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Determination of the extraction frequency of green tea leaves by the antioxidant method

Abstract

Aim. To determine the optimal extraction frequency of green tea leaves with 60 % ethanol by the antioxidant method.

Materials and methods. Chun Myn green tea leaves were the object of the study, the raw material was collected in Anhui province (China) from March to April. Dry green tea leaves were standardized according to the European Pharmacopeia 9.0. Spectrophotometry was used to quantify biologically active substances. The antioxidant activity was determined by the potentiometric method. Potentiometric measurements were performed on a HANNA 2550 pH meter (Germany) with a combined platinum EZDO 50 PO electrode (Taiwan). A UV-1000 spectrophotometer (China) was used to measure the optical density.

Results and discussion. The total content of phenolic compounds was 9.60 ± 0.17, 1.30 ± 0.03 and 0.12 ± 0.002 %, catechins – 9.20 ± 0.18, 1.20 ± 0.02 and 0.07 ± 0.002 %, flavonoids – 0.27 ± 0.005, 0.04 ± 0.001, 0.005 ± 0.001, hydroxycinnamic acids – 0.49 ± 0.01, 0.07 ± 0.002 and 0.007 ± 0.001 %, dry residue – 10.75 ± 0.11, 1.59 ± 0.02 and 0.15 ± 0.002 %, the antioxidant activity was 474.08 ± 9.48, 67.70 ± 1.35 and 7.01 ± 0.14 mmol-equiv $m_{dry\ res}^{-1}$ for the first, second and third extraction, respectively. According to the results obtained, the optimal number of extractions of the raw material with 60 % ethanol was found to be two.

Conclusions. The dynamic of extractions of biologically active substances of green tea leaves has been studied by triple extraction of the raw material to find the optimal extraction frequency; for the first time, a method for determining the extraction frequency based on the antioxidant activity of the extracts has been developed and proposed. It has been found that the optimal extraction rate is 2 times. The results obtained will be used in the further production of herbal medicines, dietary supplements, and cosmetic products with a green tea extract.

Keywords: green tea; leaves; analysis; extract; extraction; antioxidant activity

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Визначення кратності екстракції листя зеленого чаю антиоксидантним методом

Анотація

Мета. Визначити оптимальну кратність екстракції листя зеленого чаю 60 % етанолом антиоксидантним методом.

Матеріали та методи. Об'єктом дослідження було листя зеленого чаю сорту Чун Мін, зібране в провінції Анхуй (Китай) з березня до квітня. Сухе листя було стандартизовано відповідно до Європейської фармакопеї 9.0. Для кількісного визначення біологічно активних речовин використовували спектрофотометрію. Для визначення антиоксидантної активності використовували потенціометричний метод. Потенціометричні вимірювання виконували на pH-метрі HANNA 2550 (ФРН) із комбінованим платиновим електродом EZDO 50 PO (Тайвань). Для вимірювання оптичної густини використовували спектрофотометр UV-1000 (Китай).

Результати та їх обговорення. Сумарний вміст фенольних сполук становив $8,60 \pm 0,17$, $1,30 \pm 0,03$ і $0,11 \pm 0,002$ %, катехінів – $9,20 \pm 0,18$, $1,20 \pm 0,02$ і $0,07 \pm 0,002$ %, флавоноїдів – $0,27$, $0,04$ і $0,005$ %, гідроксикоричних кислот – $0,49 \pm 0,01$, $0,07 \pm 0,002$ і $0,007 \pm 0,001$ %, сухий залишок – $10,75 \pm 0,11$, $1,59 \pm 0,02$ і $0,15 \pm 0,002$ %, антиоксидантна активність становила $474,08 \pm 9,48$, $67,70 \pm 1,35$ і $7,01 \pm 0.14$ ммоль-екв. $m_{сух.зал.}^{-1}$ для першої, другої і третьої екстракції відповідно. Одержані результати свідчать, що двократна екстракція досліджуваної сировини 60 % етанолом є оптимальною.

Висновки. Для визначення оптимальної кратності екстракцій було проведено дослідження динаміки екстракцій біологічних активних речовин листя зеленого чаю шляхом трикратної екстракції сировини, а також уперше розроблено

й запропоновано метод визначення кратності екстракції, заснований на визначенні антиоксидантної активності екстракти. Виявлено, що двократна екстракція є оптимальна. Отримані результати будуть використані для створення фітопрепаратів, дієтичних добавок та косметологічної продукції з екстрактом зеленого чаю.

Ключові слова: зелений чай; листя; аналіз; екстракт; екстракція; антиоксидантна активність

■ Introduction

Oxidative stress is a part of many diseases, such as diabetes mellitus, cancer, atherosclerosis, Alzheimer's disease, and chronic obstructive pulmonary disease [1]. Literature data evidence represents that oxidative stress is caused by the imbalance between the production and degradation of reactive oxygen species (ROS). Examples of ROS include free radicals, such as the superoxide radical ($O_2^{\bullet-}$), hydroxyl radical ($OH^{\bullet}$), and nitric oxide radical ($NO^{\bullet}$) [2].

The defense system of the human body involves endogenous enzymes inactivating ROS. The members of this group are superoxide dismutase, catalase, and glutathione peroxidase [3]. However, there are cases when the endogenous defense system is exhausted by chronic diseases, stress, and not a good diet. As a result, it requires exogenous antioxidants, such as phenolic compounds, carotenoids, vitamin E, and amino acids. Among them, the leading position in the top of antioxidants is taken by catechins – a subgroup of phenolic compounds [4].

Green tea leaves are the major source of catechins. The content of catechins there varies from 25 to 35% [5]. Moreover, green tea leaves contain other subgroups of phenolic compounds. Thus, flavanols (1–2.5%), flavanones (1.5–3%), phenolic acids (2–5%); caffeine (1.5–2.5%), amino acids (1–5.5%), organic acids (1–1.8%) have been isolated from green tea leaves [6].

Owing to the chemical composition of green tea leaves, the extract from this raw material can be used in developing and creating herbal medicines, dietary supplements, and cosmetic products, which further can be used by patients with cardiovascular diseases, diabetes mellitus, cancer, polycystic ovary syndrome, infectious diseases, etc. Today, the optimal extraction frequency is determined by measuring the content of biologically active substances, as well as the dry residue in the extracts obtained. However, no one has previously described the determination of the extraction frequency by measuring the antioxidant activity of the extracts. Our recent study [3] has revealed that 60% ethanol is the optimal solvent for obtaining an extract from green tea leaves. Therefore, the current work aimed to study the dynamics of extraction of biologically active substances from green tea leaves with 60% ethanol to determine the optimal extraction frequency by the method of measuring the antioxidant activity.

■ Materials and methods

Chun Myn green tea leaves were the object of the study, the raw material was collected in Anhui province (China) from March to April. Dry green tea leaves were standardized according to the European Pharmacopeia 9.0 [7]. All solvents and other chemicals used in the study were of analytical grade.

Potentiometric measurements were performed on a HANNA 2550 pH meter (Germany) with a combined platinum EZDO 50 PO electrode (Taiwan). The quantitative analysis of biologically active compounds was performed on a UV-1000 spectrophotometer (China) with matched 1 cm quartz cells.

The weighing was done using an AN100 digital analytical balance (AXIS, Poland) with d = 0.0001 g.

Extraction procedure

Five (5.0) g of the grinded leaves was mixed with 100 mL of 60% ethanol. The extraction was carried out within 1 hour on a water bath with a condenser, then it was repeated two times with a new portion of the solvent. After that, the extracts

obtained were filtrated and concentrated to 10 mL using a rotary evaporator.

The dry residue of the extracts was determined by the gravimetric method according to the State Pharmacopeia of Ukraine (SPhU) [8].

Preparation of the standard solutions

To plot the calibration curve of the dependence of absorbance on the amount of gallic acid, the stock solution (250 mg mL^{-1}) was prepared by dissolving 50.0 mg (accurate weight) of gallic acid in 96% ethanol, and the solution was diluted to 200.0 mL with the same solvent. The stock solution was diluted with the solvent to prepare the model solutions 1–5 with concentrations of 1.0; 2.0; 3.0; 4.0; 5.0 µg mL^{-1}, respectively.

To plot the calibration curve of the dependence of absorbance on the amount of (−)-epigallocatechin gallate, the stock solution (10 mg mL^{-1}) was prepared by dissolving 250.0 mg of (−)-epigallocatechin gallate in 96% ethanol, and the solution was diluted to 25.0 mL with the same solvent. The stock solution was diluted with the solvent to prepare the model solutions 1–5 with the concentrations of 100; 150; 200; 300; 400 µg mL^{-1}, respectively.

The standard solution of rutin was prepared by weighing 10 mg (accurate weight) of rutin, transferred in a 25 mL volumetric flask, dissolved in 70% ethanol while heating on a water bath, and diluted to the volume with the same solvent. 1.0 mL of the solution prepared was transferred into a 25 mL volumetric flask and diluted to the volume with the same solvent.

The total content of phenolic compounds was measured by the Folin-Ciocalteu assay, the optical density was measured at 760 nm [9]. The calibration curve was plotted with interval concentrations of 1.0–5.0 µg mL^{-1}, the calibration equation Y = 0.1055X + 0.1745 (R^2 = 0.9951). The total content of phenolic compounds with reference to gallic acid was calculated according to the following equation:

$$X(\%) = \frac{C_x \times K_{dil} \times 100}{V}$$

where C_x – is the concentration of gallic acid according to the calibration curve, $C \times 10^{-6}$, g mL^{-1}; V – is the volume of the extract, mL; K_{dil} – is the dilution coefficient.

The vanillin reagent assay was applied to determine the total amount of catechins [5], and the absorbance was measured at 505 nm. The calibration curve was plotted within interval concentrations of $(100-400) \times 10^{-6}$ g mL^{-1}, the calibration equation Y = 0.0025X − 0.0851 (R^2 = 0.9951). The total catechins content in the extracts with reference to (−)-epigallocatechin gallate was calculated according to the following equation:

$$X(\%) = \frac{C_x \times K_{dil} \times 100}{V}$$

where C_x – is the concentration of (−)-epigallocatechin gallate according to the calibration curve, $C \times 10^{-6}$ g mL^{-1}; K_{dil} – is the dilution coefficient.

The total flavonoids were determined using the assay of the complex formation with 2% AlCl$_3$, the absorbance was measured at 417 nm [10].
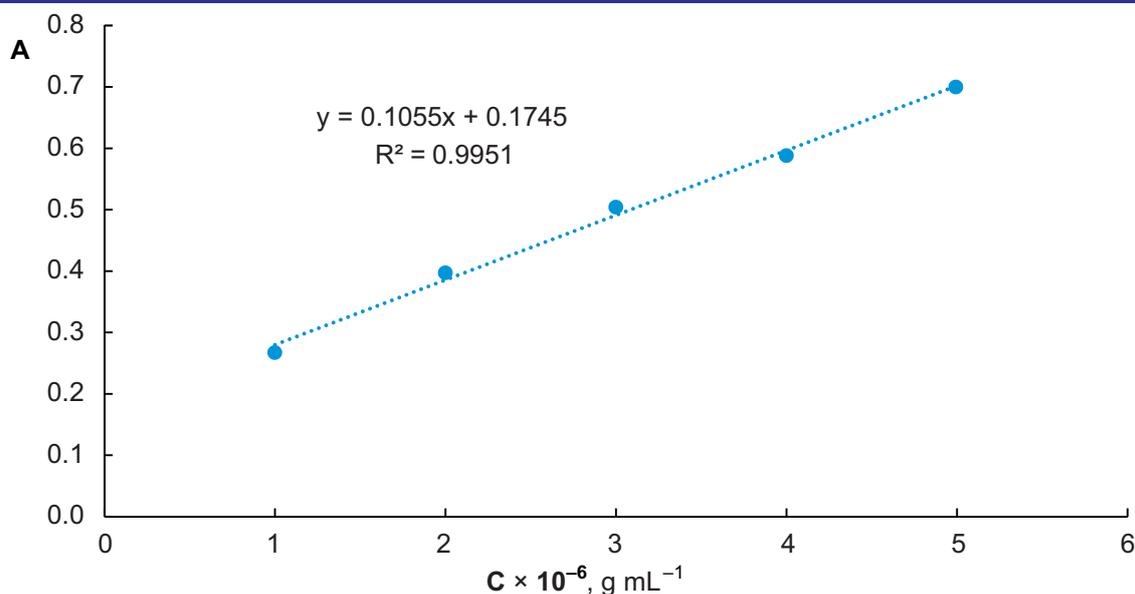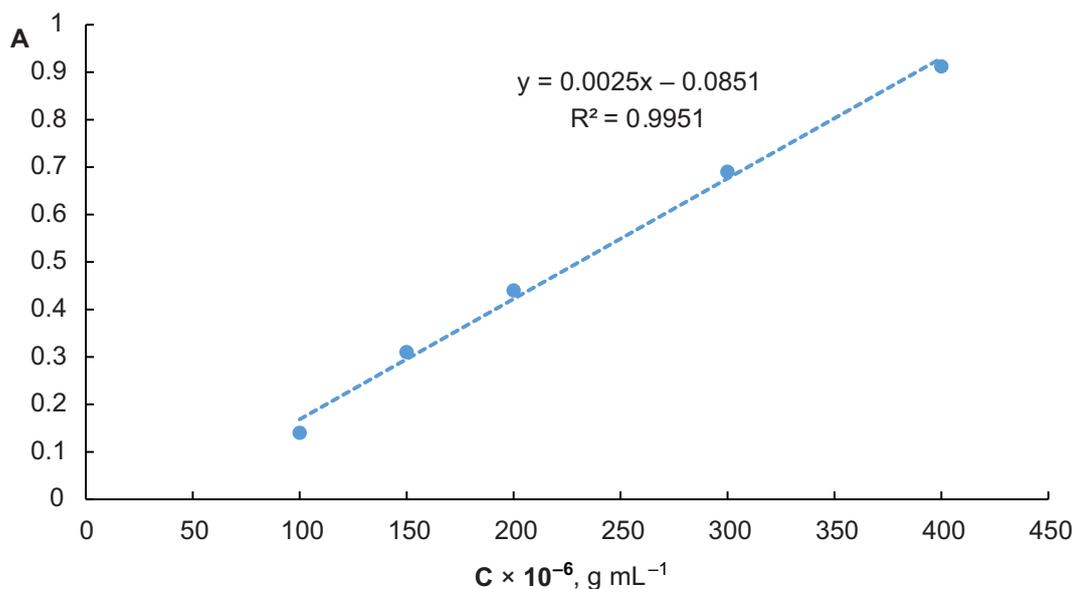

Figure 1. The calibration curve of the absorbance against the concentration of gallic acid

Figure 2. The calibration curve of the absorbance against the concentration of (−)-epigallocatechin gallate

The total content of flavonoids in the extracts with reference to rutin was calculated according to the following equation:

$$X(\%) = \frac{A \times K_{\text{dil}} \times 100}{A_{\text{st}} \times V}$$

where A – is the absorbance of the solution analyzed; A_{st} – is the absorbance of the standard solution of rutin; V – is the volume of the extract, mL; K_{dil} – is the dilution coefficient.

The total content of hydroxycinnamic acids was measured by the assay of complex formation with $NaNO_2$-Na_2MoO_4, and the absorbance was measured at 505 nm [11]. The total content of hydroxycinnamic acids in extracts with reference to chlorogenic acid was calculated according to the following equation:

$$X(\%) = \frac{A \times K_{\text{dil}}}{188 \times V}$$

where A – is the absorbance of the solution analyzed; 188 – is the specific absorption coefficient of chlorogenic acid; V – is the volume of the extract, mL; K_{dil} – is the dilution coefficient.

The antioxidant activity of the extracts was measured by the potentiometric method [12] calculated according to the following equation, and expressed as mmol-equiv $m_{\text{dry res.}}^{-1}$:

$$AOA = \frac{C_{\text{ox}} - \alpha \times C_{\text{red}}}{1 + \alpha} \times K_{\text{dil}} \times 10^3 \times \frac{m_1}{m_2}$$

where $\alpha = C_{\text{ox}}/C_{\text{red}} \times 10^{(\Delta E - E\text{ethanol})nF/2.3RT}$; C_{ox} – is the concentration of $K_3[Fe(CN)_6]$, mol L^{-1}; C_{red} – is the

concentration of $K_4[Fe(CN)_6]$, mol L^{-1}; E_{ethanol} − 0.0546×$C_{\%}$ − 0.0091; $C_{\%}$ – is the concentration of ethanol; ΔE – is the change of the potential; $F = 96485.33$ C mol^{-1} – is the Faraday constant; n = 1 – is the number of electrons in the electrode reaction; $R = 8.314$ J mol^{-1} К$^{-1}$ – is the universal gas constant; T – 298 K; K_{dil} – is the dilution coefficient; m_1 – is the mass of a dry residue; m_2 – is the mass of a dry residue in 1.0 mL of the extract.

For all experiments, five samples were analyzed, and all assays were performed 5 times. The results were expressed as mean values with confidence intervals. MS EXCEL 7.0 and STATISTIKA 6.0 were used to perform the statistical analysis.

■ Results and discussion

The total content of phenolic compounds was determined by the Folin-Ciocalteu method. According to the results obtained given in Table, the total content of phenolic compounds was 9.60±0.17% in the first extraction, whereas in the second extract the amount of phenolic compounds decreased by 86%, and in the case of the third extraction by 99%. The summary content of phenolic compounds was equal to 11.02% in the total extract.

The amount of catechins was measured using the vanillin reagent assay. Table represents that in the first extraction the content of catechins was 9.20±0.18%, in the second extraction − 1.20±0.02%, and in the third one − 0.07±0.002%. The total catechins were equal to 10.47% in the total extract. Hence, the contribution of the first

Table. The total content of phenolic compounds, catechins, flavonoids, and hydroxycinnamic acids in green tea extracts

	1st extraction	2nd extraction	3rd extraction	Sum
Dry residue, %	10.75±0.11	1.59±0.02	0.15±0.005	12.49
Total phenolic compounds, %	9.60±0.17	1.30±0.03	0.12±0.002	11.02
Total catechins, %	9.20±0.18	1.20±0.02	0.07±0.002	10.47
Total flavonoids, %	0.27±0.005	0.04±0.001	0.005±0.001	0.32
Total hydroxycinnamic acids, %	0.49±0.01	0.07±0.002	0.007±0.001	0.56
Antioxidant activity, mmol-equiv $m_{dry\ res.}^{-1}$	474.08±9.48	67.70±1.35	7.01±0.14	548.79

extraction to the overall extract was 87.87%, the second one – 11.46%, and the third one – 0.67%.

The total amount of flavonoids in extracts was relatively low compared to other biologically active substances. In the first extract their content was 0.27±0.005%, in the second one – 0.04±0.001%, and the third one – only 0.005±0.001%.

Table also shows that the total content of hydroxycinnamic acids was 0.56% in the total extract. The first extraction provided 0.49±0.01%, the second one – 0.07±0.002%, and the last one – 0.007±0.001% to the total extract. Among all phenolic compounds, hydroxycinnamic acids took second place after catechins.

The dry residue of the extracts analyzed was determined by the gravimetric method of analysis. Table shows that the dry residue in the first extraction was 10.75±0.11%, in the second one –

1.59±0.02%, and the third one – 0.15±0.005%. The total dry residue of three extractions was 12.49%.

Today the main method of finding the extraction frequency from various types of the raw material is based on determining the dry residue and the amount of biologically active substances in extracts. According to the data obtained (Table, Figure 3), the optimal extraction frequency equals two. The third extraction with a new portion of the solvent does not significantly increase the amount of biologically active substances in the total extract.

However, in our view, this way of determining the extraction frequency is not rather suitable due to several disadvantages of the method. Among others, one should note that the gravimetric method and the measuring amount of all
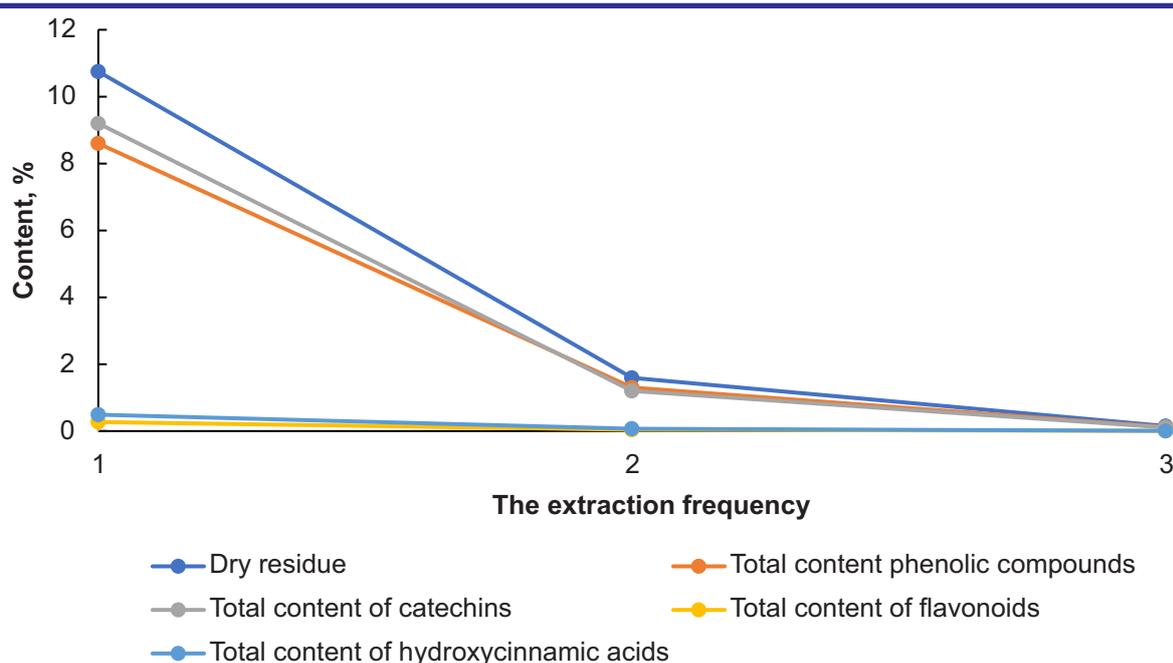
Figure 3. Curves of the dry residue, total phenolic compounds, catechins, flavonoids, hydroxycinnamic acids versus the extraction frequency
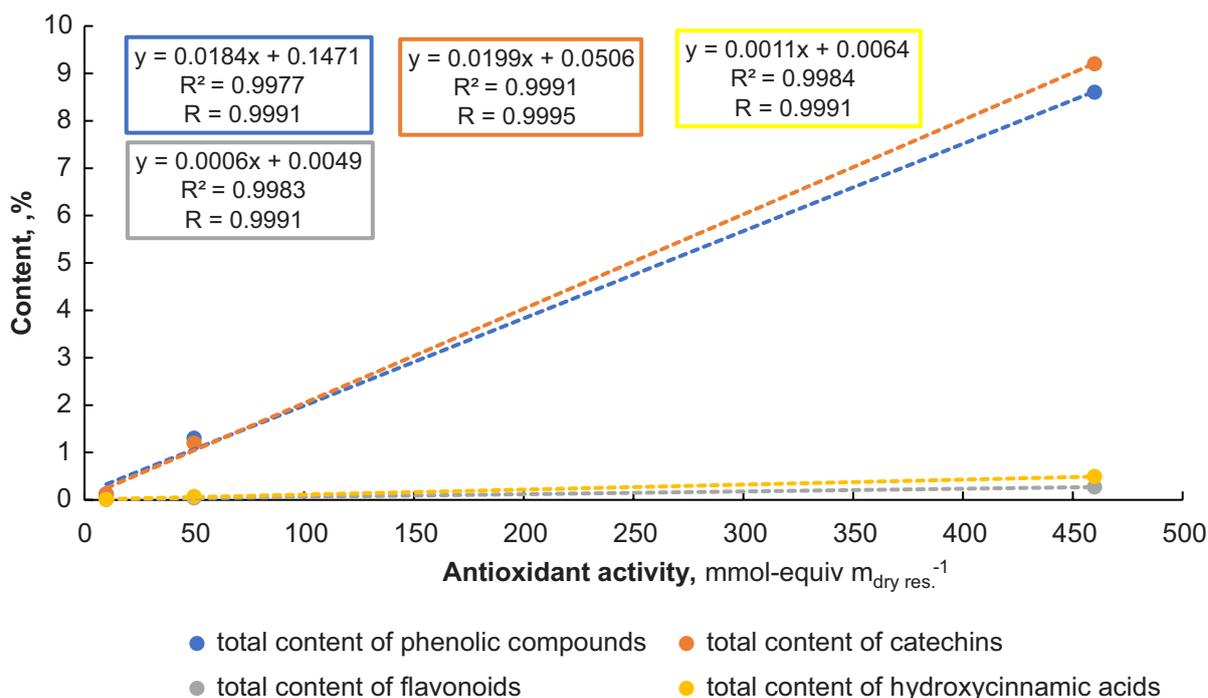
Figure 4. The correlation between the antioxidant activity and the content of total phenolic compounds, catechins, flavonoids, hydroxycinnamic acids in extracts of green tea leaves

biologically active substances in extracts are rather time-consuming processes.

Therefore, herein we suggest determining the optimal extraction frequency using the antioxidant activity of the extracts obtained and the total antioxidant activity of the raw material. There are some reasons for that. Firstly, the potentiometric method of the antioxidant activity determination is simple and not time-consuming. Secondly, the accuracy of the method is not inferior to that one of gravimetry. Moreover, it does not need expensive reagents and equipment for analysis. In addition, in many scientific studies the antioxidant activity correlates with the anti-inflammatory effect, this gives an idea of other possible effects of the extracts under research [14].

In our previous study [15] it was found that the total antioxidant activity of green tea leaves equaled 660.75 mmol-equiv $m_{dry\ res.}^{-1}$. In our opinion, the most optimal value for the acceptance criterion of the antioxidant activity is 80% of the total antioxidant activity of the raw material, which is due to the fact that, depending on the type of green tea, the antioxidant activity of its extracts may vary. Thus, in our case, the acceptance criterion equals 528 mmol-equiv $m_{dry\ res.}^{-1}$. Table represents that the antioxidant activity of the 1st extract was 474.08 mmol-equiv $m_{dry\ res.}^{-1}$, the 2nd extract – 67.70 mmol-equiv $m_{dry\ res.}^{-1}$ and the 3rd extract – 7.01 mmol-equiv $m_{dry\ res.}^{-1}$. The antioxidant activity of the total extract was equal to 548.79 mmol-equiv $m_{dry\ res.}^{-1}$ as shown in Table.

The 1st extraction contributes 86% to the total antioxidant activity, the 2nd extraction – 12.73%, and the 3rd one only 1.27%. According to the results, the total antioxidant activity is equal to 542 mmol-equiv $m_{dry\ res.}^{-1}$ after two extractions of green tea leaves, and it meets the requirement of the acceptance criteria as it is not less than 528 mmol-equiv $m_{dry\ res.}^{-1}$. Therefore, the 3rd extraction was redundant and only led to overspending of the solvent, energy, and labor resources, and decreasing the labor productivity.

To compare the methods of determining the extraction frequency, the correlation between antioxidant activity and the content of biologically active substances in green tea extracts was assessed. For this purpose, the linear regression analysis and Pearson's coefficient (R) were used. As a result, it was found that there was a very high correlation between the antioxidant activity and the amount of phenolic compounds (R = 0.9991), catechins (R = 0.9995), flavonoids (R = 0.9991), and hydroxycinnamic acids (R = 0.9991). Thus, there is no difference in the results and the method of the antioxidant activity can be applied to determine the optimal extraction frequency from green tea leaves. The results obtained are shown in Figure 4.

■ Conclusions

The dynamic of extractions of biologically active substances of green tea leaves has been studied

by triple extraction of the raw material to find the optimal extraction frequency; for the first time, a method for determining the extraction frequency based on the antioxidant activity of the extracts has been developed and proposed.

Thus, it has been found that the optimal extraction rate is 2 times. The results obtained will be used in the further production of herbal medicines, dietary supplements, and cosmetic products with a green tea extract.

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