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Method for Determining the Total Content of Phenolic Compounds in Plant Extracts with Folin–Denis Reagent and Folin–Ciocalteu Reagent: Modification and Comparison

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Abstract—The method for determining the total content of phenolic compounds in plant tissue extracts with the Folin–Denis reagent and the Folin–Ciocalteu reagent has been modified to establish the correspondence of results obtained when using these methods. The method with the Folin–Denis reagent has been adapted for determinations in microvolumes. For the method with the Folin–Ciocalteu reagent, concentration of the latter (0.4 N, a 5-fold dilution of the standard reagent) and composition of the reaction mixture were selected to obtain almost the same optical densities of the products of reduction of the Folin–Denis and Folin–Ciocalteu reagents by polyphenol-containing ethanol extracts from wheat, buckwheat and tea callus tissue. The absorption spectra of the products of reduction of these reagents by gallic acid, rutin, (–)-epicatechin, as well as ethanol extracts from wheat, buckwheat, and tea callus tissue, were in the same range (680–770 nm) and had similar characteristics. The calibration graphs of dependence of optical density of the solutions on the concentration of standard substances (gallic acid, (–)-epicatechin, rutin) constructed using the Folin–Denis and Folin–Ciocalteu reagents had a linear pattern within a concentration range of 10–100 μg/mL and nearly coincided. The results of determining the content of phenolic compounds in ethanol extracts of plants differing in the ability to accumulate them showed highly similar and statistically significant values when using these two reagents.

Keywords: method, phenolic compounds, Folin–Denis reagent, Folin–Ciocalteu reagent, ethanol extracts of plants

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INTRODUCTION

The relevant and important objectives for researchers and practitioners include determination of the levels of various metabolites in plant tissues and organs. They include phenolic compounds, or polyphenols, being among the most widespread substances of secondary metabolism with extremely diverse structure [1, 2]. Their functional role is associated with plant growth and development, regulation of photosynthesis, respiration and the hormonal system, as well as protection from different stress factors [3, 4]. The antioxidant and antiradical properties of these secondary metabolites allow them to "neutralize" reactive oxygen species [5]. This is due to the presence of hydroxyl groups in their structure, which easily interact with various reactive oxygen species, including free radicals of different origin, and thereby interrupt the processes of radical chain oxidation, which is especially important under plant cell exposure to stress factors [5, 6].

It is known that phenolic compounds are not synthesized in animal and human organisms but enter mainly with plant food and demonstrate anti-inflammatory, antiviral, anticarcinogenic, cardioprotective, and other properties [7]. The marked therapeutic effect, high physiological activity and low toxicity of plant polyphenols determine the possibility of their application in medical and pharmaceutic industries. It is precisely this aspect that is of great interest to both researchers and practitioners.

Determination of the levels of phenolic compounds in plant tissues and cells requires reliable, sensitive and simple methods. At present, it is performed using spectrophotometry (colorimetry) with the Folin–Denis or Folin–Ciocalteu reagents containing phosphotungstic and phosphomolybdic heteropoly acids, which are reduced with phenolic compounds in an alkaline medium. This leads to the formation of a blue-colored complex (tungsten blue or heteropoly blues) whose color intensity is proportional to the number of phenolic compounds [8, 9].

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The method for the quantitative determination of phenolic compounds in human urine using heteropoly acids was proposed for the first time by Folin and Denis in 1912 [10]. Later on it was modified by Swain and Hillis to determine the total content of phenolic compounds in plant objects [11]. For a long time it had been the basic (classical) method of their determination with the Folin–Denis reagent [12]. However, its disadvantage was the formation of a white precipitate that had to be removed (by filtration or centrifugation) before spectrophotometry of heteropoly blues. To eliminate this drawback, the Folin–Denis reagent was further modified by adding a lithium sulfate component (to prevent precipitate formation) and increasing the proportion of phosphomolybdic acid in the heteropoly acid complex (to increase its sensitivity). This improved version of the Folin–Denis reagent was called the Folin–Ciocalteu reagent (13). It was originally intended for determination of tyrosine, tryptophan and protein but also proved to be suitable for quantitative determination of phenolic compounds [8, 13, 14].

It should be noted that the original method for determining the content of phenolic compounds in plant tissue extracts required large volumes of reagents, i.e., their significant consumption [13]. Hence, later it underwent various modifications related to the changes in reaction mixture volume, reagent concentration and sample–reagent ratio [6, 15, 16]. It should be emphasized that this method was used for a long time with both of these reagents to determine the total content of phenolic compounds in plant extracts. However, in recent years preference has been given to the Folin–Ciocalteu reagent, especially by foreign authors [16, 17]. This raises the question of the possibility of comparing the data on the total content of phenolic compounds in plant extracts obtained with the Folin–Denis reagent and with the Folin–Ciocalteu reagent.

This work was aimed at modifying and comparing the methods for determining the total content of phenolic compounds in plant extracts using Folin–Denis reagent and Folin–Ciocalteu reagent to establish the correspondence between the results obtained.

EXPERIMENTAL

The studies were performed with ethanol extracts obtained from the heterotrophic callus culture of tea plant (*Camellia sinensis* L.), the sprouts of soft spring wheat (*Triticum aestivum* L.), Amir variety, and buckwheat (*Fagopyrum esculentum* Moench), Dasha variety, as well as gallic acid, (–)-epicatechin and rutin solutions.

The callus culture of tea plant stem was grown in the dark at +25°C and relative humidity of 70% on the basic Heller medium containing 2,4-dichlorophenoxyacetic acid (5 mg/L), glucose (2.5%), and agar (7%) [18]. Twenty-day calluses were used for the study. Wheat and buckwheat seedlings were grown in roll culture for 11 days in water under a 16-h photoperiod (illuminance of 5000 lux) and a temperature of +25°C. The material was fixed with liquid nitrogen and stored at –70°C.

Phenolic compounds were extracted with 96% ethanol from the plant material frozen with liquid nitrogen and crushed for 45 min at 45°C [19]. The homogenate was centrifuged at 16000 rpm for 2 min. The supernatant was separated and used for simultaneous determination of the total content of phenolic compounds with Folin–Denis and Folin–Ciocalteu reagents. The content of phenolic compounds in the plant material was expressed as mg eq. gallic acid/g wet weight.

Gallic acid, rutin, and (–)-epicatechin solutions were prepared by diluting commercial preparations (Serva, Germany) in 96% ethanol with a concentration range of 10–150 μg/mL. They were used to plot calibration curves for the Folin–Denis and Folin–Ciocalteu reagents. The Folin–Denis reagent was prepared by the standard procedure [12]. The Folin–Ciocalteu reagent was a commercial preparation (AppliChem. Pancreac, Germany).

The absorption spectra of heteropoly blues formed during the interaction between the Folin–Denis and Folin–Ciocalteu reagents and the gallic acid, (–)-epicatechin, rutin, and ethanol extracts from the callus cultures of tea, buckwheat, and wheat tissue were measured within a range of 600–900 nm.

MiniSpin microcentrifuges (Eppendorf, Germany), Gnom thermostats (DNA-technologies, Russia) and SF-46 (LOMO, Russia) and Specord-40 (Germany) spectrophotometers were used in the studies.

All determinations were performed in five biological and three analytical replicates. Statistical processing of the results was performed with Microsoft Excel 2010 and Sigma Plot 12.2. Table 1 shows the arithmetic mean and standard error of determined values. Superscripts denote the significance of differences of the mean values by the Student's t-test at $p < 0.050$.

RESULTS AND DISCUSSION

The modern approach to scientific research and data acquisition requires the use of reliable, reproducible and simple methods, as well as cost reduction, inter alia, due to the lower consumption of reagents. For this purpose, the classical (standard) method for determining the total content of phenolic compounds with the Folin–Denis reagent, where the reaction mixture consists of 0.5 mL of a sample (ethanol plant extract or standard reagent), 0.5 mL of the Folin–Denis reagent, 1 mL of saturated Na_2CO_3 solution and distilled water in a total volume of 10 mL, was modi-

Table 1. The total content of phenolic compounds (PC) in ethanol extracts obtained from various plant tissues and analyzed with the Folin–Denis reagent or the Folin–Ciocalteu reagent

Plant object	Total PC content (mg equiv gallic acid/g wet weight)	
	Folin–Denis reagent	Folin–Ciocalteu reagent
Wheat	0.937 ± 0.068^a	0.937 ± 0.069^a
Buckwheat	2.625 ± 0.193^b	2.490 ± 0.182^b
Tea callus culture	1.050 ± 0.076^c	1.0540 ± 0.074^c

The mean values of determinations and their standard errors are presented ($n = 5$). The values significantly different at $p < 0.05$ are denoted by different letters (a, b, c).

fied in the first place [11, 12]. In our work, the latter was reduced to 1.5 mL while maintaining the ratio and concentrations of all components, which made it possible to use Eppendorf tubes (1.7 mL), DNA-technology thermostats, and microcentrifuges. In addition, instead of saturated Na_2CO_3 solution, the concentration of which can vary depending on the ambient temperature, a 20% solution was used as noted in other works [6, 8]. Strictly speaking, its concentration is not fundamentally important and can vary within a fairly wide range (from 7.5 to 20%). It is only necessary that it should be the same for all determinations, since the function of this component in the reaction mixture is to maintain the alkaline environment within a range of pH 9–10, which is optimal for the reaction [8, 14].

As a result of the above, the reaction mixture for determining the total content of phenolic compounds by the standard method contained 0.075 mL of ethanol extract from plant material or standard reagent (when plotting the calibration curve), 0.075 mL of the Folin–Denis reagent, 0.15 mL of 20% Na_2CO_3 solution, and 1.20 mL of distilled water.

The next stage of the work was to develop the method for determining the content of phenolic compounds in ethanol extracts of plants using the Folin–Ciocalteu reagent. The main criterion in this case was to obtain the same optical density values of heteropoly blue solutions formed as a result of reaction between phenolic compounds and these two reagents. For this purpose, parallel determination was performed. The reaction mixture composition was the same in both cases, except that different concentrations of the Folin–Ciocalteu reagent were used while maintaining the volume introduced. This was achieved by diluting the commercial solution 2–10 times (data not shown). A mixture with 0.075 mL of 96% ethanol added instead of the extracts was used as a control in both cases. The reaction mixture was left to stand for 1 h and, if necessary, centrifuged to remove the precipitate, followed by the measurement of optical density of the solution at 725 nm [12]. The measurements were made in cuvettes with a narrow beam with an optical path length of 1 cm.

This approach has shown that the optical density of heteropoly blues formed during the interaction with ethanol extracts from different plant tissues in case of 5-fold dilution of the commercial Folin–Ciocalteu reagent (concentration of 0.4 N) was almost equal to that obtained with the Folin–Denis reagent. These data were used to consider the possibility of calculating and presenting the data on the total content of phenolic compounds in plants by the Folin–Ciocalteu method on the basis of data obtained by the classical method with the Folin–Denis reagent, which is important for presentation of experimental material in scientific articles.

To calculate the total content of phenolic compounds in plant material, it was necessary to plot calibration curves representing the dependence of optical density of the solution on substance concentration in the latter. For this purpose, we used gallic acid accepted as a standard for calculations in most research works (especially foreign ones), as well as (–)-epicatechin and rutin that are widespread in plant tissues and often used as standards [20, 21]. In all cases, the work was performed with the Folin–Denis and Folin–Ciocalteu reagents used in parallel. As follows from Fig. 1, all calibration curves for the dependence of optical density on the concentration of standard substances are linear in the range of 10–100 μg and almost completely coincide.

Consequently, the method for determining the total content of phenolic compounds with the Folin–Ciocalteu reagent that we have developed is comparable with that for the Folin–Denis reagent. This is confirmed by the results of determining the total content of phenolic compounds in ethanol extracts from wheat, buckwheat, and the callus culture of tea plant tissue (Table 1). When using these two reagents, their amounts in samples under study were sufficiently close to statistically significant values. Hence, significant differences in the content and composition of

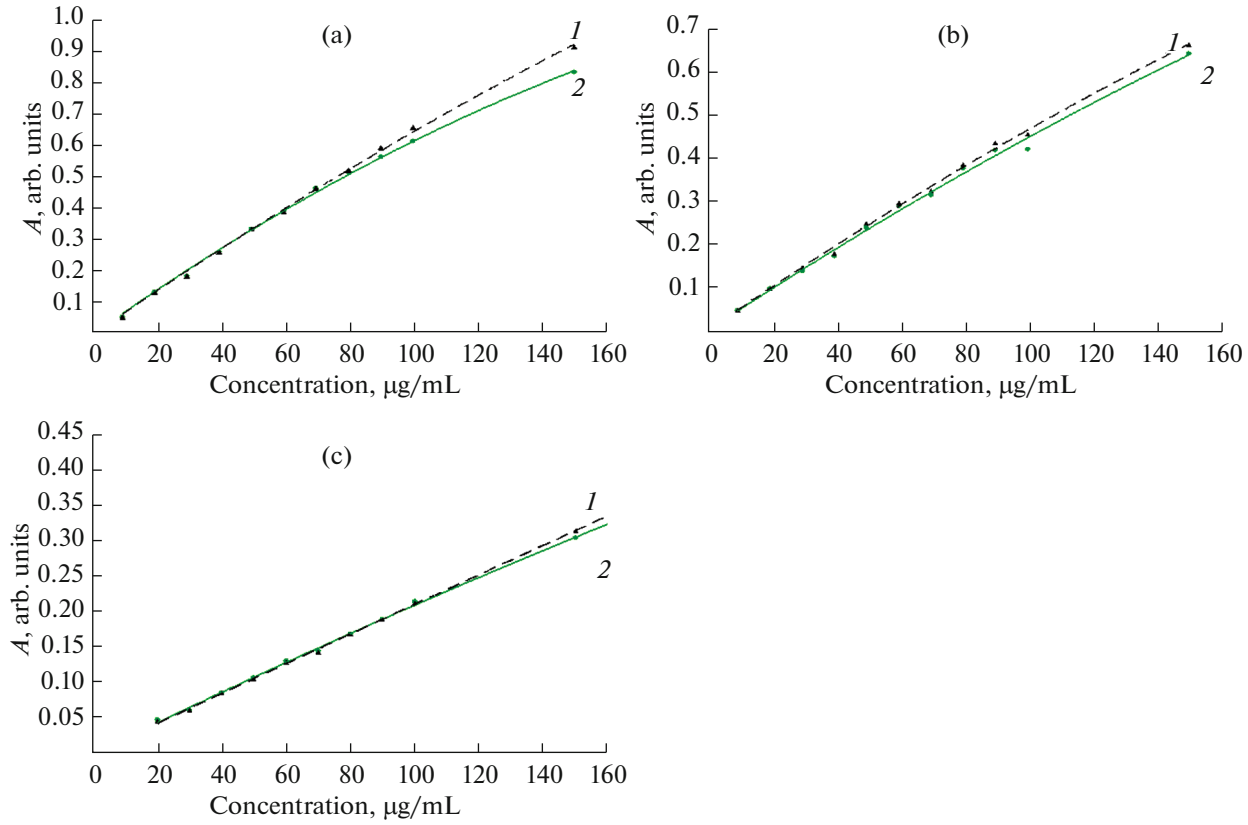

Fig. 1. The calibration curves of dependence between the optical density of heteropoly blues formed with the Folin–Denis (*1*) and Folin–Ciocalteu (*2*) reagents and the concentration of gallic acid (a), (–)-epicatechin (b), and rutin (c). Measurements were made at 725 nm in a 1-cm cuvette with a narrow beam.

phenolic complexes in these plant objects [18, 19, 22] had no effect on determination and calculation of the total content of phenolic compounds when using the Folin–Denis reagent or the Folin–Ciocalteu reagent.

As mentioned above, the optical densities of heteropoly blues were measured in all cases at 725 nm, the standard wavelength for the Folin–Denis reagent, whereas the wavelength range in the standard method with the Folin–Ciocalteu reagent is 760–765 nm. It is known that the absorption spectra of heteropoly blues formed with the Folin–Denis reagent and with the Folin–Ciocalteu reagent have rather broad maxima within wavelength ranges of 560–750 nm and 740–800 nm, respectively [8, 14]. These differences can be accounted for by the fact that the structure of the formed heteropoly blues depends on the phenolic compound–reagent ratio. An increase in the proportion of phenolic compounds leads to the formation of heteropoly blues of different structure, with the maximum of the absorption spectrum gradually shifting towards the shortwave region [23, 24]. This was observed when using the standard methods with the Folin–Denis and Folin–Ciocalteu reagents, when the phenolic compound–reagent ratio was 1 : 1 and 1 : 5, respectively. When using the standard method with the Folin–Ciocalteu reagent, the optical density of heteropoly blues is measured at 760–765 nm in most cases [6, 13, 25]. When working with the Folin–Ciocalteu reagent, we used the reaction mixture composition identical to that for the Folin–Denis reagent and, accordingly, the phenolic compound–reagent ratio was 1 : 1.

In this context, it was necessary to analyze the absorption spectra of heteropoly blues formed with the Folin–Ciocalteu reagent at the concentration used vs. the spectra of heteropoly blues formed with the Folin–Denis reagent and to find out how much they coincide.

We have analyzed the spectra of heteropoly blues formed by these reagents with phenolic compounds from the ethanol extracts of wheat, buckwheat and the callus culture of tea plant tissue, as well as gallic acid, rutin and (–)-epicatechin (Fig. 2). All absorption maxima of these solutions are within the same spectral region (680–770 nm) and have similar patterns.

Thus, despite the differences in the content and composition of phenolic compounds of the plant objects used in our work, the heteropoly blues formed with the Folin–Denis and Folin–Ciocalteu reagents

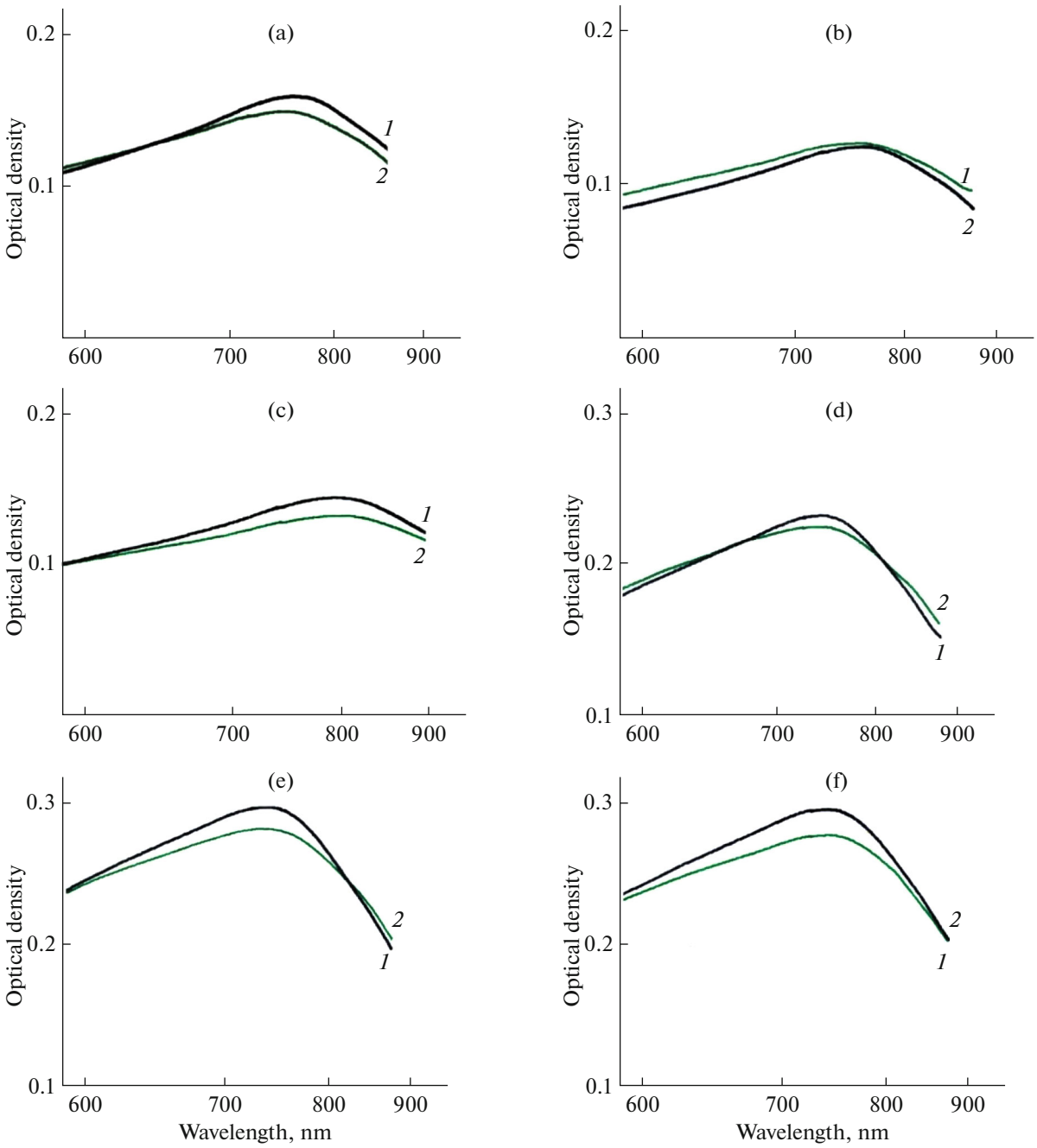

Fig. 2. The absorption spectra of heteropoly blues formed with the Folin–Denis (*1*) and Folin–Ciocalteu (*2*) reagents during oxidation of gallic acid (a), rutin (b), (–)-epicatechin (c) and polyphenol-containing ethanol extracts from wheat (d), buckwheat (e) and callus tea culture (f).

are probably similar in structure, which depends largely on the phenolic compound–reagent ratio but does not depend on the composition of reagent used in reaction mixture. The shift in the absorption maximum of heteropoly blues formed with the Folin–Ciocalteu reagent upon a change in the proportion of phenolic compounds relative to the latter has also been reported by other authors [16, 26, 27].

CONCLUSIONS

(1) The method for determining the total content of phenolic compounds in ethanol extracts from plant tissues using the Folin–Denis reagent has been adapted for the measurement in microvolumes.

(2) The method for determining the total content of phenolic compounds in ethanol extracts from plant

tissues using the Folin–Ciocalteu reagent (measurement in microvolumes) has been modified.

(3) The comparability of results of determining the content of phenolic compounds in ethanol extracts from plants using the modified method with the Folin–Denis reagent or the Folin–Ciocalteu reagent has been demonstrated.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflicts of interest. This article does not contain any studies involving animals or human participants performed by any of the authors.

REFERENCES

1. Zaprometov, M.N., *Fenol'nye soedineniya. Rasprostranenie, metabolizm i funktsii v rasteniyakh* (Phenolic Compounds. Distribution, Metabolism and Functions in Plants), Moscow, 1993.
2. Ramawat, K.G. and Merillon, J.M., *Natural Products: Phytochemistry, Botany and Metabolism of Alkaloids, Phenolics and Terpenes,* Heidelberg: Springer, 2013.
3. Cheynier, V., Comte, G., Davies, K.M., Lattanzio, V., and Martens, S., Plant phenolics: Recent advances on their biosynthesis, genetics and ecophysiology, *Plant Physiol. Biochem.,* 2013, vol. 72, pp. 1–20. https://doi.org/10.1016/j.plaphy.2013.05.009
4. Naikoo, M.I., Dar, M.I., Raghib, F., Jaleel, H., Ahmad, B., Raina, A., and Naushin, F., Role and regulation of plants phenolics in abiotic stress tolerance: An overview, in *Plant Signaling Molecules,* Netherlands: Woodhead, Springer, 2013, pp. 15–30. https://doi.org/10.1016/B978-0-12-816451-8.00009-5
5. Belscak-Cvitanovi, A., Durgo, K., Hudek, A., Bacun Druzina, V., and Komes, D., Overview of polyphenols and their properties, in *Polyphenols: Properties, Recovery, and Applications,* Duxford, UK: Woodhead, 2018, pp. 3–44.
6. Dudonne, S., Vitrac, X., Coutiere, Ph., Woillez, M., and Merillon, J.-M., Comparative study of antioxidant properties and total phenolic content of 30 plant extracts of industrial interest using DPPH, ABTS, FRAP, SOD, and ORAC assays, *J. Agric. Food Chem.,* 2009, vol. 57, pp. 1768–1774. https://doi.org/10.1021/jf803011r
7. Tungmunnithum, D., Thongboonyoul, A., Pholboon, A., and Yangsabai, A., Flavonoids and other phenolic compounds from medicinal plants pharmaceutical and medical aspects: An overview, *Medicines,* 2018, vol. 5, p. 93. https://doi.org/10.3390/medicines5030093
8. Singleton, V.L. and Rossi, J.A., Colorimetry of total phenols with phosphomolybdic-phosphotungstic acid reagents, *Am. J. Enol. Vitic.,* 1965, vol. 16, pp. 144–158.
9. *Rukovodstvo po metodam kontrolya kachestva i bezopasnosti biologicheski aktivnykh dobavok k pishche* (Guidance on Methods of Quality Control and Safety of Biologically Active Food Supplements), Moscow, 2004.
10. Folin, O. and Denis, W., On phosphotungstic-phosphomolibdic compound of a color reagents, *J. Biol. Chem.,* 1912, vol. 12, pp. 239–243.
11. Swain, T. and Hillis, W.E., The phenolic constituents of Prunus Domestica. 1. The quantitative analysis of phenolic constituents, *J. Sci. Food Agric.,* 1959, vol. 10, pp. 63–68.
12. Zaprometov, M.N., *Phenolic compounds and methods for their study, in Biokhimicheskie metody v fiziologii rastenii* (Biochemical Methods in Plant Physiology), Moscow, 1971, pp. 185–197.
13. Folin, O. and Ciocalteu, V., On tyrosine and tryptophane determination in proteins, *J. Biol. Chem.,* 1927, vol. 73, pp. 627–650
14. Singleton, V.L., Orthofer, R., and Lamuela-Raventos, R.M., Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin–Ciocalteu reagent, *Methods Enzymol.,* 1999, vol. 299, pp. 152–178.
15. Li, M., Pare, P.W., Zhang, J., Kang, T., Zhang, Zh., Yang, D., Wang, K., and Xing, H., Antioxidant capacity connection with phenolic and flavonoid content in chinese medicinal herbs, *Rec. Nat. Prod.,* 2018, vol. 12, pp. 239–250. https://doi.org/10.25135/rnp.24.17.08.138
16. Cicco, N., Lanorte, M.T., Paraggio, M., Viggiano, M., and Lattanzio, V., A reproducible, rapid and inexpensive Folin–Ciocalteu micro-method in determining phenolics of plant methanol extracts, *Microchem. J.,* 2009, vol. 91, pp. 107–110. https://doi.org/10.1016/j.microc.2008.08.011
17. Wabaidur, S.M., Obbed, M.S., Alothman, Z.A., Alfaris, N.A., Badjah-Hadj-Ahmed, A.Y., Siddiqui, M.R., Altamim, J.Z., and Aldayel, T.S., Total phenolic acids and flavonoid contents determination in yemeni honey of various floral sources: Folin–Ciocalteu and spectrophotometric approach, *Food Sci. Technol.,* 2020, vol. 40, pp. 647–652. https://doi.org/10.1590/fst.33119
18. Zagoskina, N.V., Nechaeva, T.L., Nikolaeva, T.N., Lapshin, P.V., and Goncharuk, E.A., Carbohydrates in the culture medium and their influence on growth and accumulation of phenolic compounds in tea plant callus culture (*Camellia sinensis* L.), *Nauch. Vedom. Belgor. Univ., Ser.: Estestv. Nauki,* 2016, vol. 37, no. 5 (246), pp. 45–55.
19. Olenichenko, N.A. and Zagoskina, N.V., Response of winter wheat to cold: production of phenolic compounds and L-phenylalanine ammonia lyase activity, *Appl. Biochem. Microbiol.,* 2005, vol. 41, no. 6, pp. 600–603.
20. Podolina, E.A., Khanina, M.A., Rudakov, O.B., and Nebol'sin, A.E., Spectrophotometric determination of phenol compounds from herbs of cornflower blue,

Khim. Rastit. Syr'ya, 2019, no. 3, pp. 145–152. https://doi.org/10.14258/jcprm.2019034675

21. Zubova, M.Yu., Nikolaeva, T.N., Nechaeva, T.L., Malyukova, L.S., and Zagoskina, N.V., About the content of pigments, phenolic compounds and antiradical activity of young tea shoots (Camellia sinensis L.), *Khim. Rastit. Syr'ya,* 2019, no. 4, pp. 249–257. https://doi.org/10.14258/jcprm.2019046065
22. Zagoskina, N.V., Kazantseva, V.V., Fesenko, A.N., and Shirokova, A.V., Accumulation of phenolic compounds at the initial steps of ontogenesis of fagopyrum esculentum plants that differ in their ploidy levels, *Biol. Bull.,* 2018, vol. 45, no. 2, pp. 171–178.
23. Wu, H., Contribution to the chemistry of phosphomolybdic acids, phosphotungstic acids, and allied substances, *J. Biol. Chem.,* 1920, vol. 43, pp. 189–220.
24. Denisenko, T.A., Vishnikin, A.B., and Tsyganok, L.P., Reaction features of 18-molibdodiphosphate and Folin–Ciocalteu reagent with phenolic compounds, *Anal. Kontrol',* 2015, vol. 19, no. 3, pp. 242–251. https://doi.org/10.15826/analitika.2015.19.3.001
25. Fuentes, J., Montoya, P., Vio, F., and Speisky, H., Total phenolics and antioxidant capacity of vegetables grown in the southwestern andes region of south america, *J. Food Nutr. Res.,* 2016, vol. 4, no. 12, pp. 760–772. https://doi.org/10.12691/jfnr-4-12-1
26. Swieca, M., Hydrogen peroxide treatment and the phenylpropanoid pathway precursors feeding improve phenolics and antioxidant capacity of quinoa sprouts via an induction of l-tyrosine and l-phenylalanine ammonia-lyases activities, *J. Chem.,* 2016, vol. 2016, p. 1936516. https://doi.org/10.1155/2016/1936516
27. Ferreira, V.B., Costa da Silva, T.C., Silvia Regina Magalhães Couto, S.R.M., and Srur, A.U.O.S., Total phenolic compounds and antioxidant activity of organic vegetables consumed in Brazil, *Food Nutr. Sci.,* 2015, vol. 6, pp. 798–804. https://doi.org/10.4236/fns.2015.69083

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