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DETERMINATION Of ANTIOXIDANTS In HERBAL SUPPLEMENTS By HPLC and X-RAY ELECTROMAGNETIC FIELD DETECTOR In A SCANNING ELECTRON MICROSCOPE SYSTEM

Elena Kuznetsova[1,], Svetlana Motyleva[2], Ján Brindza[3], Houssam Alhusseini[4], Galina Zomiteva[1], Elena Kuznetsova[4], Elvira Pyanikova[5], Anna Kovaleva[5], Maria Zaikina[5], Maksim Rebezov[6], Vladimir Masalov[7], Oksana Evdokimova[7], Natalia Berezina[7], Mohammad Ali Shariati[9,*]*

Address(es):

[1] Orel State University named after I.S. Turgenev, 95 Komsomolskaya str., Oryol, 302026, Russia.
[2] Laboratory of Physiology and Biochemistry and Biotechnology of Functional Products, Federal center of vegetable production, 4 Zagorievskay str., Moscow, 115598, Russia.
[3] Institute of biological conservation and biosafety, Faculty of agrobiology and food resources, Slovak University of Agriculture in Nitra, 2 Trieda Andreja Hlinku, 94976, Nitra, Slovakia.
[4] Research Department, Orel State University named after I.S. Turgenev, 95 Komsomolskaya str., Oryol, 302026, Russia.
[5] Southwest State University, 94, 50 years of October Av., Kursk, 305040, Russia.
[6] V. M. Gorbatov Federal Research Center for Food Systems, 26, Talalikhin Str., Moscow, 109316, Russia.
[7] Oryol State Agrarian University named after N.V. Parakhina, 69 General Rodin str., Oryol, 302019, Russia.
[8] Konkuk University, Seoul, 05029, Republic of Korea.
[9] Kazakh Research Institute of Processing and Food Industry, Semey Branch of the Institute, 238G Gagarin Ave., Almaty, 050060, Kazakhstan.

*Corresponding author: elkuznetcova@yandex.ru ; shariatymohammadali@gmail.com

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ABSTRACT

An herbal supplement made with thyme, mint, cilantro, and rowan and viburnum fruit has been used to fortify wheat bread with antioxidants. The antioxidant activity of raw materials and bread was determined by the spectrophotometric method. To determine substances with antioxidant properties, the HPLC method was used. The chromatogram of the alcoholic extract of the herbal supplement showed that the supplement contains organic acids, flavonoids and anthocyanins. The HPLC method was used to determine the amount of flavonoids in the supplement and bread. Micrographs of the surface of slices of bread with a phytonutrient were obtained by scanning electron microscopy. The presence of chemical elements with antioxidant properties was determined using an X-ray EMF detector in the scanning electron microscope system. The use of phytonutrients in bread technology has been found to lead to an increase in the quality of the product and its antioxidant activity.

Keywords: Herbal supplements; Antioxidants; Spectrophotometric method; HPLC; Organic acids; X-ray EMF; Antioxidant activity

INTRODUCTION

Antioxidants are known to play an important role in promoting human health and protecting against many diseases. Therefore, bread with high antioxidant content is in high demand by consumers. Typically, the antioxidant activity of enriched bread is mainly associated with the presence of phenols **(Gnanavinthan *et al.*, 2011)**. To increase antioxidant activity, bread is enriched with natural ingredients with antioxidant properties **(Nilova *et al.*, 2018)**. In the group of phenolic substances, the most common are flavonoids and phenolic acids, which have a wide spectrum of biological and pharmacological activity **(Dajas, 2012; Becut *et al.*, 2018)**. It is known that phenolic acids and flavonoids are the protective mechanisms of the cell to prevent any lethal effects caused by stress **(Manquián-Cerda *et al.*, 2018; Davies *et al.*, 2018)**. Flavonoids are part of the human diet and have a positive effect on the body. They act as natural antioxidants and have anti-tumor properties **(Falcone Ferreyra *et al.*, 2010; Cho *et al.*, 2014)**, anti-inflammatory drugs **(Cheng *et al.*, 2014)**, anti-allergic **(Cristea *et al.*, 2021)**, antithrombotics **(Lee *et al.*, 2014)**, antidiabetic activities **(Gaur *et al.*, 2014)** and anti-atherosclerotic **(Salvamani *et al.*, 2014)**.

Flavonoids, due to their antioxidant properties, maintain the redox state in cells. The antioxidant activity of flavonoids is linked to the structure of the molecule: the presence of conjugated double bonds and the appearance of functional groups in the rings **(Seyoum *et al.*, 2006; Amić *et al.*, 2003)**. It has been shown that flavonoids are located in the membrane layer between the lipid bilayer and the aqueous phase and can affect both enzymatic and non-enzymatic lipid peroxidation **(Mierziak *et al.*, 2014)**. It has been found that flavonoids also interact directly with biological membranes, reducing their fluidity, making them more resistant to many oxidizing factors and inhibiting the diffusion of free radicals **(Arora *et al.*, 1998)**. Phenolic compounds, phytates and certain trace elements are responsible for the overall antioxidant activity **(Jeszka-Skowron *et al.*, 2015)**. It has been found that the heavier metals there are, the more phenols there are in plant tissues **(Petukhov *et al.*, 2019)**. The correlation between chemical elements (Fe, Cu, Cd, Pb, Co, Ni, Zn, Mn, Cr, Na, K, Ca and Mg), antioxidant activity and the amount of flavonoids in fungi has been confirmed experimentally **(Buruleanu *et al.*, 2019)**. It is known that flavonoids are capable of chelating the cation Fe 2+, Fe 3+, Cu 2+, Zn 2+, Al 3+ and Mg 2+, but they are unable to bind Na +, K + and Ca 2+ **(Packer, 2011)**. They can stop Fe 2+ and Fe 3+ dependent lipid peroxidation **(Arora *et al.*, 2000)**. Chelation of metals such as Fe 2+, Fe 3+, Cu 2+ and Cu + is another free radical neutralization mechanism. Copper and iron are oxidized to their Fe 3+ and Cu 2+ states, so antioxidants such as quercetin and hydroxycinnamic acids return them to a stable form, which inhibits radical formation **(Končić *et al.*, 2011)**. The HPLC method is fast and precise. It allows the simultaneous separation of complex samples into their constituent components, the determination of most of the components and the measurement of their concentrations. Currently, methods have been developed which allow the qualitative and quantitative determination by HPLC of flavonoids and other biologically active compounds. Many of these substances determine the antioxidant status of biological objects. Combining chromatography with other precise methods allows you to study the relationship between various indicators of biological systems. The use of an energy dispersive spectrometer in a scanning electron microscopy system allows quantitative x-ray microanalysis with a predetermined analysis area: at a point and obtaining element distribution maps, y including the microstructural image of the sample under study, spectra and histograms.

The aim of this work was to investigate the possibility of using the HPLC method and an X-ray EMF detector in a scanning electron microscope system to assess the antioxidant status of herbal supplements and bread enriched with them.

MATERIAL AND METHODS

The selection of raw materials for use in baking was carried out on the basis of the characteristics of the chemical composition and the concentration of biologically active substances.

We used an herbal supplement made from powders of dry extracts of thyme, mint, cilantro, and rowan and viburnum fruits, taken in equal proportions. To obtain dry phytoextracts, the prepared herbs and fruits were dried in a laboratory drying cabinet ShS-80-01 (OOO AEROTUBE, Russia) at 50-55®C to a residual moisture content of 8%. Then, the plant material was ground to a particle size of 5 mm.

The extraction of biologically active substances from herbs and plant fruits was carried out using an 80% hydroalcoholic solution of ethyl alcohol at a temperature of 80 ° C in two stages. At the first contact of the phases, the charge: extractant ratio was 1: 100, the extraction time was 120 min. At the second contact of the phases, the raw material: extractant ratio was 1:50, the extraction time was 60 minutes. The dry powder of bioflavonoids was obtained by evaporation on a rotary evaporator at a temperature of 80 ° C. Then, the thick extract obtained was dried at a temperature of 45 ° C. The powders of dry extracts ob-tained were used for further research.

The total flavonoid content was determined by spectrophotometry **(Khoo *et al*., 2013)**. To a methanol solution of the phytoextract, 2% aluminum chloride was added, incubated for 10 min and the absorbance was measured at 415 nm. The result was determined using a calibration curve made from quercetin with a methanolic solution of aluminum chloride.

The antioxidant activity was determined by spectrophotometry in an alcoholic extract described by Silva et al 2005 **(Silva *et al*., 2005)** on the basis of the percentage inhibition of the DPPH radical (2,2-diphenyl-1-picrylhydrazyl). We determined the optical density of the solutions during the interaction, Specord M40 (Carl Zeiss Industriel Messtechnik GmbH, Germany) at a wavelength of 515 nm.

The determination of the qualitative composition of the phytoextract was carried out by HPLC on a Milichrom UF-5 ap-paratus (Nauchpribor, Russia) equipped with a Multikhrom computer processing system (Ampersand, Russia). For analysis, a reverse phase HPLC version was used, a 100x2 chromatography column filled with C-18 separaon, the eluent was a solution of acetonitrile in phosphate buffer solution by volume with a pH of 3-7 in acetonitrile: phosphate buffer ratio - 82:18. Detection was performed at wavelengths of 230, 262, 272, 310 nm. The eluent flow rate is 100 µl / min.

The complex of phenolic compounds was determined by HPLC on a Milichrom-UF-5 apparatus (Nauchpribor, Russia). An alcoholic extract of phytoadditive powder was used, the eluent for the composition was acetonitrile: an aqueous solution of trifluoroacetic acid (pH 2.5, in a 15/85 ratio); the elution mode is isocratic, analysis time 12 to 25 minutes, sample volume 2 to 6 l. The digital processing of the data was carried out using the "Multichrome" software (CJSC "Ampersand", Russia).

The amount of inorganic elements was determined by atomic absorption spectrophotometry using an AAS Hitachi - 180-80 instrument (Hitachi, Japan).

EDS analysis of the chemical composition of the main components of the sample (Na, P, S, K, Mn, Fe, Mg, Ca, Al, Si, Cl, Zn, Se, Mo) by energy dispersion spectrometry (ESD) in a JEOL JSM 6390 scanning electron microscope. Samples of bread were taken from the central sample of crumb and crust weighing 10 g and their sections were prepared. The average sample of the phytoextract powder was also 10 g. Samples were coated with platinum in a JEC-3000FC benchtop vacuum unit and placed on a carbon dioxide coated tape. The resolution of the microscope is 4 Nm at an acceleration voltage of 20 kV. During elemental analysis, the working distance (WD) was 10 mm. 20 points from each sample were examined. The local scan is 3 mm, the scan-ning area is not less than 12 microns. Statistical evaluation was performed using standard methods using Statgraphics Centu-rion XVII statistical software (StatPoint Inc. USA).

The quantitative determination of the amount of free organic acids was carried out by potentiometric titration. The acids are extracted with water, titrated with a 0.1 M sodium hydroxide solution using a microburette with constant stirring using a magnetic stirrer. The EMF values were recorded and, according to the results obtained, titration curves were plotted in coordi-nates pH = f (V) to determine the equivalence point. **(Zhilkina, 2016; Marakhova, 2016)**.

ALL CHEMICALS USED ARE MANUFACTURED BY MERK (GERMANY)

The wheat flour dough was prepared without safety (Wt = 42.0%). To prepare the dough, the water was heated to 38-40 ºC, the yeast was suspended in 50% water, salt and sugar were dissolved in the remaining amount of water according to the recipe, a mixture carefully mixed flour and phytoextract was added in the amount of 5%, 7.5% and 10% by weight of flour and knead the dough, margarine is added at the end of kneading. After kneading, the dough was left to ferment in a fermentation cabinet at a temperature of 32 ºC for 120-200 minutes. The end of fermentation was determined by measuring the titratable acidity. The dough was cut as follows: for baking molded products, dough pieces weighing 300 g were placed in oiled molds and subjected to fermentation in an oven at a temperature of 35-38 ºC with humidification. The preparation of the dough for cooking was determined organoleptically. Firing was carried out in an electric laboratory oven PRSh-11 (JSC ONIIP, Russia) at a tempera-ture of 220 ºC for 25-30 minutes.

Phytoextract powder was added during kneading of the dough at the rate of 3%, 5% and 7% by weight of flour. The con-trol sample did not contain phyto-additives, as it was a sliced bread without phyto-additives, prepared according to a tradi-tional recipe without steam. The determination of the porosity of bread was carried out in accordance with **GOST 5669-96**, the specific volume of bread - in accordance with **GOST 27669-88**. The structural and mechanical properties of the bread crumb were determined using an ST-1M Structurometer (NPF Ra-dius, Zelenograd, Russia). The statistical processing of the data obtained was carried out using the statistical software Microsoft Office Excel 2007 with the determination of the arithmetic mean value (M), the mean error of the arithmetic mean value (m) and the correlation coefficient. (r). The materials and methods section should contain sufficient detail so that all procedures can be repeated. It may be divided into headed subsections if several methods are described.

RESULTS AND DISCUSSION

The seeds and green parts of plants are known to be the richest in antioxidants. The fruits and herbs that make up the phytoextract contain essential components known to have antioxidant activity associated with human health **(Ismaili *et al*., 2002; Sokmen *et al*., 2015; Fialová *et al*., 2008; Al-Tawaha *et al*., 2013; Oganesyan *et al*., 2007; Raudonis *et al*., 2014)**.

The HPLC method was used to determine the presence of biologically active substances in the phytoextract. The chromatogram of the phytoextract is shown in Figure 1.

Groups of biologically active substances have been identified by their respective retention times, which are present in the chromatogram as peaks. Taking into account the resolution of the chromatography in the phytoextract, 3 groups of biologically active substances have been isolated: organic acids, phenol carboxylic acids and anthocyanins. The retention times for the identified groups of biologically active compounds are as follows:

- up to 2 minutes - organic acids;
- from 2 to 5 minutes - phenol carboxylic acids;
- from 5 to 8 minutes – anthocyanins **(Sychev *et al*., 2006)**.

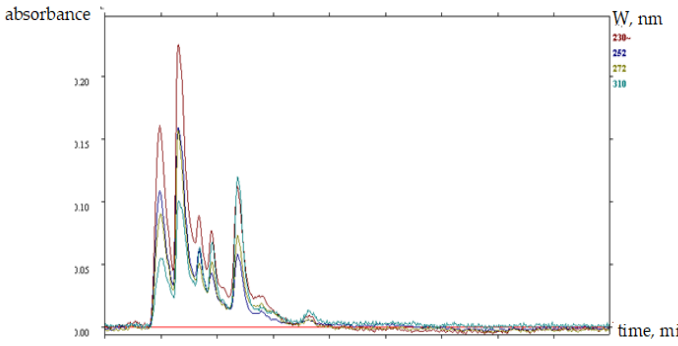

Figure 1 Chromatogram of phytoextracts

The antioxidant activity of the phytoextract was determined. To confirm the presence of phenolic compounds in the phytoextract, a spectrophotometric analysis was performed to identify the phenolic antioxidants. The amount of organic acids and mineral elements was also determined. The experimental data obtained are presented in Table 1.

Table 1 Antioxidant activity and content in the phytoextract of some compounds with antioxidant properties and trace elements

Index	Phytoextract
Antioxidant activity, % DPPH inhibition	84.7±1.2
The amount of flavonoids, %	1.88±0.03
The amount of organic acids in terms of malic acid, %	2.5±0.2
Mineral elements, mg / kg	
Calcium	34.1±1.5
Manganese	6.4±0.5
Iron	9.6±0.6
Zinc	3.7±0.3
Copper	2.5±0.1
Cobalt	0.20±0.01

The phytoextract, which includes herbs of thyme, mint, coriander, and the fruits of rowan and viburnum, was found to have high antioxidant activity (84.7 ± 1.2% inhibition of DPPH). Researchers associate the manifestation of the antioxidant activity of plant extracts with the presence of phenolic compounds which are easily soluble in the protophilic solvents (ethanol, methanol) which they contain **(Goupy *et al*., 2003)**. Rowan berries are known to contain around 1.34-1.47 g / 100 g of polyphenols and various organic acids such as malic, citric and succinic, which confer high antioxidant activity of 5.8 mmol TE / 100 g. **(Cristea *et al*., 2021)**.

The complex of phenolic compounds of the phytoextract was investigated by HPLC. The results of the chromatographic study of the phytoextract are shown in Figure 2.

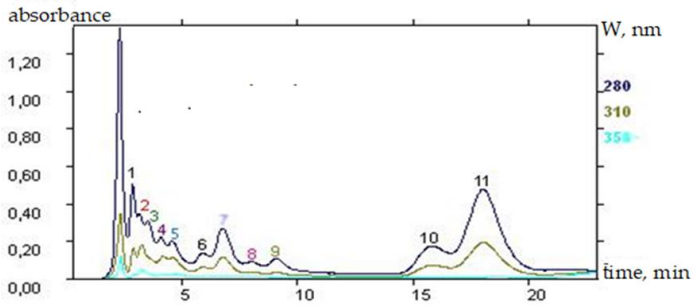

Figure 2 Chromatogram of an alcohol extract from a phytoextract

Chromatograms of extracts in automated data processing show groups of antioxidants from phenolic compounds, some of which have not been identified. As markers of the antioxidant activity in the phytoextract, are listed: apigenin (VR = 3.0; RS = 1.380), quercetin (VR = 4.05; RS = 0.540), ferulic acid (VR = 15.2; RS = 0.532), rutin (VR = 18.5; RS = 0.630). Table 2 presents the experimental data on the content of flavonoids identified in the dry extract of plant materials.

Table 2 Content of individual phenolic compounds in dry phytoextract

Phenolic compound	Content, %
quercetin	0,16±0,04
apigenin	0,16±0,05
ferulic acid	0,19±0,05
routine	0,08±0,01

The results obtained from the study of antioxidants in a phytoextract from the herb of thyme, mint, coriander and the fruits of rowan and viburnum are consistent with the data in the literature, which represents a study of individual plants of this combination. Raudonis et al. (2014) **(Raudonis *et al.*, 2014)** found that in addition to the usual antioxidant active phytochemicals such as ascorbic acid, tocopherols and carotenoids, rowan fruits are rich in phenolic compounds. According to Yurteri et al. (2021) **(Yurteri *et al.*, 2021)**, general content phenols in viburnum fruits ranged from 86.395 mg GE / g to 124.792 mg GE / g, and their antioxidant activity was AOA 91.79-94.21% inhibition of the DPPH radical. Fruits also contain a high content of organic acids and potassium. It is established the high antioxidant activity of cilantro herb powder. It was found that the absorption activity of DPPH radicals in mint grass is 54.84 ± 0.57% **(Fialová *et al.*, 2008)**. It was found that thyme herb has antioxidant activity associated with the presence of flavonoids, tannins and microelements **(Sokmen *et al.*, 2015)**.

X-ray energy dispersive spectrometry was used to obtain data on the mineral composition of the phytoextract powder. FIG. 3 shows a micrograph of the powder, carried out using a JEOL JSM 6390 scanning electron microscope.

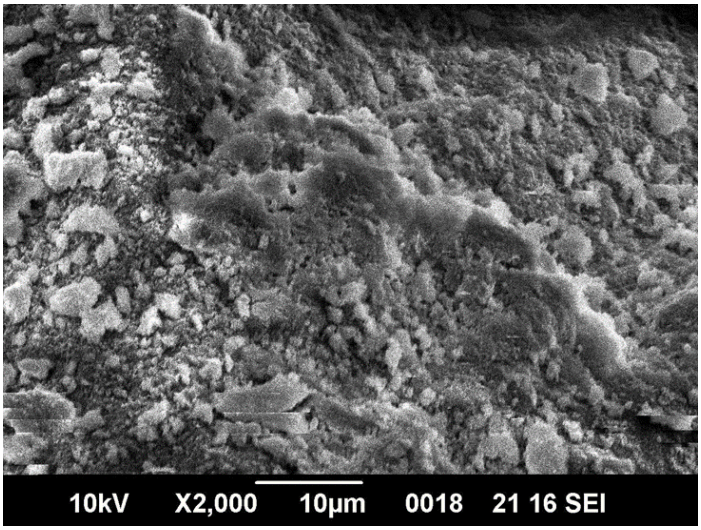

Figure 3 Micrograph of phytoextract powder (magnification 2000x)

After having prepared a sample of the phytoextract powder, a local analysis of the mineral composition was carried out using an X-ray CEM detector. An energy dispersion spectrometer makes it possible to perform a quantitative microanalysis at X-rays with a given area of analysis: at a point or area, and to obtain distribution maps of the elements. The analysis results are shown in Table 3.

The phytoextract powder is dominated by the main chemical elements of organic compounds (C + N + O). It has been established that among the mineral elements, calcium, iron, cobalt, zinc and potassium are the most by weight in the additive. The phytoextract powder can be recommended for use in food technology to enrich foods with microelements.

Table 3 Chemical composition of phytoextract powder

Chemical element	Relative content, mass%
C + N + O	94.87± 0.480
Na	0.04± 0.002
Mg	0.08± 0.005
Al	0.17±0.003
P	0.07± 0.016
S	0.13± 0.010
K	0.52± 0.022
Ca	1.00± 0.008
Cr	0.07± 0.003
Mn	0.33± 0.025
Fe	0.86± 0.028
Co	0.70 ± 0.005
Cu	0.19± 0.012
Zn	0.61± 0.056
Se	0.26± 0.011

Many plants containing biologically active compounds can be considered as good alternatives to synthetic antioxidant nutritional supplements **(Yanishlieva *et al.*, 2006)**.

A bread baking test was carried out with the addition of phytoextract powder during kneading at a rate of 3, 5 and 7%. The physicochemical and sensory characteristics of the bread were studied. It was found that the value of the porosity, compared to the control, with the introduction of 3% of phytoextract from the mass of flour increases by 1.9%, with the introduction of 5% and 7% of phytoextract - of 3.5 and 4.2%, respectively. The specific volume of phytoextract bread has also increased: with the introduction of 3% of the weight of flour of the additive - by 3.6%, 5% phyto-additives - by 10.7%, 7% phyto -additives - by 11.6%. In addition, indicators of the quality of bread such as shape stability, general compressibility of the crumb and its plastic and elastic deformation increase. The experimental data characterizing the quality indicators of bread with the addition of phytoextract are presented in Table 4.

Table 4 Influence of the powdered phytoextract on the physicochemical indicators of the quality of the finished products

The name of indicators	Quality indicators of the test and parameters of the technological process Phytoextract dosage, %			
	0	3	5	7
Porosity, %	76,8±1,0	78,7±1,0	80,3±1,0	81,0±1,0
Structural and mechanical properties of crumb, ΔH total	173,2±1,5	176,4±1,8	180,6±2,0	181,5±1,7
ΔH stratum	126,2±2,1	128,2±2,5	131,6±2,0	132, 4±2,2
ΔH ynp	47±0,4	48,2±0,5	49±0,6	49,1±0,4
Specific volume, cm3 / 100g	337,4±4,0	349,6±3,8	373,3±3,9	376,0±4,2
Output, %	146,3±2,0	150,17±2,4	151,13±2,5	152,24±2,2

As a result of the studies, it was found that the dosage of phytoextract at a rate of 5% is optimal. The introduction of a phytoextract in a dose greater than 5% leads to a slight increase in physicochemical quality indicators, however, organoleptic indicators deteriorate (the taste of medicinal plants appears). Therefore, increasing the dosage is impractical.

From the consumer's point of view, one of the important factors is the sensory value of the bread, as taste, smell and taste significantly influence consumer preferences. The results of the study of sensory indicators of the quality of wheat bread showed that the test samples with the addition of phytoextract have an elastic crumb, fine porosity, thin-walled and uniform, a smooth surface of the upper crust, the taste characteristic of bread and a delicate aroma of collection of herbs. The introduction of phytoextract powder at a rate of 3%, 5%, 7% in the mass of flour has a positive effect on the structural and mechanical properties of the bread crumb, its freshness and its shelf life: the rate of stale slows down.

Along with the quality indicators of bread with herbal additives, the antioxidant properties and the distribution of mineral elements that determine the antioxidant activity of bread were studied.

The antioxidant activity of the average sample of enriched wheat bread was on average 23.8% inhibition of the DPPH radical, while in the control bread it was 8.5%. The antioxidant activity of the crust ranged from 25.6 to 28.4% inhibition of

the DPPH radical, and the crumb ranged from 19.8 to 22.3% inhibition of the DPPH radical.

A local analysis of the mineral composition of the crust and bread crumb was performed using an X-ray CEM detector (Table 5) and micrographs of a section of local areas were obtained (figure 4).

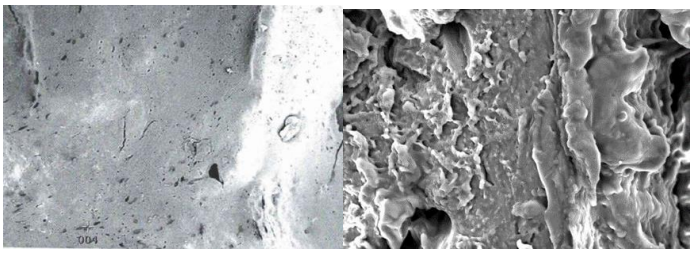

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Figure 4 Micrographs of wheat bread with 5% phytoextract powder added: crust / 1 / crumb / 2 /, 950x magnification

In the crumb, the proportion of mineral elements is 35.7% by weight. The crust of wheat bread is less enriched with chemical elements.

Studies have shown that the order of accumulation of mineral elements in the crust of bread elements is as follows: Zn > Al > Cu > Co > P > Ca > Mn > K > S > Cr ≈ Se ≈ Fe > Mg≈ Na. In the crumb of bread, the order of accumulation of chemical elements is different: Mn > Cu > Fe > Zn > P > S > Al > Co > S > K ≈ Ca > Mg≈ Na.

The chemical elements which determine the antioxidant properties of the product manganese, iron, copper are prevalent in the breadcrumbs. Zinc is evenly distributed between the crumb and the crust.

Mn is a cofactor and activator of many enzymes (pyruvate kinase, decarboxylase, siperoxide dismutase) is involved in the synthesis of glycoproteins and proteoglycans and has antioxidant activity. Fe is found in the composition of active centers, hemoproteins and iron-sulfur proteins, it determines the spatial structure, activity and participates in redox reactions. Zn stabilizes the structure of molecules, plays an important role in protein synthesis, signal transduction processes within the cell and is part of oxidative enzymes. Cu is one of the enzymes and controls a number of biological and chemical processes in a living organism **(Gins *et al*.,2018)**.

Table 5 Chemical composition of bread with phyto-additive

Chemical element	Relative content, mass%	
	crust	crumb
C + N + O	82.32± 0.480	63.4±0.510
Na	0.03±0.002	0.02±0.001
Mg	0.05± 0.005	0.08±0.004
Al	3.62±0.003	1.62±0.009
P	0.86± 0.013	3.34±0.020
S	0.36± 0.011	2.33±0.18
K	0.43± 0.020	0.83±0.009
Ca	00.76± 0.009	0.87±0.006
Cr	0.12± 0.005	-
Mn	0.60± 0.029	9.18±0.025
Fe	0.11± 0.020	5.98±0.012
Co	2.98 ± 0.009	1.49±0.010
Cu	3.37± 0.015	6.48±0.035
Zn	4.27± 0.042	4.38±0.026
Se	0.12± 0.010	-

High correlation coefficients (r) were recorded for the concentration of Cu, Fe, Mn and the content of flavonoids and the antioxidant activity in herbal supplement (from - 0.74 to - 0.82).

CONCLUSION

Using HPLC, spectroscopy, and X-ray electromagnetic fields in a scanning electron microscope, certain components of a herbal supplement prepared from the herbs of thyme, mint, cilantro, and rowan fruits and viburnum, which determine its antioxidant properties, have been determined. The chromatogram of the alcoholic extract of the herbal supplement showed that the supplement contains organic acids, flavonoids and anthocyanins. Also, in the powder of the phytoextract, the vital mineral elements are determined. The possibility of using X-ray spectral analysis during energy dissipation in a scanning electron microscope system to predict the antioxidant properties of plant additives developed for use in food technologies is considered.

The phytoextract powder has been used in the production of wheat bread. An increase in antioxidant levels has been identified as an important beneficial effect of the fortification of wheat bread. It has been found that the addition of phytoextract to wheat bread at the level of 5.0% is the optimal level of introduction of additives to improve the quality of the bread and enrich it with antioxidants. Using energy dispersive x-ray spectrometry, new data was obtained on the diversity of the mineral composition of fortified wheat bread, the proportion of elements in the crust and crumb was determined and the coefficients of correlation were calculated. The deficiency of macro and microelements in the diet is extremely dangerous for human health. Therefore, the fortification with antioxidants and microelements of bread, as a product of daily consumption, is very important.

Data Availability: A data availability statement is compulsory for research articles and clinical trials. Here, authors must describe how readers can access the data underlying the findings of the study, giving links to online repositories and providing deposition codes where applicable. For more information on how to compose a data availability statement, including template examples, please visit: https://www.hindawi.com/research.data/#statement.

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