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Effect of Grinding and Successive Sieving on the Distribution of Active Biological Compounds in the Obtained Fractions of Blackthorn Berries

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Abstract: The current study evaluated the effect of powder fractionation based on particle size on the chemical composition of macronutrients such as proteins and sugars, on the phytochemical properties (total content of polyphenolic compounds, vitamin C, and antioxidant activity), on preservation capacity (water activity), powder functional properties (water absorption capacity and water solubility index), and physicochemical properties (particle size distribution and moisture content) of blackthorn berry (*Prunus spinosa*) powders. The fruits were separated from the plant material and seeds, dried, and then ground using an universal mill for dry materials. Eight fractions were obtained after sieving on sieves with different mesh sizes, such as 1 mm, 0.8 mm, 0.630 mm, 0.450 mm, 0.315 mm, 0.200 mm, and 0.125 mm. The grinding/sieving procedure was effective in separating *Prunus spinosa* powder into sufficiently different size classes. The maximal moisture content and water activity were 5.61% and 0.250, respectively, showed good preservation from a microbiological point of view, and ensured the prevention of oxidation of biologically active compounds of blackthorn berry powders. For samples with reduced particle sizes, the powder functional properties were greatly improved. The total phenolic content, carbohydrates, and antioxidant activity showed significantly different values for some particle size classes compared to the un-sieved sample. A considerable content of vitamin C was presented in the fraction with large particle sizes, precisely because they did not undergo intense degradation processes. Therefore, the technique of grinding and successive sieving proved effective in enhancing the physicochemical and functional characteristics of powdered blackthorn berries, particularly for smaller particles.

Keywords: blackthorn berries; grinding; sieving; active biological compounds; phenolic content; antioxidant activity

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1. Introduction

Wild plants are becoming more recognized as important sources of naturally occurring bioactive compounds for use in functional food products. The health advantages of wild fruits, including their ability to scavenge free radicals and their antioxidant, anti-inflammatory, antibacterial, and anticancer properties, have been demonstrated by several research studies [1–3]. The crucial role of nutrition and food science is highlighted by the transformation of phytotherapeutic products into powders with multiple applications such as improving food quality, extending shelf life, and reducing the amounts of synthetic additives used [4].

The fruits of *Prunus spinosa* L. appear to be a promising candidate, because the fruits contain a variety of bioactive compounds that need further research, despite their inedible character and low industrial use. *Prunus spinosa* L. is a tree or shrub in the Rosaceae

family that is categorized under the Prunus genus. It grows in the Mediterranean, Europe, West Asia, and West Africa and is sometimes known as "wild plum", "blackthorn", or "sloe" [5–7]. *P. spinosa* L. is found in the plain areas of Romania, but it is more common in hilly areas that reach altitudes of 900–1000 m. It is found on the edges of agricultural lands, embellishing the landscape, on pastures that have been abandoned, and on the edge of forests of oak and beech. It is a 2–3 m shrub that grows well in sunny areas. It has dense, stiff, spiky branches and dark blue-violet bark. This shrub does not require care and is an endless source of tiny, spherical, blackish fruits, with a diameter of around 10–12 mm, that have both medicinal and functional qualities [4,7–9]. Its late ripening and seasonality may have hampered its widespread usage. Also, these fruits cannot be eaten directly because of the bitter taste and the information regarding their content in active substances is relatively limited and still unknown [5,10–12]. In addition to being preserved and utilized to create herbal teas, they are typically used to produce syrups jams, juices, compotes, wine, spirits, pickled like olives, vinegar, and other traditional products [13–15].

The fruits of *P. spinosa* L. are rich in organic acids (malic, citric, and fumaric), fatty acids (oleic, linoleic, arachidonic, and linolenic) anthocyanins, polyphenols, tannins, sugars, proteins, pectins, coumarin derivatives, vitamins (C, E), and calcium and magnesium salts [16,17]. Furthermore, several studies have noted fluctuations in the fruit nutrient content of *P. spinosa* L., which have mostly been attributed to environmental conditions [4,18]. In some studies, it was identified that the powder obtained by grinding the fruit of blackthorn berries can be used as a natural coloring and preservative for obtaining functional drinks, gummy bears, pastry products, ice cream, yogurt, and in fermented meat products, improving the nutritional, physical–chemical, and sensory value of these products [4,12].

To use the fruits produced by wild shrubs, they are often processed using traditional techniques (aqueous or dry extracts, powders) and by current extraction techniques made precisely for the extraction of bioactive compounds [5,19]. It has also been possible to isolate polyphenolic chemicals more easily recently using modern extraction techniques (accelerated solvent extraction, microwave-assisted extraction, and supercritical fluid extraction) [14]. Nevertheless, there are currently significant concerns regarding the use of solvents to extract active ingredients. These concerns focus on the dangerousness of many of the solvents used in this process (such as toluene, chloroform, dichloromethane, acetyl acetate, and petroleum ether), their effects on the environment, human health, and the atmosphere, the expense of treating the toxic wastes that are produced, and the effects on the quality and safety of the materials that are liable to be extracted [5,20]. Thus, we applied a method that consisted of drying, grinding, and sieving to concentrate the bioactive compounds in some granulometric classes of blackthorn berry powders [21–23].

The powders from the fruits of wild plants have an advantage over traditional bioactive compounds extracted from fruits using solvent extraction because this method preserves the bioactive ingredients, particularly their bioactivity, for human consumption [24]. The process of grinding results in a decrease in particle size, a widening of the dispersion of particle sizes, and an increase in the specific surface area, all of which enhance the functional properties of the material [21]. Given that one of the key factors influencing extraction is particle size, this can create confusion regarding the extraction of bioactive compounds that can occur either following the extraction processes or from the sample grinding stage [25].

Fruit powders are separated by granulometric differentiation using decreasing mesh sieves during the sieving process, which results in the controlled distribution of bioactive compounds in the various granulometric fractions [24]. The relationship between variety of particle sizes in plant powders, the bioactive ingredient amounts, the physicochemical characteristics, and antioxidant activity has been documented in several recent research studies [21–24]. From an industrial perspective, the functional qualities of powders, such as their flowability, preservation potential, and functional properties are crucial because they dictate how they should be produced, handled, stored, and reconstituted to preserve their bioactive components. The particle size and physical structure can have a significant

influence on the physicochemical properties of powders [26–28]. Although the blackthorn fruits have been examined in the specialized literature, there are still only a few references addressing the detailed composition and fraction of the crushed products in terms of chemical composition and biological activity [1–17].

There are a number of articles on the extraction of bioactive compounds and those with antioxidant activity from blackthorn berry fruits, focusing on extraction with solvents of different polarity, or with eutectic solvents using techniques such as ultrasound (US), pulsed electric field (PEF), and the simplex lattice mixture design method [5,29–31]. Increased understanding of the impact of particle size on the phytochemical characteristics, powder functional properties, and preservation ability of powdered blackthorn berries was the goal of the current study. There are also research studies [32–35] showing that the particle size distribution provides a complete description of a powder, and sieving is the operation that can produce a sample with a uniform particle size distribution, facilitating the development of products with improved functional properties.

This study aimed to evaluate the characteristics of the blackthorn berry ground powder with different particle sizes, such as several relevant chemical and powder functional properties, as well as the main parameters reflecting powder quality suitability for storage purposes. This research is intended to expand our understanding of the importance of grinding and sieving in order to obtain vegetal powders with specific characteristics that could be used as valuable ingredients to develop functional foods.

2. Materials and Methods

2.1. Materials

2.1.1. Plant Material

The fruits of *Prunus spinosa* L. were harvested in the Râmnicu Vâlcea region, located in the central–south area of Romania (latitude: 44.9697628, longitude: 23.876168, altitude 335 m according to Google Earth version 10.57.0.4), in November 2023. Fruits were hand-picked, at optimal ripeness, from several shrubs within the same area, and carefully cleaned under running water and patted dry with a paper towel. Using a knife, the fruits' flesh and peel were carefully separated from the kernel, and then they were placed in an oven at a temperature of 40 °C, until they reached a moisture content of less than 6%.

2.1.2. Chemicals

In order to obtain the standard curves and to perform the actual tests, the following chemicals were used: sodium hydroxide (NaOH), sodium carbonate (Na_2CO_3), potassium sodium tartrate ($KNaC_4H_4O_6 \cdot 4H_2O$), copper sulphate ($CuSO_4$), Folin–Ciocalteu reagent, bovine serum albumin (BSA), 3,5-dinitrosalicylic acid ($C_7H_4N_2O_7$), sodium sulfite (Na_2SO_3), phenol (C_6H_6O), hydrochloric acid (HCl), potassium iodate (KIO_3), potassium iodide (KI), 2,2-difenil-1-picrilhidrazil (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and gallic acid.

2.2. Methods

2.2.1. Grinding of Dry Plant Material

A quantity of 200 g of dry plant material was ground using the universal mill Fritsch Pulverisette 19 based on the cutting principle between the edges of the rotor and the fixed knives in the grinding chamber, this being equipped with a sieve of 1 mm with trapezoidal perforations. In order to prevent the loss of the bioactive compounds, the grinding process was conducted at a speed of 2800 rpm at room temperature. The resulting powder from the grinding step was then separated into two equal amounts of 100 g (un-sieved powder sample and the sample subjected to sieving), in sealable polyethylene bags. It was then kept at 10 °C until determination of characteristic parameters and the sieving.

2.2.2. Sieving Plant Powder

One of the two aliquots was sieved for five minutes at a vibration amplitude of 1.5 mm using an Analysette 3 Spartan Vertical Vibrating Laboratory Sieve Shaker (Fritsch, Idar-Oberstein, Germany).

The particle size was screened by evaluating the mass percent of particles with sizes between the mesh sizes of the sieves that frame a certain fraction of sieved material.

Then, by means of the granulometric curves, the cumulative mass percentage of particles larger/smaller than the mesh size of a certain sieve was determined.

To maximize the effectiveness of the sieving process, a batch of about 100 g of fruit powder was sieved with seven sieves with different mesh sizes.

The mesh sizes of the sieves were 1 mm, 0.8 mm, 0.630 mm, 0.450 mm, 0.315 mm, 0.200 mm, and 0.125 mm. These were placed one under the other in ascending order of mesh size. The equation used to describe the particle size distribution was the Rosin–Rammler. The average size of the material was calculated with the following equation:

$$d_m = \frac{\sum a_i\ (\%) \times d_i}{100}\ (\text{mm}) \quad (1)$$

The specific surface area (S_{sm}) of a set of particles was calculated using the following formula:

$$S_{sm} = \frac{6}{100 \times \rho} \sum_{di}^{n+1} \frac{a_i}{d_i} \left(\frac{m^2}{kg} \right) \quad (2)$$

The empty sieves and the particle size classes obtained on each sieve were weighed and then the samples were stored under vacuum in sealed bags at 10 °C for analysis. The sample obtained from the sieve with a mesh size of 1 mm was not subjected to analysis as it was an insufficient amount.

2.2.3. Moisture Content and Water Activity

Moisture content was determined using a DBS 60-3 halogen lamp moisture analyzer based on the thermogravimetric principle at 105 °C and water activity was determined using a Rotronic HygroLab2 measurement system, in standard mode using about 10 g powder in a thermostatic room.

2.2.4. Hydration Properties

The water absorption capacity (WAC) and water solubility index (WSI) were determined using the respective methods of Miganeh Waiss et al. [26] and Deli et al. [28] with slight modifications. The following procedure was used to calculate WAC: 1 g of blackthorn berry powder from each sample and 10 mL of distilled water were placed in stoppered test tubes, then the mixture was vortexed at 300 rpm for 30 min, and the sample was filtered on filter discs with a cellulose nitrate membrane with a pore size of 0.45 µm, using a vacuum filtration system for 30 min. The filter disc was weighed before the sample was poured. At the end of the filtration, the sample together with the disc was weighed, dried in an oven at 103 °C for 6 h, cooled in a desiccator, and weighed again. The WAC was calculated using the following formula (Equation (3)) [26].

$$WAC \left(\frac{g}{g} \right) = \frac{Weight\ of\ wet\ sediments}{Weight\ of\ dried\ sediments} \quad (3)$$

In the above relation, the mass of wet and dry material is expressed in grams.

The water solubility index (WSI) of powder samples was determined using the following procedure: 2.5 g of blackthorn berry powder from each sample and 20 mL of distilled water were placed in stoppered test tubes, then the mixture was vortexed at 300 rpm for 30 min and the sample was filtered on filter discs with a cellulose nitrate membrane with a pore size of 0.45 µm using a vacuum filtration system. The filter disc was weighed before

the sample was poured. At the end of the filtration, the sample together with the disc was weighed, dried in an oven at 103 °C for 6 h, cooled in a desiccator, and weighed again. The WSI was calculated using the following formula (Equation (4)) [26]:

$$WSI(\%) = \frac{Dry\ weight\ of\ supernatant\ (g)}{Dry\ weight\ of\ blackthorn\ berries\ powder\ (g)} \times 100 \quad (4)$$

2.2.5. Preparation of Extracts and Analysis of the Absorption Spectrum

All seven samples (collected from the sieves) were mixed with distillated water (DL), with a sample-to-DL ratio of 1:10. At the same time, a series of seven other samples were mixed with ethanol, with a sample-to-ethanol ratio of 1:10.

2.2.6. Total Phenolic Content

Spectrophotometry was used to determine the total phenolic content (TPC), with gallic acid serving as a standard, in accordance with Singleton and Rossi's method [36]. In simple terms, 1.0 mL of a 1/10 dilution of Folin–Ciocalteu's reagent in water was placed in tubes together with 0.2 mL of the diluted sample extract. The sample was treated with 0.8 mL of a 7.5% w/v sodium carbonate solution after a 10-min waiting period. The absorbance at 743 nm was then measured after the tubes had been let to remain at room temperature for 30 min. The gallic acid equivalent (GAE) was used to express the TPC. The polyphenol concentration in the samples was calculated using a standard gallic acid curve with concentrations ranging from 20 to 200 µg/mL. The calibration curve was determined by linear regression, obtaining an R^2 coefficient of 0.9938, and the statistical package used was Microsoft Excel. UV-Vis analyses were carried out with the T92+ Spectrophotometer [36–39].

2.2.7. Antioxidant Activity: DPPH Test

The extract was tested with the most used method, based on the substance called DPPH, i.e., 1,1-diphenyl-2-picryl hydrazyl. To obtain the DPPH stock solution, 4 mg of DPPH were weighed and dissolved in 100 mL of ethanol. Also, 400 microliters of each working sample were pipetted into clean test tubes, over which 6 mL of DPPH solution was added, vortexed, and left in the dark for 30 min. The absorbance was determined at 515 nm [37,40–43].

2.2.8. Proteins by the Lowry Method

This method is based on the reduction in phosphormolybdates and phosphotungstates from the Folin–Ciocalteu reagent by the phenolic compounds in the protein.

Alkaline solution A was prepared by dissolving 2 g Na OH, 5 g Na_2CO_3, and 0.1 g potassium sodium tartrate in distilled water, and brought to the 0.5 l mark in a volumetric flask. Solution B, namely that of $CuSO_4$ 0.5%, was obtained by dissolving 0.125 g of $CuSO_4$ in distilled water and brought to the 50 mL mark in a volumetric flask. The Folin–Ciocalteu reagent was diluted 1:2 with water before use.

To obtain the standard curve, dilutions were made from the standard solution of bovine serum albumin (BSA). For the formation of copper proteinate, 1 mL of protein preparation was mixed with 1 mL of alkaline Cu reagent and allowed to stand for 10 min at room temperature. After that, 3 mL of Folin–Ciocalteu reagent (1:10) was added. The samples were shaken vigorously and incubated for 10 min at 50 °C, cooled, and the absorbance of each tube was measured using a spectrophotometer at 660 nm. Using the findings of many standard solutions, a standard curve for protein was drawn, and the estimated protein content of unknown variables (extracted from samples) was determined [37,44,45].

2.2.9. Carbohydrates by Dinitrosalicylic Acid (DNSA) Technique

The alkaline solution that successfully absorbs sunlight at 540 nm was used to lower DNSA to 3-Amino, 5-Nitrosalicylic acid. Depending on the amount of reducing sugar

present in the sample, the color changes from yellow to orange and reddish–brown. Using the absorbance found in a spectrophotometer, the level was estimated. In order to prepare the 1% DNSA solution, 2.5 g of NaOH was used, which was added to 175 mL of deionized water and mixed while 45.4 g of sodium potassium tartrate was added. When the solution dissolved, 2.5 g of 3.5 dinitrosalicylic acid was further added, followed by the addition of 0.125 g of Na_2CO_3 and 0.5 g of phenol. To prepare the glucose solution, 0.1 g of glucose was dissolved in distilled water and the solution was brought to the mark in a 100 mL volumetric flask. To create the standard curve, 11 test tubes were used in which different volumes of glucose solution and distilled water were added so that the final volume was 10 mL, of which 3 mL were transferred to another clean test tube. Test tubes were appropriately labeled with P1, P2, . . ., P11. A total of 3 mL of DNS was added, and the mixture was heated to 90–100 °C for the development of the reddish–brown color. After cooling to room temperature, the absorbance (OD) measurements of every test tube were determined at 540 nm. A calibration curve including carbohydrates was created using the results of the several standard solutions that were produced, and the carbohydrate concentration of the samples was evaluated [37,46–48].

2.2.10. Amount of Vitamin C by the Iodometric Method

This technique uses a redox titration using potassium iodate in the presence of potassium iodide to measure the content of vitamin C in a sample. Solutions of 2% hydrochloric acid, the KIO_3 solution, and the aqueous KI solution were prepared. A total of 10 cm^3 of filtrate was pipetted into a 100 mL Erlenmeyer flask, and 30 mL of distilled water, 5 mL of KI, 5 mL of HCl, and 1.5 mL of starch solution were added [49–52].

It was titrated with a solution of KIO_3 until the blue color was attained. When potassium iodate was added to the mixture that also contained potassium iodide, iodine was generated that oxidized the vitamin C present. After vitamin C was completely oxidized, the generated iodine formed with the starch presented as a deep blue-colored inclusion complex. The vitamin C content was generally calculated in mg/100 g of material [49–52].

2.2.11. Data Analysis

All determinations were made in duplicate, and the results are presented as an average of their values. An ANOVA was performed to analyze the data using a statistical package Microsoft Excel Version 2406 64-bit. Since the ANOVA test with a single factor (one-factor analysis of variance) indicated the existence of significant differences, a least significant difference (LSD) test was also performed to identify which of the groups differed significantly from each other according to their means.

3. Results

3.1. Particle Size Distribution of Samples

There are also parameters that influence the separation of particles by sieving such as amplitude, frequency, angle of vibration direction, opening and size of the sieving holes, average particle size, and the properties of the material taken for analysis [53,54]. The ground powder with different particle sizes is shown in Figure 1.

The initial step was to confirm that the established grinding/sieving process would produce granulometric fractions with distinct particle sizes. Table 1 shows the results of the particle size distribution by illustrating the masses of the sieves used and the masses of material left on each sieve following the sieving process.

Using data from the particle size distribution table (Table 1), a number of characteristics were determined corresponding to the sieving mesh size (l_i), such as the average particle size of fraction (d_i), the cumulative percentage of material with a size between dimensions l_i and l_{i+1} of the adjacent sieves (a_i), the percentage of material smaller than the size of the sieve mesh (T_i), and the cumulative percentage of material larger than the size of the sieve mesh (R_i). All these resulting characteristics can be observed in Table 2.

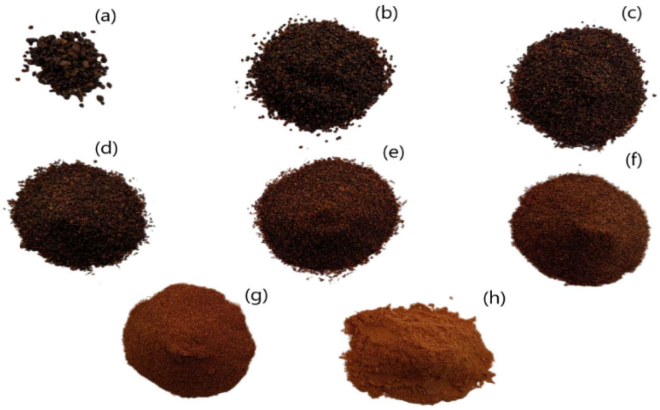


Figure 1. Ground blackthorn berries and the different particle sizes resulting from sieving: (**a**) >1000 µm; (**b**) 800–1000 µm; (**c**) 630–800 µm; (**d**) 450–630 µm; (**e**) 315–450 µm; (**f**) 200–315 µm; (**g**) 125–200 µm; (**h**) 0–125 µm.

Table 1. The masses of the sieves used and the masses of material left on each sieve following the particle size distribution.

Mesh Size of the Sieve (mm)	Sieve Mass (g)	The Sample Mass Found on the Sieve (g)	The Mass of the Sample Taken for Analysis (g)
1.000	392.2	1.4	100
0.800	474.8	21.0	
0.630	458.6	14.7	
0.450	305.9	13.6	
0.315	413.9	12.5	
0.200	335.3	10.1	
0.125	391.9	14.3	
0	342.5	12.4	

Table 2. Variation in ground material characteristics according to mesh size (l_i).

Mesh Size of the Sieve l_i (mm)	The Average Particle Size of Fraction d_i (mm)	The Percentage of Material with a Size between Dimensions l_i and l_{i+1} of the Adjacent Sieves a_i (mm)	The Percentage of Material Smaller Than the Size of the Sieve Mesh T_i (%)	The Percentage of Material Larger Than the Size of the Sieve Mesh R_i (%)
0	0.063	12.40	0.00	100.00
0.125	0.163	14.30	12.40	87.60
0.200	0.258	10.10	26.70	73.30
0.315	0.383	12.50	36.80	63.20
0.450	0.540	13.60	49.30	50.70
0.630	0.715	14.70	62.90	37.10
0.800	0.900	21.00	77.60	22.40
1.000	1.207 *	1.40	86.20	13.80

* It was supposed that the mesh sizes of the sieves followed a ratio $l_i = l_{i-1} \times \sqrt{2}$.

For a more suggestive illustration of the data in Table 2, the data were represented graphically; T and R(%) were represented on the ordinate on a conveniently chosen scale, and on the abscissa, the size l_i of the mesh sizes. The data obtained above were also used to calculate the average particle size of the initial material. Granulometric distribution curves, along with the Rosin-Rammler equation, can be seen in Figure 2.

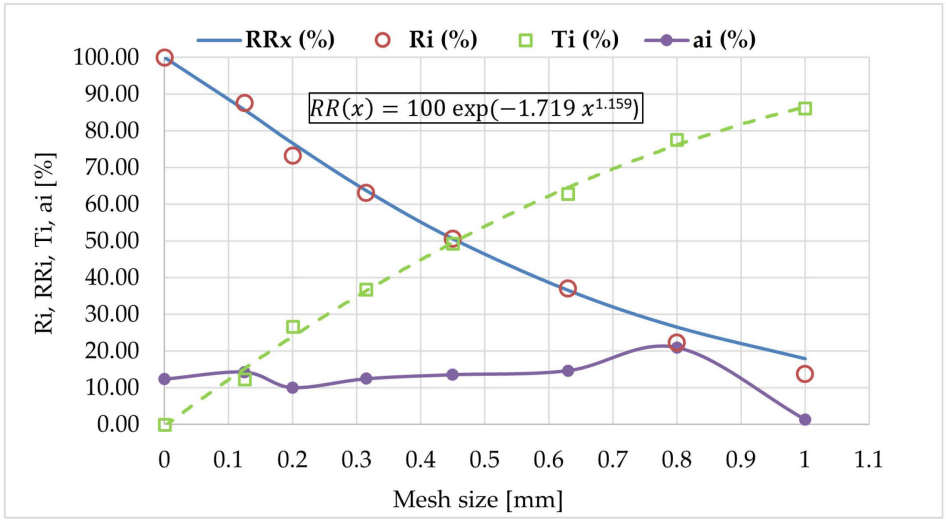


Figure 2. Granulometric distribution curves.

Furthermore, the results of the sieve fractionation process and the particle size characteristics of the powder fractions (Table 2) demonstrated showed that the grinding/sieving procedure was effective in separating *Prunus spinosa* powder into sufficiently different size classes. It is crucial to note that the vegetal material was sticky and cohesive during the sieving study, so it cannot be claimed that the results of the sieving analysis were accurate in terms of particle size.

In another organization of the experimental data, based on the values obtained by calculation with relation 2, the graph in Figure 3 was obtained, and represents the distribution of the specific surface area of the material particles corresponding to the size class of the particles.

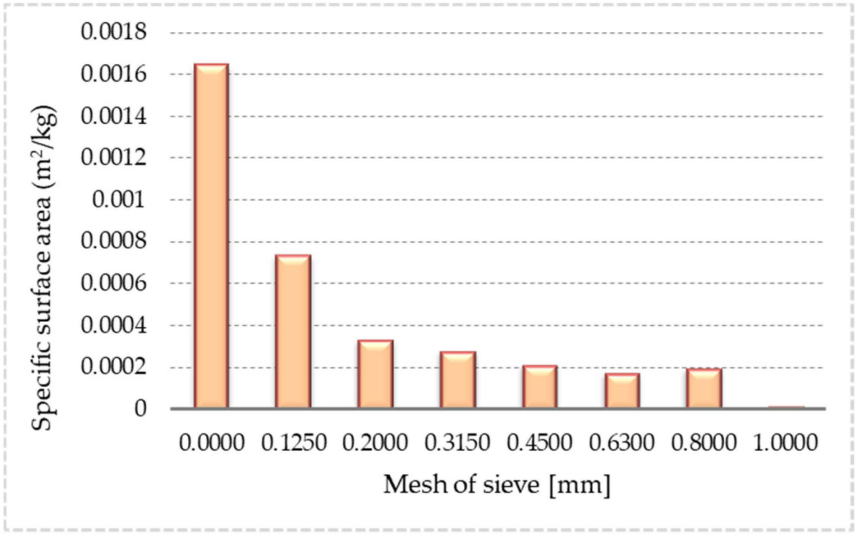


Figure 3. The distribution of the specific surface area of the particles of the ground material corresponding to the particle size class.

3.2. Analysis of the Absorption Spectrum of Extract

The UV-vis spectra of the blackthorn berry powder extracts in the two solvents (ethanol and water) are shown in Figures 4 and 5, respectively.

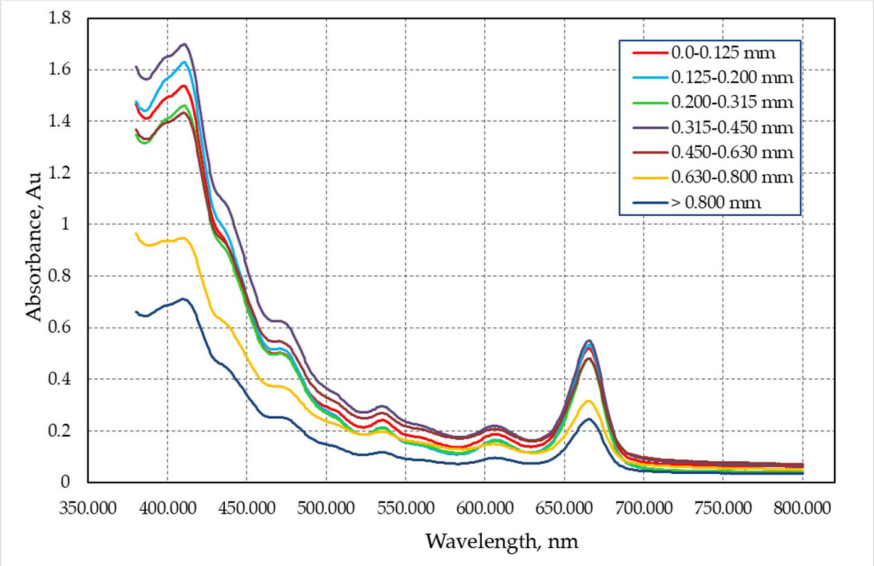


Figure 4. UV-vis spectra of the extracts of the seven granulometric classes in ethanol.

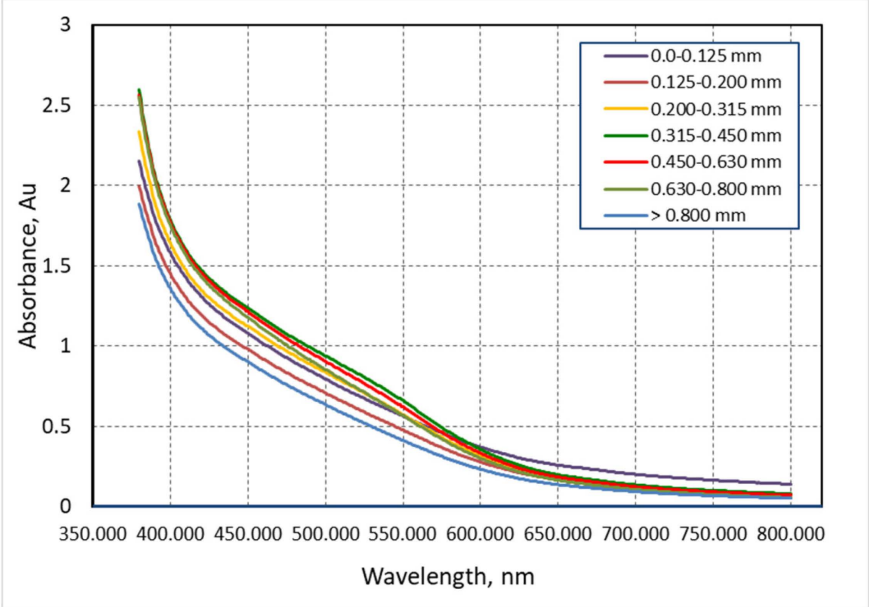


Figure 5. UV-vis spectra of the extracts of the seven granulometric classes in water.

Firstly, the absorption spectra in ethanol and water differ significantly due to the solubility of the colored compounds. Due to the weak polar character of ethanol, in this solvent, the less polar compounds were extracted. The highest absorbance value for the ethanolic extract, 1.537, was recorded for the powder with a particle size between 0.315 and 0.450 mm, at the wavelength of 410 nm, which corresponds to a mixture of yellow–orange compounds. For all samples obtained by sieving, the same characteristic maximum, but with different values, was recorded at the wavelength between 411 and 666 nm. This means that in all the fractions, there is a mixture of yellow–orange-colored compounds, probably flavonic compounds, in different amounts. All the recorded spectra also present other lower absorption maxima at the wavelengths of 536 nm, 606 nm, and 631 nm, in the specific range of compounds colored in red, violet, and blue. The highest value in this wavelength range was observed for the wavelength between 660 and 665 nm, specific to the blue color.

Therefore, in all the sieved samples, a mixture of colored compounds with characteristic values of the wavelengths corresponding to the maxima was extracted in ethanol. As

expected, the lowest absorbance values were recorded in the case of samples with the largest particle sizes due to the smaller extraction surface. Also, the extract from the sample with the smallest particle sizes had lower absorbance values due to the fact that the peel of the dried fruit was more difficult to grind, and was found in the fractions with intermediate sizes.

The extracts in the water even after filtration on filter paper were not perfectly clear, and the absorbance spectra all showed a decrease in absorbance from low wavelengths to higher wavelengths.

The mixture of colored compounds, and most likely the anthocyanins, gave the extracts an orange color.

3.3. Moisture Content and Water Activity

Determining the moisture content of powders is important because it influences a number of factors, including stickiness, flowability, stability, drying effectiveness, oxidation of bioactive agents, and the growth of microbial colonies [43]. Deterioration of the product in powder form and the growth of microorganisms are caused by higher moisture content—typically more than 10% [55]. From the moisture content results, a clear trend can be deduced regarding the influence of particle size, i.e., the smaller the particle size, the lower the moisture content, which is due to temperature-induced evaporation, which will be compensated for during the particle size distribution analysis, as smaller particles are more hygroscopic. Also, the results of the moisture content values of granulometric classes and un-sieved samples ranged from 3.31% to 5.61%. The powder's structure also contributes to the water supply for microorganisms and degrading reactions; therefore, moisture content alone is insufficient to characterize the powder's stability during storage [26].

The following factors are significantly impacted by water activity: microbiological safety, glass transition, scent, taste, caking and clumping, and chemical degradation [55,56].

The results obtained in Table 3 show that the water activity of the granulometric classes and un-sieved samples of blackthorn berry powders varied from 0.232 to 0.250, which makes the powder stable during storage and packaging, being well below the critical value (aw = 0.6) required for most microorganisms to survive and reproduce (molds, yeasts, and bacteria). As a result, all powder samples under investigation are thought to be microbiologically and biochemically stable. Similar to the water content, water activity frequently decreased as particle sizes reduced. This was because the larger surface-to-volume ratio of the particles allowed for greater interactions with the moisture in air, which improved the water absorption and raised water activity.

Table 3. Values of moisture content, water activity, water absorption capacity, and water solubility index of the seven granulometric fractions and the un-sieved powder samples.

Mesh Size of the Sieve l_i (mm)	Mean of Intervals, (mm)	Moisture Content (%)	Water Activity(-)	Water Absorption Capacity, WAC (g/g)	Water Solubility Index, WSI (g/100 g)
Un-sieved sample	-	4.52	0.241	4.905	32.672
0	0.063	3.31	0.232	5.883	41.284
0.125	0.163	3.79	0.234	5.688	40.676
0.200	0.258	4.34	0.237	3.892	42.272
0.315	0.383	4.71	0.242	3.958	37.920
0.450	0.540	5.10	0.243	3.868	35.544
0.630	0.715	5.11	0.244	3.847	38.912
0.800	1.131	5.61	0.250	3.837	36.828

3.4. Hydration Properties

Table 3 illustrates the WAC and WSI of all granulometric classes and un-sieved samples of blackthorn berry powders.

The amount of water that a food powder can absorb and retain to the maximum is indicated by the WAC [57]. A clear influence of particle size on WAC was certainly observed as the decreasing particle size resulted in higher absorption capacity. The WAC value for the un-sieved sample had an average value of 4.905 g/g. For the samples subjected to particle size distributions, the WAC value varied from 3.837% g for the particles with the largest size to 5.883% for the particles with the smallest size. Therefore, all of the components of the dried blackthorn berry hydrophilic groups were exposed once the cell walls were broken down by the grinding process. Also, the higher absorption capacity could even be due to the presence of the higher content of sugars in the powder with reduced particle sizes.

One of the most crucial functional characteristics of food powders is their solubility, which demonstrates how the particles respond when they regenerate in water. The quantity of either dissolved or undissolved particles is indicated by the solubility index [58]. The WSI values increased for the powders with small particle sizes (cf. Table 3), indicating that the interaction with water was improved in the case of these samples. This can be understood by the fact that milling causes a reduction in particle size, which improves material functions like surface energy and particle surface specific area and improves the plant material's interactions with water [26,34]. The solubility index values ranged from 32.672% for the un-sieved sample to 42.272% for the sample with particle size between 200 and 315 μm.

The current study's findings are in line with those of other authors, Miganeh Waiss et al. [26]; Bala et al. [34]; and Deli et al. [56], who noted that samples with smaller particle sizes showed improvements in WAC and WSI. Blackthorn berry powders with smaller particles should therefore be more soluble and interact with water more readily, which could help when ingesting them through food, facilitating their intestinal absorption [26].

3.5. Total Phenolic Content

Blackthorn berry powder contains polyphenolic compounds with antioxidant capacity, which is supported by the findings of Negrean et al. [14] in their paper. In addition to preserving color, flavor, and taste, these phenolic chemicals may extend the shelf life of the powder and have antibacterial qualities. They function as a barrier against reactive oxygen species (ROS), protecting molecules from damage and avoiding the negative consequences generated by insects and microorganisms [57–59]. Figure 6 shows the total content of phenolic compounds present in the un-sieved sample (C) and in the granulometric classes obtained after sieving. The results were expressed in μg GAE/mL of sample extract. The highest total phenol content was observed for the sample with a particle size between 0.315 and 0.450 mm, i.e., a phenol content of 40.670 μg GAE/mL. This content is followed by that found in the sample with particle size between 0.450 and 0.630 mm, i.e., 40.121 μg GAE/mL. According to the studies, the content of polyphenolic compounds is often associated with the content of protein fractions in the plant extract [21,26,56]. This can also be demonstrated by the current study because the granulometric classes with the highest content of phenolic compounds also have the highest protein content (cf. Figure 6 and Figure 8). The lowest content of polyphenols was observed in the sample with particle size > 0.800 mm, i.e., 25.615 μg GAE/mL, which proves that this group of chemicals is influenced by the degree of grinding.

After performing the analysis of variance, a difference of 2.97 was obtained, indicating that the data are more concentrated and therefore more predictable.

Error bars in the graphs display the standard error value, calculated as the ratio of the sample standard deviation to the square root of the sample size.

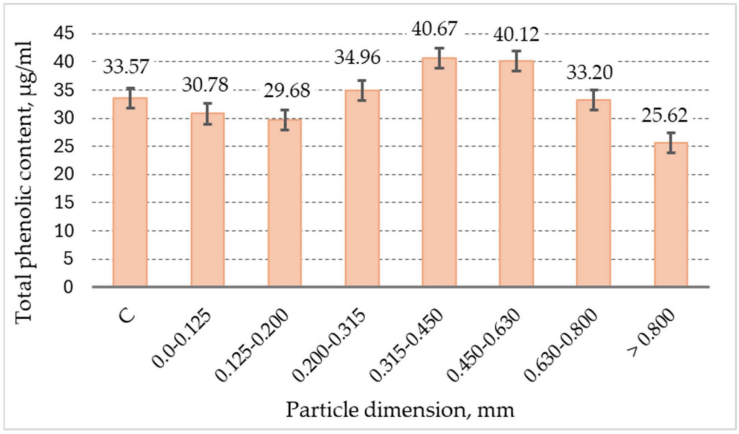


Figure 6. The total content of phenolic compounds presents in the un-sieved sample (C) and in the granulometric classes.

3.6. Antioxidant Activity

The results of antioxidant activity of blackthorn berry powders obtained by DPPH test are presented in Figure 7. Each of the analyzed extracts had the ability to scavenge DPPH radicals. Between 0–0.125 mm and 0.315–0.450 mm were the granulometric classes with higher percentages of DPPH inhibition. Also, the particles with the largest dimensions, namely over 0.800 mm, showed the lowest antioxidant activity, which can be demonstrated by the fact that the physical structure of the fruit matrix was not destroyed so that the antioxidant compounds could be released in depth. After carrying out the regression analysis for the variation in DPPH concentration according to the total content of phenols, we obtained for the linear variation an R coefficient of 0.651; for the logarithmic variation, the correlation coefficient R obtained was 0.51, and the highest correlation coefficient R = 0.754 was obtained for the polynomial variation of the third order. At the same time, the DPPH scavenging activity was relatively higher for two of the sieved samples, which showed that the sieving process has an important role in decreasing the cohesion of the powder, facilitating the dispersion and release of the antioxidant compounds in ethanol. Even the mixing of the sample with the solvent was an important step leading to an improved antioxidant activity. Phenolic compounds were considered to contribute significantly to the antioxidant activity of the tested fruit powders, which was also observed by the authors of [26], in their work. This aspect is in line with the notion that plant products' antioxidant capacity is primarily ascribed to polyphenols' capacity to scavenge radicals. The high bioactive compound contents of blackthorn berry powders are probably responsible for their significant antioxidant activity [56].

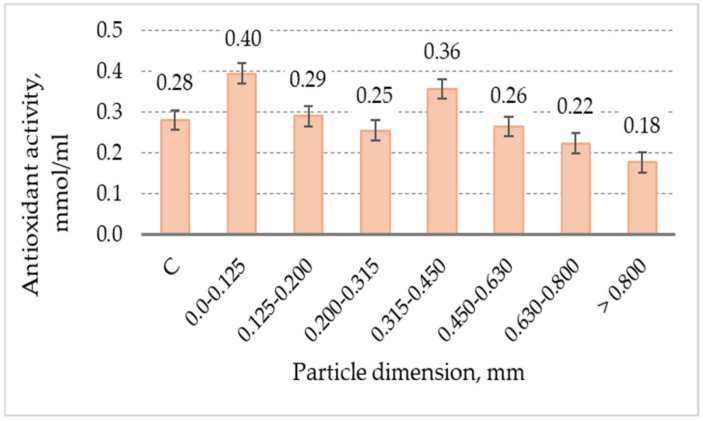


Figure 7. Antioxidant activity of the granulometric fractions and un-sieved powders (C) of blackthorn berries.

3.7. Proteins

Figure 8 shows that there are no significant differences in the protein content of blackthorn berry powder extracts with different particle sizes. It was observed that the proteins were more concentrated in the fractions with particles <0.800 mm. The protein content values ranged from 701.020 µg/mL for the sample with particle sizes >0.800 mm to 857,251 µg/mL for the sample with particle sizes between 0.630 and 0.800 mm. It can be seen that the protein content of the sample with a particle size > 0.800 varied in contrast to that of the un-sieved sample (C); therefore, as many studies (Deli et al. [56]; Becker et al. [14]) have claimed in this respect, the particle size has a particular influence on the chemical composition of plant powders.

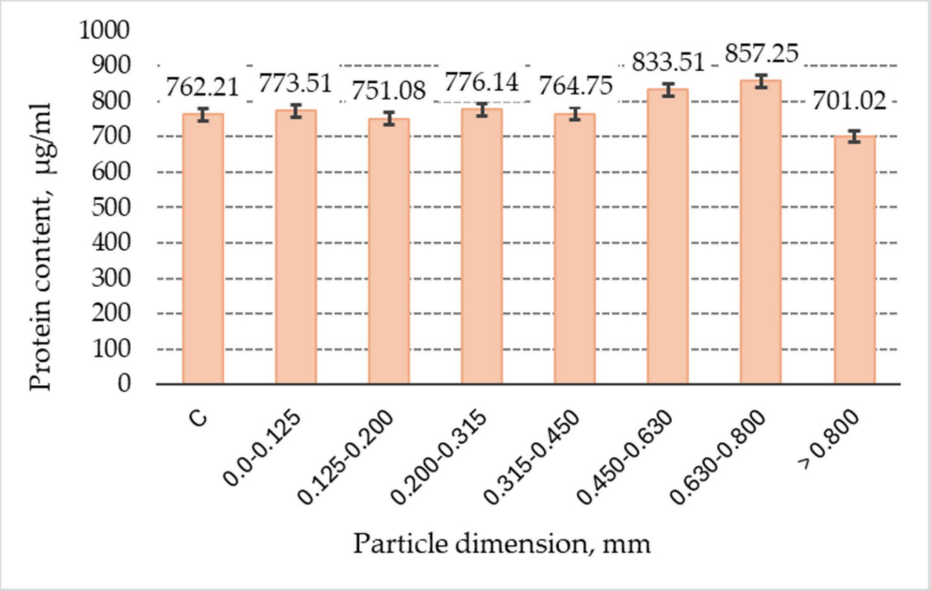


Figure 8. Protein content of the granulometric fractions and un-sieved powders (C) of blackthorn berries.

In the case of protein content, the difference between the variance calculated for the sample of 2350.78, and the variance calculated for the population of 2056.93 is approximately 293.85 and indicates a greater scatter in the data, which may suggest high uncertainty.

3.8. Carbohydrates

Regarding studies on the composition of blackthorn berry fruits, most attention has been paid to phenolic compounds; however, recently, Capek et al. [12] conducted research on the carbohydrate content of these fruits. According to their research, the carbohydrate content of fresh blackthorn berries is only 8.64g/100g. The current study agrees with this, Figure 9 claiming that the highest carbohydrate content was obtained for the sample with a particle size between 0.125 and 0.200 mm, namely 5.429 µg/mL. The lowest carbohydrate content was obtained for the sample extract with a particle size of > 800 mm, a sign that these carbohydrates predominate in the pulp of the fruit and less in the peel. The small difference of variation of 0.04 provides confidence in the validity and reliability of our data on the carbohydrate content of the extract.

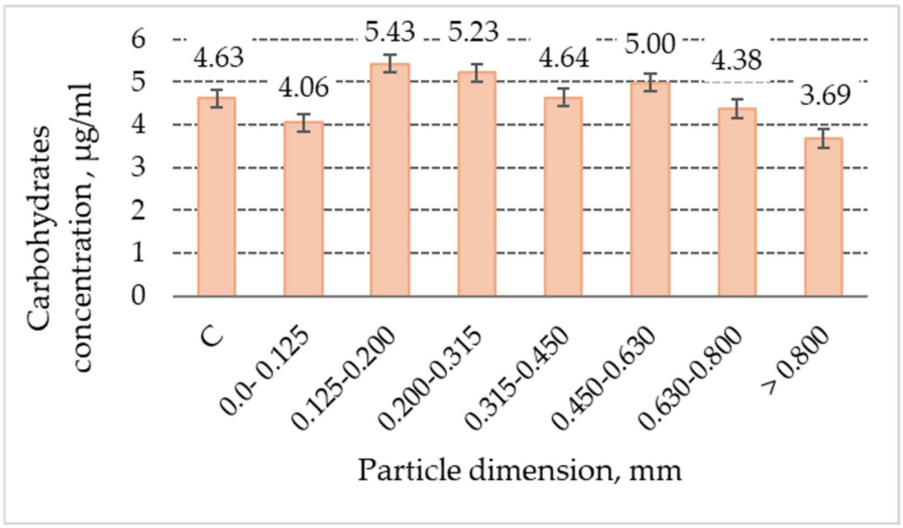


Figure 9. Carbohydrate content of the granulometric fractions and un-sieved powders (C) of blackthorn berries.

3.9. Vitamin C

When the vitamin C content of the sieved fractions and the un-sieved sample was compared, it was found that it reduced as the degree of grinding increased. This aspect can be seen in Figure 10. Vitamin C is prone to oxidation and has a low thermal stability, so it is lost throughout any procedure involving high temperatures. Depending on several factors such as temperature, processing time, and oxygen exposure, these losses might range from 20% to nearly 90% [60]. Furthermore, breaking the dried fruit's cells mechanically results in an immediate loss of vitamin C. At the same time, the small-sized particles were prone to the degradation of vitamin C, precisely because of the large specific surface in contact with the air in the laboratory where the grinding and sieving were carried out. Thus, the highest vitamin C content was obtained for the sample with a particle size >0.800 mm, precisely because those particles did not undergo intense shearing processes. The results were confirmed with the help of those found in the study by Negrean et al. [14], claiming that blackthorn berries are rich sources of vitamin C, with a content of 11.27 mg/100 g − 1 fw, predominating in dehydroascorbic acid.

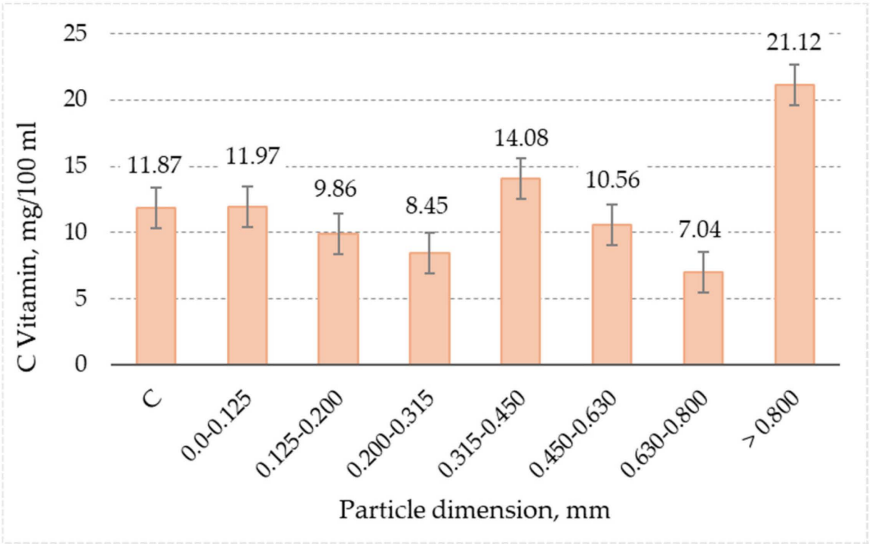


Figure 10. Vitamin C content of the granulometric fractions and un-sieved powders (C) of blackthorn berries.

In the case of the vitamin C content of the extract, we found a high level of confidence in the data obtained, the difference between the variances calculated for the whole population and the sample being only 2.34.

3.10. Statistical Data Analysis

The ANOVA test with a single factor, also known as the unifactorial variation analysis, was the statistical method used to compare the means of the four independent groups represented by the concentration of the substance in the extract (polyphenols, protein content, vitamin C, carbohydrates) depending on the size of the particle size class, to determine if there are significant differences between them. The test results are presented in Table 4.

The assumptions assumed to ensure the validity of the results were:

- Null hypothesis (H_0): there are no significant differences between group means (all means are equal).
- Alternative hypothesis (H_1): there is at least one significant difference between the group means.

Table 4. One-factor ANOVA test results.

Groups	Count	Sum	Average	Variance
Phenolic content [µg GAE/mL]	8	269.59	33.69	23.79
Protein content [µg/mL]	8	6219.53	777.44	2350.78
Vitamin C [µg/100 mL]	8	94.94	11.86	18.74
Carbohydrates [µg/mL]	8	37.06	4.63	0.34

The calculation of the F-statistic value, the ratio of the mean variance between groups to the mean variance within groups (F = 1936.07), suggests that the variance between groups is significantly greater than the variance within groups; the p value is less than 0.05 ($p = 1.48^{-32}$), so we removed the null hypothesis and concluded that there are significant differences between the groups. This aspect can be observed in Table 5.

Table 5. ANOVA test results.

Source of Variation	SS	df	MS	F	p-Value	F Crit
Between Groups	3,475,723.33	3	1,158,574	1936.07	1.48^{-32}	2.94
Within Groups	16,755.63	28	598.41	/	/	/
Total	3,492,478.97	31	/	/	/	/

Since the one-factor ANOVA method can tell whether there is a significant difference between groups, but does not indicate between which groups these differences exist, a post hoc test is needed to identify pairs of groups between which there are significant differences. The least significant difference (LSD) test can identify which groups differ significantly in terms of their means.

The critical value named "Least Significant Difference" ($LSD_{Crit\ one\text{-}tail}$ = 23.17 and $LSD_{Crit\ two\text{-}tail}$ = 28.92) of the test was calculated based on the standard error of the difference between the group means and the critical coefficient derived from the t distribution ($t_{Crit\ one\text{-}tail}$ = 1.89 and $t_{Crit\ two\text{-}tail}$ = 2.36), corresponding to a level of significance p = 0.05 and the number n = 8 degrees of freedom. Through comparison of each pair of group means, it was found that the difference between the group means of protein content (777.44) and the other group means of vitamin C (11.86), carbohydrates (4.63), phenolic content (33.69) is greater than the LSD values, and then, we can consider that there is a significant difference between those pairs of two groups, protein versus vitamin C, carbohydrates and phenolic content.

4. Conclusions

The experimental findings of this study demonstrated a significant correlation between the particle size of blackthorn berry powder and its total phenolic content, protein content, carbohydrates, antioxidant activity, moisture content, water activity, water absorption capacity, and water solubility index by combining the milling and sieving processes. Sieving proved to be a very important stage in order to obtain fractions with characteristics that recommend their use as valuable ingredients for functional food products; however, additional studies are necessary to deepen our understanding of the biotechnical processes and to further optimize the grinding processing parameters to obtain blackthorn berry powders with suitable characteristics that recommend their use as valuable ingredients with functionalized properties, as well as with appropriate biochemical and microbiological stability when introduced into food products. The results of this study are expected to benefit the milling industry, as well as the other food industry operators, to valorize in a sustainable way the autochthonous insufficiently explored wild fruits, with the final scope to further validate and up-scale these innovative solutions.

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