

The influence of raw material preparation on the yield of bioactive substances in lingonberry (*Vaccinium vitis-idaea* L.) fruits

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Phenolic compounds are well-known phytochemicals found in many types of plants. Phenolic compounds and flavonoids are potential substitutes for bioactive agents in pharmaceutical and medicinal sections to promote human health and prevent and cure different diseases. Lingonberry (*Vaccinium vitis-idaea* L.) belongs to the Ericaceae family. It is widely spread in the northern hemisphere in such countries as Russia, Lithuania, Latvia, Iceland, Scandinavian countries, etc. Lingonberry fruits are beneficial due to their anti-inflammatory, antibacterial, antioxidative activities and effect on the cardiovascular system. Although a number of studies are done on phenolic compound extraction methods, there is a high need of evidence on the most efficient way of raw material preparation. In this research, various lingonberry (*Vaccinium vitis-idaea* L.) fruit raw material preparation methods will be compared.

Keywords: lingonberry, phenolic compounds, procyanidins, anthocyanins, antioxidant activity, raw material preparation

INTRODUCTION

Phenolic compounds are well-known phytochemicals found in many types of plants. They consist of simple phenols, benzoic and cinnamic acid, coumarins, tannins, lignins, lignans and flavonoids. Substantial developments in research fo-

cused on the extraction, identification and quantification of phenolic compounds as medicinal and/or dietary molecules have occurred over the last 25 years [1].

Phenolic compounds and flavonoids are potential substitutes for bioactive agents in pharmaceutical and medicinal sections able to promote human health and prevent and cure different diseases [2]. There are plenty of studies of

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phenolic compound extraction from various plant materials [3, 4].

A number of studies show the impact of the phenolics on human general wellbeing, such as the ability to cure and prevent diseases. The main investigated effects are antioxidant and antibacterial impacts, cardioprotective effects, anticancer impacts, the promotion of immune system and anti-inflammatory effects. Recent research shows potential activity against various human viruses, immunomodulatory and anti-inflammatory activity [5, 6].

Lingonberry (*Vaccinium vitis-idaea* L.) belongs to the Ericaceae family. It is widely spread in the northern hemisphere in such countries as Russia, Lithuania, Latvia, Iceland, Scandinavian countries, etc. Lingonberry fruits have anti-inflammatory [7], antibacterial, antioxidative [8, 9] activities and effect on the cardiovascular system [10]. Their anticancer effect was also investigated [9].

Recent research shows that a number of plants is a potential source of phenolic compounds and may be used for the reason of health promotion as a source of dietary/food supplements production [11].

A number of studies show that the proportion of the population who reported the use of dietary supplements is growing among different sociodemographic groups and countries [12, 13].

That is the reason why the question of selecting the raw material preparation method is still relevant as it is important to choose an appropriate one in order to receive the highest yield of phenolic compounds from the sample matrix [14].

Therefore, this research mainly focuses on the phenolic compounds and several methods of raw material preparation that could be used to obtain them from lingonberry fruit materials [15].

Nevertheless, there are not so many studies how different preparation methods of raw material could influence the amount of bioactive substances in herbal raw material.

Many problems related to the enhancing the yield of bioactive substance could be solved by finding the most efficient way of raw material preparation [16, 17].

In the present work, we aimed to investigate the influence of different methods of lingonberry fruit preparation on the yield of bioactive substances.

EXPERIMENTAL

Materials

Distilled water, rectified ethyl alcohol 96% V/V, sodium carbonate (Carl Roth GmbH & Co, Karlsruhe, Germany), Folin–Ciocalteu reagent (Sigma Aldrich, St. Louis, USA), gallic acid monohydrate (Sigma Aldrich, St. Louis, USA), hydrochloric acid (Sigma Aldrich, Germany), DMAC (4-dimethylaminocinnamaldehyde) (Sigma Aldrich, Germany), epicatechin (Sigma Aldrich, Germany), ABTS (2,2'-Azino-di(3-ethyl-benzthiazoline sulphonic acid (6)), ammonium salt (Alfa Aesar, USA), potassium persulfate (Alfa Aesar GmbH & Co, Germany), trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (Sigma Aldrich, St. Louis, USA), iron (III) chloride hexahydrate (Sigma Aldrich, Germany), sodium carbonate (Carl Roth GmbH & Co, Karlsruhe, Germany), glacial acetic acid, TPTZ (2,4,6-tripyridyl-triazine) (Alfa Aesar, Germany).

Processing and extraction

The following devices were used for processing and extraction: an electric grinder (coffee grinder, First, Austria) for crushing the raw material, an analytical balance (Sartorius CP6M-0CE, Germany) for weighing, an ultrasonic bath (Bandelin Sonorex Digital 10 P, Germany) for extraction, a mechanical shaker (laboratory shaker 358S, Poland) for mixing liquids, a spectrophotometer (CamSpec M550, United Kingdom) to determine the amount of biologically active compounds, a moisture measuring device (Presica HA 300, Dietikon, Switzerland) to perform loss on a drying test, a fruit dryer (Rospec, China) to dry fruits at constant temperature.

Lingonberry fruits were dried at ambient temperature in a well-ventilated room protected from direct sunlight.

Lingonberry fruits were dried in a special fruit dryer at a constant temperature of 55°C. The fruits were dried in that dryer for 6 days.

Lingonberry fruits were frozen in a freezer with a constant temperature of –18°C.

Since lingonberries have a thick skin, they were crushed in a mortar before lyophilising. The crushed fruits were placed in a freezer equipped with air circulation at –35°C temperature. The fruits were kept in the freezer for one day. Afterwards, they were transferred to a freeze-dryer where 0.05 mba pressure and –50°C condenser

temperature were maintained. The first 24 h the fruits were lyophilised at -20°C , the next 12 h at -5°C and the last 12 h at -1°C .

Lingonberry fruits prepared in different ways (except lyophilised) were crushed with an electric grinder before extraction. Ethanolic extracts are prepared from crushed raw materials in a ratio of 1:10. For the preparation of extracts, 2 g (± 0.01) of plant material is weighed and transferred to 25 mL brown glass bottles and 20 mL (± 0.01) of 70% V/V ethanol is poured in. After these steps, the brown glass vials are transferred to an ultrasonic bath and extracted in it for 15 min.

Measurement of total phenolic content

The colorimetric Folin–Ciocalteu method was applied for the determination of the total phenolic content of lingonberry fruit samples prepared by different methods. The principle of this method is that the phenolic compounds present in lingonberry fruits react with the specific Folin–Ciocalteu reagent to form a blue complex which can be quantified using the spectrophotometric method. First, the Folin–Ciocalteu reagent is prepared. It is obtained by diluting the stock Folin–Ciocalteu reagent 10 times with distilled water.

The test solution is prepared by mixing 1 mL of the extract with 4 mL of the 7.5% sodium carbonate solution and the 5 mL stock Folin–Ciocalteu reagent. The prepared test solution is kept at ambient temperature in a dark place for 1 h. Then, a reference solution is prepared. The reference solution is prepared by mixing 1 mL of 70% V/V ethanol with 4 mL of the 7.5% sodium carbonate solution and the 5 mL stock Folin–Ciocalteu reagent. The absorbance of the obtained solutions is measured with a spectrophotometer at a wavelength of 765 nm.

The total phenolic content is expressed as gallic acid equivalents per gram of raw material. The total amount of phenolic compounds is evaluated by comparing the obtained absorbance values according to the standard gallic acid calibration curve. The data are evaluated according to the linear regression equation of the gallic acid calibration curve [18].

Measurement of total proanthocyanidins

The total content of proanthocyanidins in lingonberry fruits is determined using the 4-dimethylaminocinnamaldehyde (DMAC) solution acidified with hydrochloric acid.

The test solutions are prepared by mixing 20 μL of the lingonberry fruit extract and 2 mL of the 0.1% DMAC reagent. The ready-made test solutions are kept at ambient temperature for 15 min. Finally, a reference solution is prepared by mixing 20 μL of distilled water and 2 mL of the 0.1% DMAC reagent. The absorbance of the solutions is measured with a spectrophotometer at a wavelength of 640 nm. The total amount of proanthocyanidins is expressed as epicatechin equivalents per gram of raw material.

Epicatechin solutions (0.0125, 0.025, 0.05, 0.075, 0.1 mg/mL) are being prepared. After preparing these solutions, epicatechin standard solutions are made mixing together 2 mL of the 0.1% DMAC reagent with 20 μL solutions of the mentioned concentrations. After measuring the absorbance of these solutions, a calibration curve for epicatechin is generated [19].

Measurement of total anthocyanins

Determination of the total anthocyanin content is performed by the spectrophotometric method. Ethanolic extracts of lingonberry fruits are acidified with 0.1% hydrochloric acid.

For analysis, 1 mL of the prepared extract is diluted with ethanol, acidified with 0.1% hydrochloric acid in a 50 mL volumetric flask. Acidified with 0.1% hydrochloric acid ethanol is used as a reference solution. The absorbance of the prepared solutions is measured with a spectrophotometer at a wavelength of 528 nm [20].

Measurement of ABTS radical scavenging activity

Free radical scavenging capacity is determined by the ABTS method. An ABTS solution is prepared by dissolving 0.0548 g of 2,2'-azinodi[3-ethylbenzthiazoline]-6-sulfonic acid (ABTS) powder and 0.0095 g of potassium persulfate in 50 mL of distilled water. The prepared solution is transferred to a dark glass bottle and kept in the dark place for 16 h at ambient temperature. After the specified period of time, an ABTS stock solution is produced from the prepared solution. It is prepared according to the following principle: the prepared solution must be diluted until the absorbance of this solution is equal to 0.800 (± 0.003) measured by a spectrophotometer at a wavelength of 734 nm. Distilled water is used as a reference solution.

The test solutions are obtained by mixing 20 μL of the tested extract and 3 mL of the ABTS stock solution. The prepared test solutions are kept in a dark place for 1 h. Then, absorbance of the test solutions at a wavelength of 734 nm is measured with a spectrophotometer. A standard trolox calibration curve is generated. Trolox solutions with concentrations of 200, 400, 800, 1200 and 1600 $\mu\text{mol/L}$ are prepared. After preparing these solutions of known concentration, 20 μL of these solutions are mixed with 3 mL of the ABTS stock solution. The absorbance values of the obtained solutions are measured with a spectrophotometer at a wavelength of 734 nm. After measuring the absorbance values, a trolox calibration curve is generated [21].

FRAP (ferric reducing antioxidant power) assay

The FRAP reagent is prepared by mixing the acetate buffer, TPTZ solution and iron (III) chloride hexahydrate solution in a ratio of 10:1:1.

Preparation of acetate buffer

3.1 g of sodium acetate is added to 16 mL of glacial acetic acid and everything is diluted to 1000 mL with distilled water in a 1000 mL volumetric flask.

Preparation of TPTZ solution

50 mL of distilled water is added to 0.1695 mL of concentrated hydrochloric acid and 0.1562 g of TPTZ powder is also added to it. The solution is mixed well. A 10 mM TPTZ solution is prepared.

Preparation of iron (III) chloride hexahydrate solution

0.2703 g of iron (III) chloride hexahydrate is dissolved in 50 mL of distilled water. The solution is mixed well. A 20 nM solution of iron (III) chloride hexahydrate is prepared.

The test solutions are prepared by mixing 20 μL of the tested lingonberry fruit extract and 3 mL of the prepared FRAP solution. The reference solution is prepared by mixing 20 μL of 70% V/V ethanol and 3 mL of the FRAP solution. The resulting solutions are kept in a dark place at ambient temperature for 1 h. After that, the absorbance of these solutions is measured with a spectrophotometer at a wavelength of 593 nm. The obtained data are compared with the calibration curve of trolox standard solutions.

The reducing capacity of the solutions is expressed in equivalents of the standard antioxidant trolox ($\mu\text{mol TE/g}$). Trolox solutions of different

concentrations are prepared (200, 400, 800, 1200, 1600 $\mu\text{mol/L}$). Then, 20 μL of solutions of different concentrations are mixed with 3 mL of the FRAP solution. The resulting solutions are kept for 1 h in the dark place at ambient temperature. After that, the absorbance of these solutions at a wavelength of 593 nm are measured with a spectrophotometer. Finally, a trolox calibration curve is generated [22].

Statistical analysis

The analysis of the data obtained during this research was performed using the following programs: SPSS Statistics 27 (IBM, USA) and Microsoft Office Excel 2013 (Microsoft, USA). All tests were repeated three times and the arithmetic means and standard deviations of those repetitions were calculated. For the evaluation whether the differences between the comparative data are statistically significant ($p < 0.05$) the one-factor analysis of variance ANOVA was performed.

RESULTS AND DISCUSSION

The total amount of phenolic compounds ranged from 6.75 ± 0.04 to 42.11 ± 0.15 mg/g depending on the raw material preparation method. The obtained results were statistically significantly different ($p < 0.005$). The highest total phenolic content was determined in the frozen lingonberry fruit samples – 42.11 ± 0.15 mg/g. The total amount of phenolic compounds in the frozen lingonberry fruits was even 6.45 times higher compared to lingonberry fruits dried at 55°C . The total amount of phenolic compounds in them was only 6.75 ± 0.04 mg/g. The total amount of phenolic compounds in the lingonberry fruits dried at ambient temperature was 13.37 ± 0.06 mg/g, and in the lyophilised lingonberry fruits it was 27.19 ± 0.08 mg/g. Since the fruits for all samples were collected at the same time, from the same cenopopulation, it can be assumed that the method of preparation of the raw material has a significant influence on the amount of bioactive substances. The variation in the amount of phenolic compounds in differently prepared lingonberry fruit raw materials is presented in Fig. 1.

Polish scientists conducted a study during which the total amount of phenolic compounds

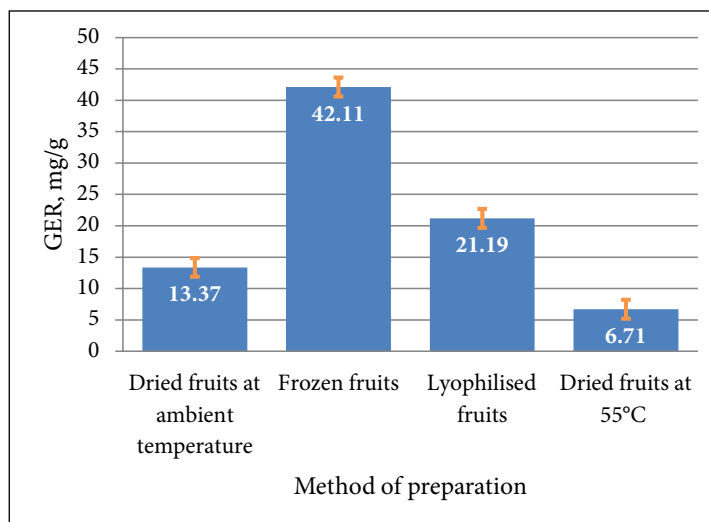


Fig. 1. Total phenolic content variation in lingonberry fruit samples prepared by different methods

in cold-stored, frozen and lyophilised lingonberry fruits was determined based on the Folin–Ciocalteu colorimetric method as well. The results of the study were also expressed on a dry raw material basis. It was determined that the total amount of phenolic compounds in frozen raw lingonberry fruits was 52.83 ± 0.88 mg/g, and in lyophilised ones 24.28 ± 1.49 mg/g. Compared to fresh raw material, the total amount of phenolic compounds in frozen fruits increased by 1.3 times, while in lyophilised fruits it decreased even by 2.18 times [6].

Latvian scientists also conducted a study in which they determined the total amount of phenolic compounds in lingonberry fruit juice based on the same colorimetric Folin–Ciocalteu method. The latter prepared the raw material in the following ways – lyophilised and dried at different temperatures of 40, 60 and 80°C. Initially, the raw material was frozen at a temperature of $-20 \pm 2^\circ\text{C}$, then thawed and prepared by different methods. Thawed lingonberry fruits were used as a control group. It was determined that the total amount of phenolic compounds in chilled lingonberry fruits was 681.54 mg/g, in freeze-dried fruits 122.3 mg/g, in 40°C dried ones 132.60 mg/g, in 60°C dried fruits 122.4 mg/g and in 80°C dried ones 139.6 mg/g [17]. According to the data of this study, there is no direct relationship between the raw material processing temperature and the total amount of phenolic compounds.

After examining lingonberry fruit samples prepared in different ways, it was discovered that

the content of total proanthocyanidins in the samples varied from 2.76 ± 0.13 to 11.48 ± 0.37 mg/g. The obtained results were statistically significantly different ($p < 0.005$). The highest amount of proanthocyanidins was determined in the frozen lingonberry fruit samples (11.48 ± 0.37 mg/g), and the lowest in the samples dried at 55°C (2.73 ± 0.11 mg/g). Almost twice the total amount of proanthocyanidins was detected in the lingonberry fruits dried at ambient temperature – 4.21 ± 0.08 mg/g. The total amount of proanthocyanidins in the lyophilised lingonberry fruits was 7.23 ± 0.10 mg/g.

A scientific study was conducted in which the diversification of proanthocyanidins content in the raw material of lyophilised lingonberry fruits was determined. The total amount of proanthocyanidins was 9.04 ± 0.41 mg/g in the lingonberry fruits collected in August, which were later lyophilised for the study. Comparing the results, it can be seen that the amount of proanthocyanidins in this work was 1.2 times lower [23]. The slightly lower amount of proanthocyanidins in this study could be due to different geographical factors of raw material collection, weather conditions, plant genotype and age.

After determining the amount of anthocyanins in lingonberry fruit samples prepared in different ways, it was discovered that the highest amount of anthocyanins was determined in the frozen lingonberry fruit samples (1.74 ± 0.04 mg/g). The content of anthocyanins was somewhat

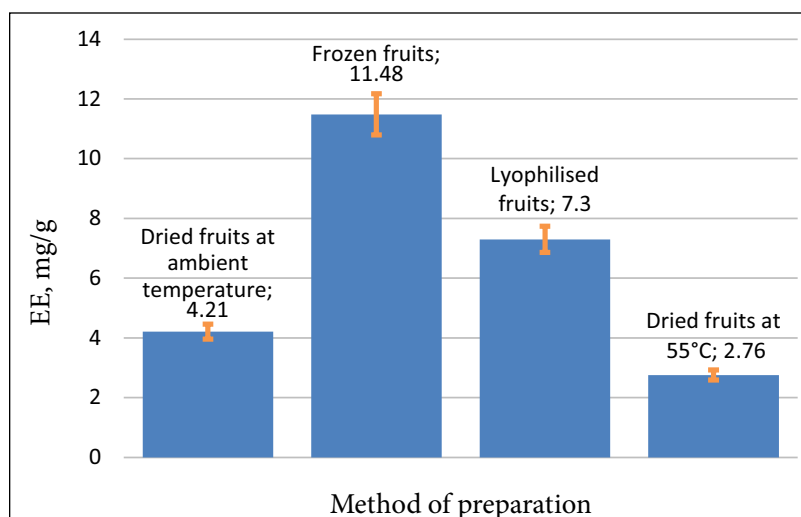


Fig. 2. Total proanthocyanidin content variation in lingonberry fruit samples prepared by different methods

lower in the lyophilised lingonberry fruits, 1.33 ± 0.01 mg/g. The lowest yields of the total anthocyanin content were determined in the fruit samples dried at ambient temperature (0.33 ± 0.01 mg/g) and the samples dried at 55°C (0.62 ± 0.01 mg/g).

Latvian scientists also determined the total anthocyanin content in differently prepared lingonberry raw materials. It was assessed that the total anthocyanin content in frozen, later thawed lingonberry juice was 5.66 ± 2.44 mg/g, in lyophilised fruits 3.96 ± 0.34 mg/g, in fruits dried at 40°C 3.87 ± 0.50 mg/g, in fruits dried at 60°C 3.43 ± 1.45 mg/g and in fruits dried at 80°C it was 3.06 ± 1.83 mg/g. According to the data of this study, it is possible to observe regularity in

the diversification of the anthocyanin content in lingonberry fruit juice processed at different temperatures. As the temperature increased, the content of anthocyanins in the raw material decreased consistently. This study practically confirms the statement that anthocyanins are thermolabile compounds [17].

After performing the free radical scavenging capacity assay by the ABTS method, it was discovered that it ranged from 680.3 ± 1.27 to 3248.67 ± 4.22 $\mu\text{mol/g}$. The obtained results were statistically significantly different from each other ($p < 0.05$). According to the obtained data, frozen lingonberry fruits demonstrated the highest antioxidant power. Free radical scavenging capacity reached 3248.67 ± 4.22 $\mu\text{mol/g}$. Compared

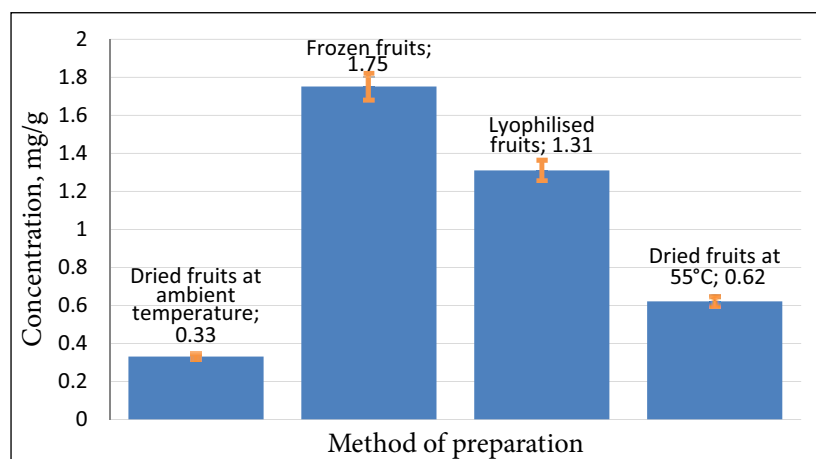


Fig. 3. Total anthocyanin content variation in lingonberry fruit samples prepared by different methods

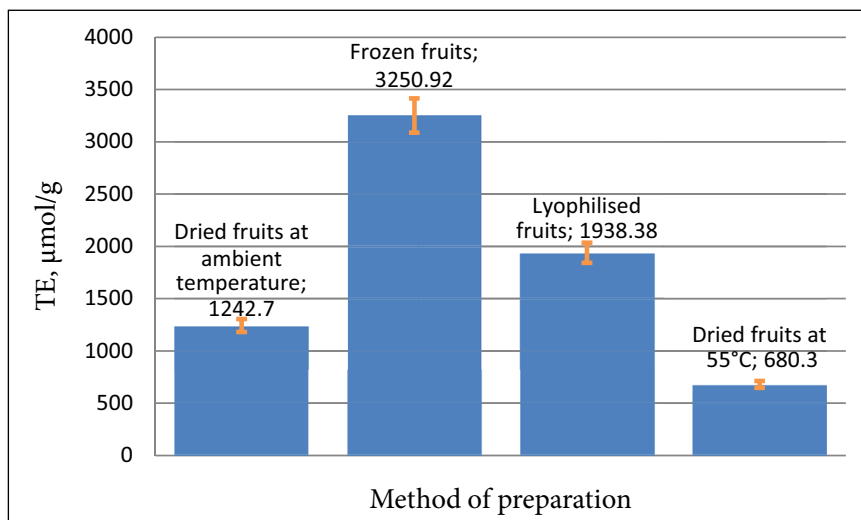


Fig. 4. Free radical scavenging capacity (ABTS) variation in lingonberry fruit samples prepared by different methods

to the frozen lingonberry fruits, the free radical scavenging capacity in the lingonberry fruit samples dried at 55°C was 4.78 times lower ($680.3 \pm 1.27 \mu\text{mol/g}$). The free radical scavenging capacity of the lingonberry fruits dried at ambient temperature was $1242.7 \pm 2.4 \mu\text{mol/g}$, and in the lyophilised ones it was $1936.45 \pm 3.49 \mu\text{mol/g}$. The free radical scavenging capacity of lingonberry fruit extracts prepared by different methods is well illustrated in Fig. 4.

According to the data obtained during this study, the ferric reducing antioxidant power (FRAP) in differently prepared lingonberry fruit samples ranged from 164.5 ± 0.08 to $840.9 \pm 0.75 \mu\text{mol/g}$.

The results were statistically significantly different from each other ($p < 0.05$). The frozen lingonberry fruits showed the greatest reducing capacity ($840.9 \pm 0.75 \mu\text{mol/g}$). The lingonberry fruits dried at 55°C demonstrated the lowest reducing capacity – $164.5 \pm 0.08 \mu\text{mol/g}$. A slightly higher reducing capacity was assessed in the lingonberry fruit samples dried at ambient temperature – $277.59 \pm 0.14 \mu\text{mol/g}$. The reducing antioxidant power of lyophilised lingonberry fruits reached $550.67 \pm 3.90 \mu\text{mol/g}$. The variation of reducing power in differently prepared lingonberry fruit samples is shown in Fig. 5.

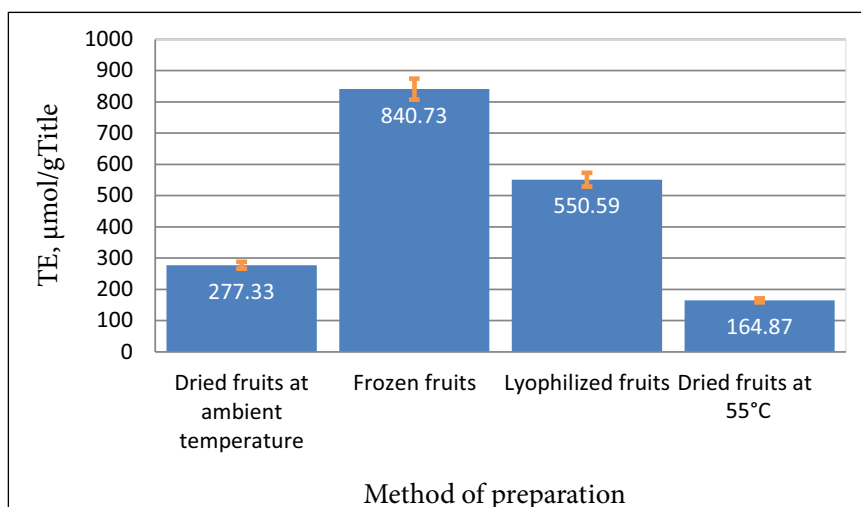


Fig. 5. Ferric reducing antioxidant power (FRAP) variation in lingonberry fruit samples prepared by different methods

Table. Dependence of the total phenolic content on the reducing antioxidant capacity in differently prepared lingonberry fruits

Raw material preparation	Total phenolic content, mg/g	Ferric reducing capacity (FRAP), $\mu\text{mol/g}$	Correlation coefficient, r
Dried fruits at ambient temperature	13.37	277.33	0.792
Dry fruits at 55°C	6.71	164.86	0.782
Frozen fruits	42.11	840.73	0.899
Lyophilised fruits	21.19	550.59	0.645

The relationship between the total phenolic content and reducing antioxidant capacity in differently prepared lingonberry raw materials was evaluated (Table). A strong correlation was estimated between the total phenolic content and reducing antioxidant capacity in the frozen lingonberry fruits ($r = 0.899$). The correlation between the total phenolic content and the lingonberry fruits dried at ambient temperature ($r = 0.792$), lyophilised fruits ($r = 0.645$) and fruits dried at 55°C ($r = 0.782$) was determined. Based on these data, we can assume that the reducing antioxidant capacity depends on the amount of phenolic compounds in the raw materials of lingonberry berries.

Scientist M. M. Bratu and his research group also aimed to evaluate the influence of the total phenolic content and trace elements on the antioxidant capacity. For this, the researchers used the correlation coefficient. A strong correlation was assayed between the total phenolic content in dried lingonberry fruits and the reducing power ($r = 0.9465$). This proves that phenolic compounds contribute significantly to the antioxidant power of lingonberry fruits. This study also evaluated the correlation coefficient between the reducing antioxidant capacity and anthocyanin content of lingonberry fruits. A weak correlation was determined ($r = 0.209$) [24]. This means that the relationship between the antioxidant capacity and anthocyanins is weaker compared to the content of total phenolic compounds.

Taking into account the results of this study, in order to get the maximum benefit from this plant raw material, it is recommended to choose the freezing method for the preparation of raw materials, because the yield of bioactive substances is highest in raw materials prepared in this way. It is a cheap, fast and simple method of raw material preparation.

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ŽALIAVŲ PARUOŠOS ĮTAKA VEIKLIŲJŲ MEDŽIAGŲ IŠEIGAI BRUKNIŲ (*VACCINIUM VITIS - IDAEA L.*) VAISIUOSE

Santrauka

Remiantis šio atlikto mokslinio tyrimo duomenimis bei palyginus juos su kitų mokslininkų darbais, nustatyta, kad bruknių vaisių žaliavos paruošos būdas turi didelę reikšmę suminei fenolinių junginių išeigai. Nustatyta, jog suminis fenolinių junginių kiekis skirtingai paruoštose bruknių vaisių žaliavose statistiškai reikšmingai skyrėsi. Jis įvairavo nuo $6,72 \pm 0,58$ mg/g iki $43,25 \pm 0,29$ mg/g. Didžiausias suminis fenolinių junginių kiekis buvo nustatytas šaldytoje bruknių vaisių žaliavoje, o mažiausias 55 °C temperatūroje džiovintuose bruknių vaisiuose. Suminis proantocianidinų kiekis, atsižvelgiant į žaliavų paruošos būdą, varijavo nuo $2,73 \pm 0,11$ mg/g iki $11,33 \pm 3,18$ mg/g. Didžiausias šių junginių kiekis nustatytas šaldytoje žaliavoje, mažiausias – 55 °C laipsnių temperatūroje džiovintuose bruknių vaisiuose. Suminis antocianinų kiekis skirtingai paruoštose bruknių vaisių žaliavose įvairavo nuo $0,33 \pm 0,01$ mg/g iki $1,74 \pm 0,04$ mg/g. Gauti rezultatai statistiškai reikšmingai skyrėsi. Didžiausias antocianinų kiekis nustatytas šaldytoje žaliavoje ($1,74 \pm 0,04$ mg/g). Tyrimo metu antioksidantinis aktyvumas įvertintas ABTS ir FRAP metodais. Apskaičiavus koreliacijos koeficientus, nustatyta, kad antioksidantinis aktyvumas priklauso nuo suminio fenolinių junginių kiekio.