DETERMINATION OF ANTIOXIDANTS IN HERBAL SUPPLEMENTS BY HPLC AND X-RAY ELECTROMAGNETIC FIELD DETECTOR IN A SCANNING ELECTRON MICROSCOPE SYSTEM

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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 (Manquín-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 (Falconcet Fereyrea et al., 2010; Cho et al., 2014), anti-inflammatory drugs (Cheng et al., 2014), anti-allergic (Cristea et al., 2021), antiatherosclerotics (Lee et al., 2014), antidiabetic activities (Gurne et al., 2014) and anti-atherosclerotic (Salvanami 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; Amini 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 Fe2+, Fe3+, Cu2+ and Cu++, Al 3+ and Mg 2++, but they are unable to bind Na+, K+ and Ca 2+ (Packer, 2011). They can stop Fe2+ and Fe3+ dependent lipid peroxidation (Arora et al., 2000). Chelation of metals such as Fe2+, Fe3+, Cu2+ and Cu++ is another free radical neutralization mechanism. Copper and iron are oxidized to their Fe3+ and Cu2+ 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 SS-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 charge: extractant ratio was 1: 150, 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 (Khoz 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, Spectord M40 (Carl Zeiss Industrial 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 apparatus (Nauchpriboi, Russia). For analysis, a reverse phase HPLC version was used, a 1000.2 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: phosphoric 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 (Nauchpriboi, Russia). An alcoholic extract of phytoadditive poweder was used, the eluent for the composition was acetonitrile: water solution by volume with a pH of 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 (EDS) 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-ron 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 micro burette 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 °C, 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%, 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 °C 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 on a plastic tray, impregnated with an aqueous extract at a temperature of 35-38 °C 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 °C 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 crust were determined using an ST-1M Structomter (NPF Ra-dios, 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](image)

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>
<thead>
<tr>
<th>Index</th>
<th>Phystoextract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antioxidant activity, % DPPH inhibition</td>
<td>84.7±1.2</td>
</tr>
<tr>
<td>The amount of flavonoids, %</td>
<td>1.88±0.03</td>
</tr>
<tr>
<td>The amount of organic acids in terms of malic acid, %</td>
<td>2.5±0.2</td>
</tr>
<tr>
<td>Mineral elements, mg / kg</td>
<td></td>
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<tr>
<td>Calcium</td>
<td>34.1±1.5</td>
</tr>
<tr>
<td>Manganese</td>
<td>6.4±0.5</td>
</tr>
<tr>
<td>Iron</td>
<td>9.6±0.6</td>
</tr>
<tr>
<td>Zinc</td>
<td>3.7±0.3</td>
</tr>
<tr>
<td>Copper</td>
<td>2.5±0.1</td>
</tr>
<tr>
<td>0.20±0.01</td>
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![Figure 1 Chromatogram of phytoextracts](image)

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 protopholic 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.

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

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

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

<table>
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<tr>
<th>Quality indicators of the test and parameters of the technological process</th>
<th>Phytorextract dosage, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Porosity, %</td>
<td>0</td>
</tr>
<tr>
<td>Structural and mechanical properties of crumb, ΔH total</td>
<td>173,2±1,5</td>
</tr>
<tr>
<td>ΔH stratum</td>
<td>126,2±2,1</td>
</tr>
<tr>
<td>ΔH vmp</td>
<td>47±0,4</td>
</tr>
<tr>
<td>Specific volume, cm³/100g</td>
<td>337,4±4,0</td>
</tr>
<tr>
<td>Output, %</td>
<td>146,3±2,0</td>
</tr>
</tbody>
</table>

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 introduction 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 staling 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 EDS detector (Table 5) and micrographs of a section of local areas were obtained (figure 4).

In the crumb, the proportion of mineral elements is 35.7% by weight. The crust of wheat bread is less enriched with macro and microelements. Studies have shown that the order of accumulation of mineral elements in the crust of bread elements is as follows: Zn > Al > Cu > P > Ca > Mn > 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 > Cr > 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, determines the spatial state, 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).

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 of wheat bread 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/statatement.

REFERENCES


TERSTATE STANDARD GOST 5669-96. BAKERY PRODUCTS. METHOD FOR DETERMINATION OF POROSITY. MOSCOW: IPK PUBLISHING HOUSE OF STANDARDS, 2001

INTERSTATE STANDARD GOST 27669-88. WHEAT BREAD FLOUR. METHOD FOR EXPERIMENTAL. LABORATORY BREADMAKING. MOSCOW: IPK PUBLISHING HOUSE OF STANDARDS, 2007


