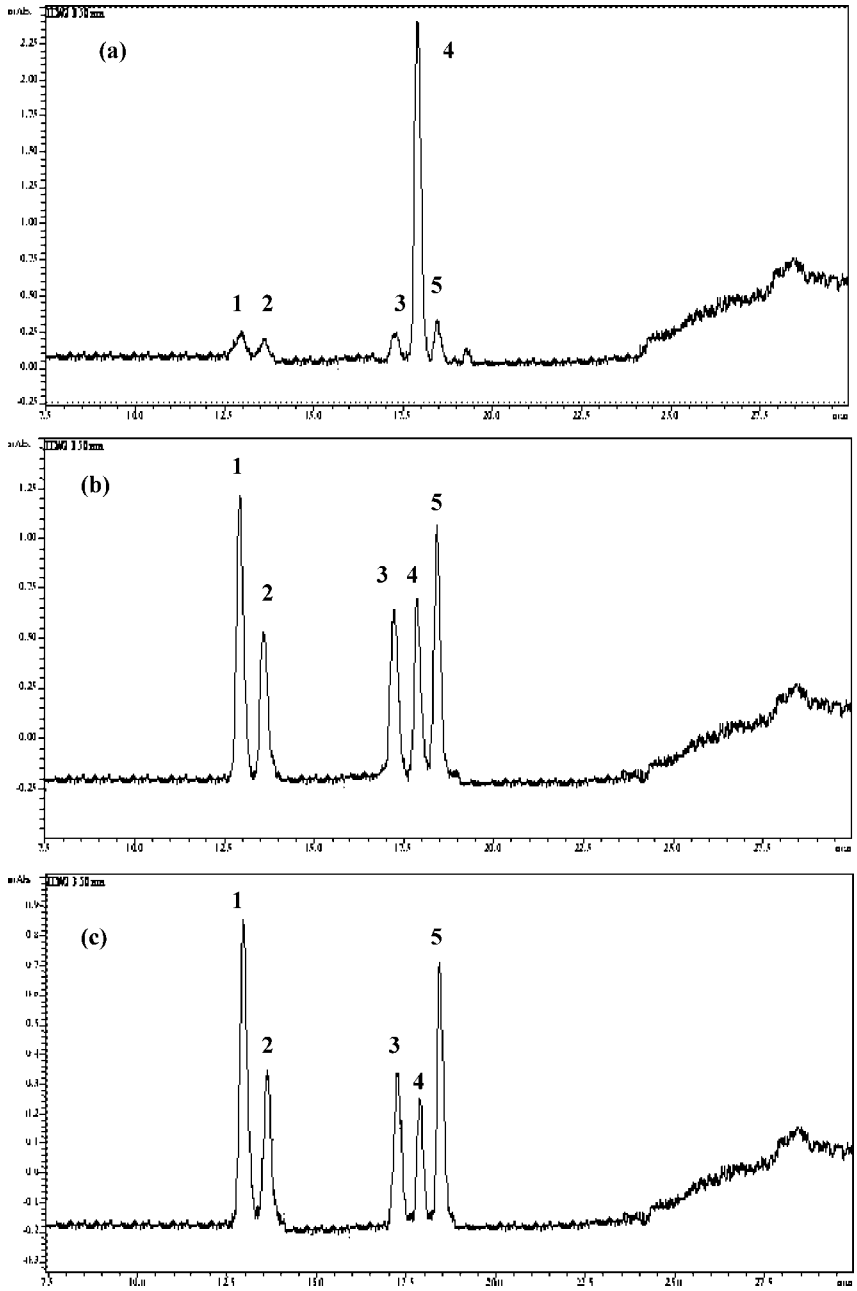

Figure 5. Typical HPLC chromatogram of buckwheat flavonoids recorded at 330 nm: (1) homoorientin, (2) orientin, (3) vitexin, (4) rutin, and (5) isovitexin. (**a**) Buckwheat seeds, (**b**) 8 DAS buckwheat sprouts produced in the dark, and (**c**) 8 DAS buckwheat sprouts produced under light. The preparation of extracts injected into the HPLC column was as described in Materials and Methods.

sures the reducing capacity of the sample (*31*), whereas the same assay based on the reaction of FCR is usually recognized as the "total phenolic assay". The popularity of the latest method can mainly be attributed to its simplicity and speed of analysis. The major disadvantage of this assay is its low specificity, as the color reaction can occur with any oxidizable group, principally phenolic hydroxy. FCR was initially intended for the analysis of proteins, taking advantage of the reagent's activity toward protein tyrosine (containing a phenol group) residue. Many years later, Singleton and co-workers extended this assay to the analysis of total phenols in wine; since then, the assay has found many applications (*32*). In this paper, to avoid any misunderstanding of the actual meaning of "total phenolic content", the FCR reducing capacity was used as suggested Huang et al. (*31*).

The FCR reducing capacity of the sprouts produced for up to 8 days under dark or light conditions increased almost 7 or 9 times, respectively, when compared to that of ungerminated buckwheat seeds (**Table 3**). The FCR reducing capacities of sprouts produced under dark or light conditions were highly correlated with those provided by the voltammetric experiments and PCL ACW and ACL ($r = 0.99$). In this study, we converted the FCR reducing capacity data into antioxidant capacity using the experimentally determined TEAC of rutin. For this purpose, FCR reducing capacity was expressed as micromoles of rutin per gram of dry matter, and then it was multiplied by the TEAC of rutin equal to 2.4 mM Trolox. In this case, the antioxidant capacity values corresponded to those obtained with the PCL ACL assay (**Table 2**), while it was almost 2 times higher when compared to PCL ACW results. The FCR reducing capacity data converted into antioxidant capacity were higher than those provided by the TEAC assay (**Table 3**). Comparison of the CV with PCL and TEAC assays has shown that these methods yielded considerably different chemical information. The anti-

Table 4. Concentration of Flavonoids, Antioxidant Capacity Derived from Identified Flavonoids, and Their Percentage Contribution to Total Flavonoid Content in Buckwheat Sprouts

material	total flavonoids (μmol/g of dry matter)	TEAC derived from flavonoids (μmol of Trolox/g of dry matter)	percentage contribution (%)				
			homoorientin	orientin	vitexin	rutin	isovitexin
		Dark Conditions					
ungerminated seeds	0.32	0.77	–	–	–	100	–
6 DAS sprouts	29.94	71.86	27.4	18.8	18.3	14.7	20.8
8 DAS sprouts	39.64	95.14	27.2	18.2	18.8	15.2	20.6
		Light Conditions					
ungerminated seeds	0.32	0.77	–	–	–	100	–
6 DAS sprouts	53.42	128.21	24.5	14.3	21.2	20.8	19.2
8 DAS sprouts	60.82	145.97	23.4	16.8	20.8	20.8	18.4

oxidant capacity of sprouts produced under dark or light conditions evaluated by the PCL ACW assay gave values 2-fold or 4 times higher, respectively, than the results provided by cyclic voltammetry. Moreover, the PCL ACL assay provided values almost 5 or 6 times higher, respectively, than those from the CV tracing. The antioxidant capacities of sprouts produced under dark and light conditions evaluated by the TEAC assay were 3 or 4 times higher, respectively, than those provided by the CV assay. In contrast, excellent agreement was noted between data provided by FCR reducing capacity and antioxidant capacity obtained by cyclic voltammetric experiments.

Flavonoid Profile. A typical HPLC chromatogram of buckwheat sprout flavonoids recorded at 330 nm is shown in **Figure 5a–c**. Column chromatography on a C18 support allowed us to separate flavonoid compounds (**a**) from whole buckwheat seeds (**b**) and from 8 DAS buckwheat sprouts produced in the dark (**c**) and 8 DAS buckwheat sprouts produced under light. The ungerminated whole buckwheat seeds contained only rutin (**Figure 5a**), whereas in sprouts produced under dark or light conditions, the following flavonoids were identified: homoorientin, orientin, vitexin, rutin, and isovitexin (**Figure 5b,c**). This finding is in agreement with recently published evidence that buckwheat germination increased the content of not only rutin but also other flavonoids such as orientin (3′,4′,5,7-tetrahydroxyflavone 8-glucoside), homoorientin (3′,4′,5,7-tetrahydroxyflavone 6-glucoside), vitexin (4′,5,7-trihydroxyflavone 8-glucoside), and isovitexin (4′,5,7-trihydroxyflavone 8-glucoside) (*33*). The obtained buckwheat sprouts contained a high level of flavonoids when compared to the ungerminated grain (**Table 4**). Moreover, flavonoid content in sprouts produced under light was almost 2 times higher than those of sprouts produced in the dark. The percentage contribution of homoorientin, orientin, vitexin, rutin, and isovitexin to the total flavonoid content in buckwheat sprouts is compiled in **Table 4**. The concentration of flavonoids in ungerminated buckwheat seeds and 6 and 8 DAS sprouts produced under dark or light conditions was highly correlated with antioxidant capacity evaluated by the voltammetric method, PCL ACW and PCL ACL, TEAC, and FCR reducing capacity, and the respective correlation coefficients were 0.964, 0.982 and 0.986, 0.994, and 0.997 for sprouts produced in the dark and 0.999, 0.997 and 0.999, 0.997, and 0.996 for sprouts produced under light, respectively. In this work, the TEAC of rutin was used to calculate a pool of antioxidant capacity derived from identified flavonoids (**Table 4**). In this case, the identified flavonoids in 6 and 8 DAS buckwheat sprouts produced under dark or light conditions formed 58.2 and 69.2% or 60.2 and 66.8%, respectively, of the antioxidant capacity shown in **Table 3**. The antioxidant capacity derived by flavonoids was almost 2 times higher than that from cyclic voltammetry (**Table 1**); it was comparable to data obtained from the PCL ACW assay (**Table 2**), and it was almost 3 times lower than the FCR reducing capacity converted into antioxidant capacity when the TEAC of rutin equal to 2.4 mM was used (**Table 3**). The calculation was based on the equal antioxidant activity of rutin and homooreintin, orientin, vitexin, and isovitexin, and further research on the antioxidant activity of these compounds is needed. Our findings are supported by recent reports on the characterization of natural antioxidants following their oxidation (*22*, *34*), thus providing a qualitative assessment of antioxidant capacity of buckwheat sprouts as a new vegetable based on reducing strength.

Concluding Remarks. The use of cyclic voltammetry, photochemiluminescence (PCL ACW), and a spectrophotometric method based on the free radical scavenging activities of the extracts against $ABTS^{\bullet+}$ radical cation (TEAC) was fully applicable for the evaluation of the antioxidant capacity of buckwheat sprouts. The sprouts produced under light conditions exhibited higher antioxidant capacity evaluated by voltammetric, chemiluminescence, and spectrophotometry assays when compared to those harvested in the dark. If we take into account the recent evidence with respect to their production (*8*), buckwheat sprouts should be more widely recommended in human nutrition.

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