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DIPLOMOVÁ PRÁCE

Vliv způsobu přípravy konopné mouky na její kvalitu

Effect of hemp meal preparation on its quality

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ABSTRAKT

Semena konopí jsou považovaná za jeden z nejlepších zdrojů plnohodnotných živin rostlinného původu pro lidskou výživu. Za studená lisovaný konopný olej je dnes nejběžnějším produktem v konopné výrobě. Za účelem splnění moderních požadavků udržitelného zpracování je reutilizace konopných zbytku velmi vyžadovaná. Zejména proto, že zbylé konopné výlisky jsou bohaté na antioxidanty, vlákninu, polyfenoly a považována za jeden z nejcennějších zdrojů bílkovin. Tato studie byla zaměřena na stanovení složení konopné mouky zbylé po odtučnění dvěma různými způsoby extrakce. Byl prostudován účinek odtučňování lisování za studena a sekundární extrakce petroletherem na složení a vlastnosti konopné mouky. Vzorky byly rozděleny na dvě frakce: <315 μm a 700-1000 μm. Tato studie prokázala příznivý účinek použití nepolárního rozpouštědla jako petrolether pro odtučnění konopné mouky a zvýšení úrovně funkčních parametrů jako rozpustnost, WHC a FAC. Diferenciace konopných mouk podle frakcí ukázala dvojnásobný účinek na výtěžek surového proteinu v případě použití <315 um velikostní frakce. A v případě použití velikostní frakce 700-1000 um prokázalo pozitivní účinek na obsah zbytků, polyfenolu a změřenou antioxidační aktivitu.

Klíčová slova: *za studena lisované konopí, konopná mouka, extrakce konopných semen, konopné bílkoviny.*

ABSTRACT

The hemp seeds are indicated as a source of valuable nutrients, so it is recognized as one of nature's perfect balanced foods. Cold-pressed hemp seed oil is the most common commodity from hemp manufactory nowadays. In order to fulfill the requirements of sustainable food processing, the reutilization of food waste is a common issue especially due to the fact that food waste is rich in proteins, dietary fibers, antioxidants, colorants and other nutrients. In that sense, oilseed meals that remain after oil cold-pressing have been recognized as one of the most valuable sources of proteins. This study was focused on the composition determination of the hemp meals reminded after defatting process by different way of extraction. It studied properties of compounds and effect of defatting by cold-pressing process and secondary extraction by petroleum ether on two fraction size: <315μm and 700-1000 μm. This study proved the beneficial effect of using non-polar solvent as petroleum ether for defatting of hemp meal and raising the level of functional parameters as solubility, WHC and FAC. Differentiation of hemp meals by fractions showed significant effect on raising for two times crude protein yield in case of using <315μm fraction size. Otherwise, in case of using 700-1000 μm fraction size, it showed a positive effect on residues and polyphenol content and measured antioxidant activity.

Key words: *cold-pressing hemp, hemp meal, hemp seed extraction, hemp protein.*

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1. Introduction

Hemp seeds are traditionally used as a source of human nutrition or feed for poultry, fish and other animals. It is due to its fast, easy cultivation, high quality and amount of yields. Hemp meal has its rich composition of nutrients and special flavor.

Recent scientific research indicated that hemp seed is a source of valuable nutrients, so it is recognized as one of nature's perfect balanced foods. In order to fulfill the requirements of sustainable food processing, the reutilization of food waste is a common issue especially due to the fact that food waste is rich in proteins, dietary fibers, antioxidants, colorants and other nutrients. In that sense, oilseed meals that remain after oil cold-pressing have been recognized as one of the most valuable sources of proteins.

However, the hemp was very common and necessary plant in daily life of humans, nowadays it is considered as an alternative crop and modern research is required.

2. Aim of study

The aim of this thesis was to evaluate the impact of hemp meal preparation reminded after oil cold-pressing on its quality. Compared samples of hemp meal were divided by different factors as variety, size fractionation and defatting by only cold-pressing and secondary followed using non-polar organic solvent (petroleum ether) extraction. All of these samples were tested for the content of residual fat, dry matter, crude protein and electrophoretic profile of proteins. Furthermore, samples of meals were evaluated for selected functional properties as water solubility, water and fat binding, polyphenol content and antioxidant activity.

3. Literature review

3.1. Tradition of cultivation

Hemp is one of the oldest plants cultivated by humans. With rice, wheat and soybeans it has very significant place in human history and development. *Cannabis sativa* and other species of *Cannabis* genus were traditionally utilized in many different ways and industries. Straw was used for constructing, paper, textile, production of ropes and cords or as bedding material. Flowers and leaves were used in medicine, religious acts, for making cosmetics or in cuisine as spices and for preparing hot beverages. Seeds were a part of nutrition for humans and their domestic animals due to its quality source of proteins and oil. With developing of technologies, it opened new applications for hemp. Nowadays it could be used as beneficial resource for bioenergy industry, modern constructing, plastic and car industry, for producing paint and varnish. The global market for hemp has been estimated to consist of more than 25,000 products (RUMAN, 2008). Whole scale of hemp seed processing and products showed on the Figure 1 below.

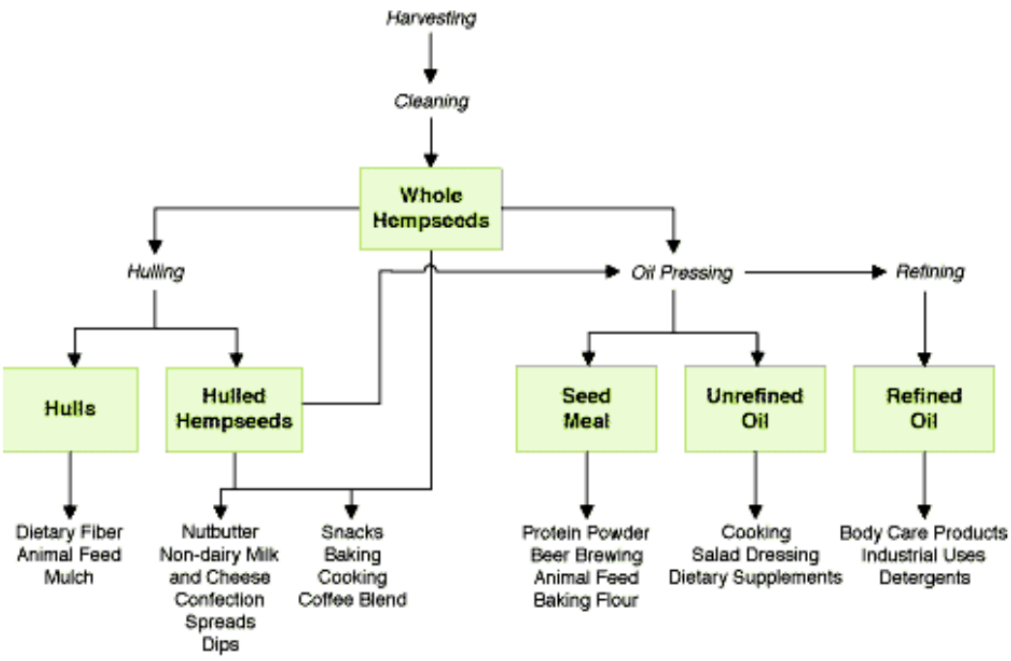


Figure 1. Processing and products of hemp seed (SELTHOFNER, 2010)

3.2. Botany of plant

Figure 2. Cannabis sativa (FUCHS, 1542).

Hemp is native to Central Asia (RUMAN, 2014). It is originally dioecious, mainly wind-pollinated plant and produce either male (pollen) flowers or female (seed) flowers. Male plants are taller and thinner, with lighter leaves and a gray-green top. They start to flower 3-15 days earlier than female plants. Female plants are lower, thicker, they have more leaves, which are darker. In normal growth, there is about 53% of male and 47% of female plants (STRAŠIL, 2011). The plant has taproot system, which extending to a depth of 30-40 cm. It has about 2 m (or even 4 m) tall steam, which is soft in the beginning of growth, but later wooding from below. It contains 13.5-19.5% of fiber. The leaves are alternate, palmate three to thirteen, and short peduncles. The fruit is an achene, with HTS from 8 to 26 g (20 g in average) (STRAŠIL, 2011).

Due to tall strong steam, harvesting of hemp for fiber needs special machinery. Although, on market are available varieties of hemp, which have lower habit with thinner softer stem. These varieties are selected for seed production. In general, in Czech Republic, cultivation of hemp is permitted by law number 167/1998 Sb. with reporting obligation. In the year 2018 in Czech Republic were 50 numbers of growers and yield were harvested from 437.03 ha (Celní správa ČR). In condition of Czech Republic yields of seeds are varying from 0.5 to 1.4 t/ha, with standard about 0.8 t/ha (STRAŠIL, 2011).

Hemp seed is achene; it has a rounded shape. It is enclosed in gray-green husk with fine marbling. It is solid but crispy. A layer of irregular columnar cells in the endocarp is responsible for the crustaceous character of the shell (REED, 1914). Under it there is another seed coat, its dark green color, containing chlorophyll. Inside is a soft, oily pulp of cream color (NARAIN et al., 2019).

Figure 3. Hemp seed (DEFERNE, PATE, 1996).

3.3. Seed composition and quality

Oil content in seed is about 25-35%, with approximate content of proteins 20-25%, 20-30% carbohydrates, 10-15% fibers, along with a variety of trace minerals (P, K, Mg, Ca, Fe, Na, Mn, Zn) and vitamins (A, B1, B2, B6, C and E). It also contains lecithin, phytin and canabidiolic acid with a strong antibacterial effect. (CALLAWAY, 2004).

Hemp oil is a rich source of essential fatty acids: 50–70% linoleic acid (LA; C18:2, an omega-6 fatty acid) and 15–25% linolenic acid (ALA; C18:3, an omega-3 fatty acid) (HAZEKAMP et al., 2010), which provides a favorable ratio of 2-3:1, required for proper human nutrition, in addition to a significant contribution of gamma-linolenic acid of potential therapeutic efficacy (DEFERNE, PATE, 1996). Furthermore, hemp oil provides significant amounts of some higher fatty acids such as GLA (C18:3; omega-6) and stearidonic acid (SDA; C18:4; omega-3).273 Oleic acid (C18:1) and saturated fatty acids (mainly palmitic, stearic acids) both make up about another 10% of the oil (HAZEKAMP et al., 2010). Typically, cultivars, which bred mainly for seed (for example Finola) contain the greatest amount of seed oil and crude protein, while dual-purpose cultivars show lower content of both compounds (VONAPARTIS, 2015).

Figure 4. Typical nutritional content of hempseed, variety Finola (CALLAWAY, 2004).

	Whole seed (%)	**Seed meal (%)**
Oil	35.5	11.1
Protein	24.8	33.5
Carbohydrates	27.6	42.6
Moisture	6.5	5.6
Ash	5.6	7.2
Energy (kJ / 100g)	2 200	1 700
Total dietary fiber	27.6	42.6
Digestible fiber	5.4	16.4
Non-digestible fiber	22.2	26.2

Hemp seed is a rich source of proteins. Two-thirds of proteins composed by globular edestin, which is responsible for functional immunity and proper growth of children. One third of the protein content is made up of albumin, which promotes proper kidney and liver function (HOUSE, 2010).

Edestin accounts for approximately 60% to 80% of the total protein content. The globular edestin is located inside the aleurone grains as large crystalloidal substructures. Using crystallographic techniques, edestin is shown to have a structure similar to that of the hexamer of soy glycinin; it is composed of six identical subunits, each consisting of an acidic (AS) and a basic subunit (BS) linked by one disulfide bond. The molecular weight (MW) of edestin is estimated to be approximately 300 kDa. The AS is approximately 34.0 kDa and relatively homogeneous, while BS consists mainly of two subunits of about 20.0 and 18.0 kDa (WANG, XIONG, 2019). Hemp seed legumin consist of mainly 11S and 7S protein types, which can be separated using the pH shifting technique. Edestin protein was isolated and characterized from Korean Cheungsam variety by KIM, LEE (2011). The first seven and six amino acid residues of the acid subunit had a sequence of Ile-SerArg-Ser-Ala-Val-Tyr in the N-terminus, wtwo constituents of basic subunit showed an identical N-terminus of Gly-Leu-Glu-Glu-Thr-Phe.

The albumin fraction constitutes about 25% of hempseed storage protein. The peptides with MW of less than 18.4 kDa correspond to the albumin components. It contains few disulfide-bonded proteins and hence a less compact structure with greater flexibility than the globulin fraction. Further analyzing illustrated greater exposures of tyrosine residues when compared with globulin. It contributes to the high solubility and foaming capacity of albumin comparison to the more compact or aggregated edestin (PIHLANTO et al., 2020).

Numerous factors are known to influence the nutritional quality of plant proteins, which can be measured by their amino acid composition and the digestibility of the protein. The amino acid composition may be affected by variety/genetics, agronomic conditions and postharvest processing that alters the ratio of seed components, such as shelling. The digestibility of proteins may be affected by protein structure, the presence of antinutritional compounds and high- temperature processing (FAO, 1990).

A direct comparison of protein amino acid profiles from egg white, hempseed and soy bean shows that hempseed protein is comparable to these other high quality proteins. Hempseed protein has good amounts of the sulfur-containing amino acids methionine and cystine, in addition to very high levels of arginine and glutamic acid (CALLAWAY, 2004).

Figure 5. Content of amino acids in hemp seed (mg/g) (CALLAWAY, 2004).

Amino acid	Content	Amino acid	Content
Alanine	9	Lysine	4.3
Arginine	18.8	Methionine	2
Aspartic acid	2.78	Phenylalanine	3.5
Cystine	19.8	Proline	7.3
Glutamic acid	34.8	Serine	8.6
Glycine	9.7	Threonine	3.7
Histidine	2.5	Tryptophan	0.6
Isoleucine	1.5	Tyrosine	5.8
Leucine	7.1	Valine	3

Carbohydrates are mainly found in hemp seed in the form of fiber. Carbohydrate content is about 52.67%, of which simple sugars make up only about 2.47% (CONRAD, 2001). Fiber significantly increases the nutritional value of hemp seeds. Whole cannabis seeds contain 320 g/kg of neutral detergent fiber, which affects protein digestibility (HOUSE, 2010).

Plant sterols, phytosterols, are natural compounds found in foods of plant origin. In human body, they compete with cholesterol for position in mixed micelles, necessary for its absorption in the small intestine. As a result, the absorption of cholesterol from food and bile acids decreases by ~ 50%, its concentration in the blood decreases by ~ 10%, despite an increase in synthesis in the liver (BITZUR, 2013). Phytosterols accumulated in hemp seeds represented by β - sitosterol. Hemp seeds contain 100-150 mg/kg (MIOVSKY, 2008).

As antinutritional substances, hemp seeds contain phytic acid, that reduces the intake of minerals. Its content is similar to flax, mustard, rapeseed or soybean seeds. And tannins, which are phenolic compounds that interact with proteins. They have a negative effect on the digestibility of nitrogenous substances (MATTHAUS, 1997).

3.4. Hemp seed processing

There are thousands of hemp food products available on market today. Commercially offered whole seeds or shelled seeds, hemp milk, energy bars, roasted seed snacks, hemp flowers with leaves and seeds for making infusions.

There are two ways for hulling process of hemp seeds. The method of utilizing hitting system or hot steam system: on the grounds of increasing temperature, seeds start to explode and kernels disengage from shall. Nevertheless, high temperature could decrease nutritional quality of seeds. Another method is utilizing mechanical power: compressing hemp seeds between two panels or two rotation discs. Due to right pressing on surface of seeds, it crunches. According to the variety of hemp (size of seeds, thickness, strength of shall) and water content of seeds, a hulling equipment needs to be adjust for the level of pressure and the frequency rate. After kernels and shells can be separated on the grounds of differences in weight. Although, by this process the small pieces of kernels are excluded as well, in general it increases the kernels percentage content in final production. Separated shells on its own can be used as dietary fiber, as feed for animals or for mulching.

Hemp oil can be extracted from whole seeds or from hulled seeds. Hemp seeds are rich on high quality oil, which can be used in food industry, medicine and cosmetics and as commodity for technical industries. There are different extraction methods.

Cold-pressed oils refer to oils that are extracted from plant seed with a screw press or hydraulic press. Cold-pressed oils are considered as healthy oils that are important to human nutrition due to their favorable polyunsaturated fatty acid content, notably

a-linolenic acid and linoleic acid. Cold-pressed seed oils contain other bioactive compounds that exert health benefits. For example, tocopherol contents in the oils function as powerful antioxidants, vitamin E, anticancer and anti-cardio-vascular disease agents. In addition, polyphenols such as phenolics and flavonoids exhibit therapeutic properties such as anti-microbial, anti-inflammatory, anti-thrombotic, anti-allergenic, anti-artherogenic, antioxidant.

Due to the high content of polyunsaturated fatty acids, hemp seed oil is very susceptible to oxidative degradation. The oxidation stability of an oil is determined by its fatty acid composition in addition to several minor components that have antioxidant properties (DA PORTO et al., 2012). One of the most important antioxidants in seeds are tocopherols and they are presented in amount of 800 mg/kg of hemp cold-pressed oil (OOMAH et al., 2002).

Cold- pressing is used to extract oil from plant seed instead of conventional solvent extraction method because cold-pressing does not require the use of organic solvent or heat. Hence, cold-pressing is able to retain bioactive compounds such as essential fatty acids, phenolics, flavonoids and tocopherol in the oils (TEH, BIRCH, 2013).

The rate efficiency of the cold-pressing of the hemp seed is about 60–80% obtained oil, and influenced by the characteristics of the seeds (MATTHAUS, BRUHL, 2008). Compared to the processing of other oleaginous seeds by cold-pressing, the productivity of the press for hemp seeds processing is lower as for sunflower seed or rape seed processing (MORAR et al., 2010). From 10% and up to 35% of the available oil can remain in the seed cake (DEFERNE, PATE, 1996; TEH et al., 2013).

Food industry is always looking for processes that can maximize efficiency, increase the quality of product and also minimize the environmental impact, decrease toxic residues.

Supercritical carbon dioxide extraction is alternative green technology method, which has many advantages over traditional methods, especially in preservation of thermosensitive compounds using low temperatures. Supercritical fluids are characterized by high mass transfer rates, liquid like density, and variable selectivity (temperature and pressure) for chosen compounds. Temperature, pressure and the size of hemp particles are important factors that influence the hemp seed oil yield in supercritical carbon dioxide extraction (DA PORTO et al., 2012). This method is still relatively new in the extraction of edible oils mainly due to very high investment costs of its equipment (ALADIC et al., 2015).

Soxhlet extraction method can be utilized for hemp seed as well. This method efficiently recycles a small amount of solvent to dissolve a large amount of material. As solvents, can be used n-hexane and methanol (DA PORTO et al., 2012, OOMAH et al., 2002). Due to this extraction method, a higher oil yield can be obtained without affecting the fatty acids composition. The extraction yield increases with decreasing particle size because grinding process not only increases the interfacial area but also releases oil from the broken cells (DA PORTO et al., 2012). The optimum extraction conditions providing the highest hempseed oil yield are: extraction temperature 70°C, solvent : seed ratio 10:1 and extraction time 10 min, under which the hempseed oil yield is 29.56 g/100 g which agreed with the yields obtained experimentally for the same conditions. The hempseed oil is rich in unsaturated fatty acids, and its physicochemical properties stay in the range previously reported values by other researchers. (KOSTIC et al., 2013).

Oil extraction remains seed cake, that can be milled into hemp flour and be offered commercially as a source of vegetable protein and dietary fiber in the form of hemp protein powder, hemp flour, and in shake drinks. The utilization of this valuable source of protein through extraction from oilseed cakes can contribute to better use of resources for economic growth and to meet food demand worldwide for an increasing human population. Hemp meal contains different percentage of residual oil (depending on the method of extraction) and high amount of fiber and phytate (MALOMO, ALUKO, 2015). The protein contents in hemp seed cakes were reported to be 33.6 % (TEH et al., 2013).

The hemp meal is used as food additives in breads, pastas, yoghurts, cookies, meat cutlets, pork loaves and other products (ZAJAC et al., 2019). Breads supplemented with hemp flour has higher nutritional value providing elevated intake of important nutrients such as proteins and macro- and microelements, especially iron, while reducing the metabolize energy from carbohydrates (POJIC et al., 2015). The high fiber and phytate contents of hemp seed cakes been reported to cause protein functionality reduction, which limits their use as new food ingredients (MALOMO, ALUKO, 2015). Hemp protein isolates show a high degree of digestibility, including the absence of antinutritional factors and the presence of bioactive compounds, which relatively unlikely cause an allergic reaction (MAMONE et al., 2019).

3.5. Hemp protein isolate

Current hemp protein isolates are generally prepared by alkaline extraction, which is then followed by isoelectric precipitation and is the most commonly used traditional procedure for plant protein isolate production (MALOMO, ALUKO, 2015). Protein composition and functionality are influenced by the isolation method and purification conditions. The harsh conditions used in the traditional procedure have negative effects on protein functionalities, especially protein solubility and foaming properties (MALOMO, ALUKO, 2015). Poor solubility was reported to be lower in comparison to soy protein. Isoelectric point isolated vegetable proteins have limited food applications due to their off-flavour and color, associated with the co-extracted non-proteinaceous material (e.g. phenolic compounds) (HADNACEV et al., 2018).

4. Materials and Methods

4.1. Materials

Seeds of *Cannabis sativa* were obtained in two different varieties, USO-31 and Finola. Both of varieties were conditioned and cold-pressed by Komet CA59G (IBG Monforts Oekotec GmbH & Co.KG, Germany) in University of Chemistry and Technology, Prague. After pressed cakes were ground by laboratory knife mill Grindomix GM200 (Retsch) and by sieving process in AS200 (Retsch) were obtained two fraction size <315μm and 700-1000 μm.

After half of the samples went through the extraction by non-polar organic solvent. Defatting of hemp meals by organic solvent was performed using a petroleum ether extraction at a 1:10 meal to ether ratio. Process was done for 3 times for 15 min shaking in the ultrasound bath. After followed by air-drying in a fume hood at room temperature for 7 days for volatilization of solvent residues.

After all, the fractions of 700-1000 μm were ground again on a planetary ball mill before the analysis began. It was adjusted to make it not as coarse, but the same fineness as the <315μm fraction in the analyzes.

Thereby, were obtained 8 different samples of hemp meal for following research. Samples are divided by variety, fractioning and extraction process (CP for cold-pressing and CP+PE for cold-pressing and followed petroleum ether extraction) as shown on the Figure 6 below. On the Figure 7 and 8 are shown photos of each sample of hemp meal used in this study.

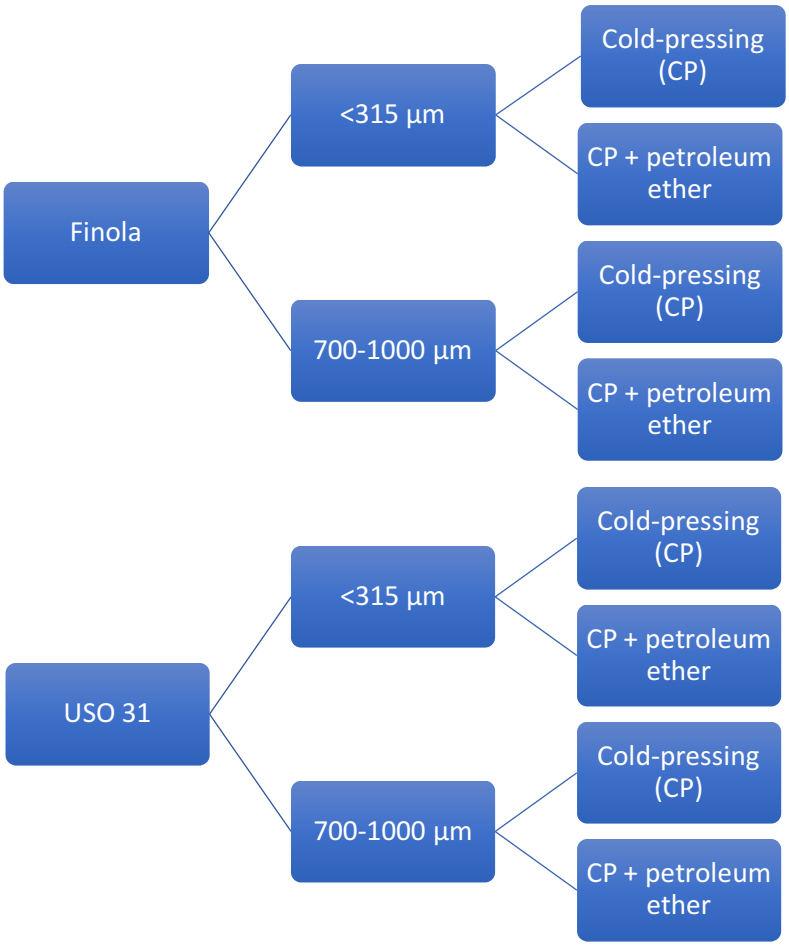
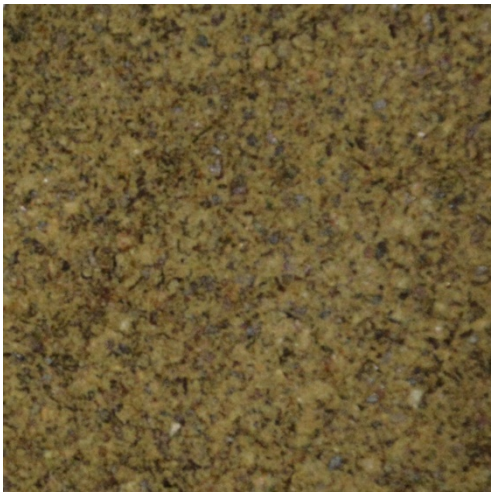


Figure 6. Scheme of hemp meal samples used for the research

Finola, <315 µm, CP

Finola, <315 µm, CP+PE

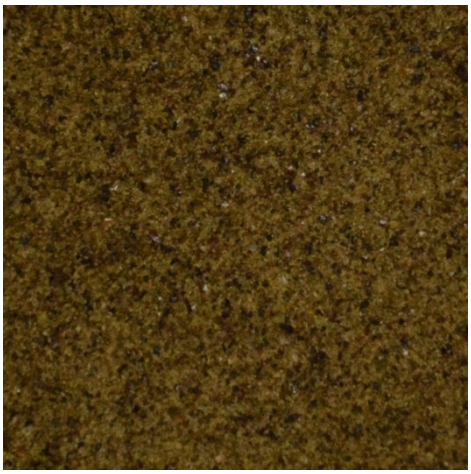


Finola, 700-1000 µm, CP

Finola, 700-1000 µm, CP+PE

Figure 7. Photo of hemp meal samples, variety Finola (Author)

(Variety, fraction size, defatting by cold-pressing (CP) and secondary extraction by petroleum ether (CP+PE))



USO 31, <315 µm, CP

USO 31, <315 µm, CP+PE

USO 31, 700-1000 µm, CP

USO 31, 700-1000 µm, CP+PE

Figure 8. Photo of hemp meal samples, variety USO 31 (Author)

(Variety, fraction size, defatting by cold-pressing (CP) and secondary extraction by petroleum ether (CP+PE))

4.2. Methods

4.2.1. Determination of moisture content

The moisture content of ground meal from compacts was determined gravimetrically. It also can be converted to the dry matter content. The meal was weighed in triplicate into special 1 g filter bags and dried for 3 h at 105 ° C in a UN 75 oven (Memmert, Germany).

4.2.2. Water solubility, water holding capacity and fat absorption capacity.

The parameter of water holding capacity (WHC) for hemp meal samples were determined according to a slightly modified version of the method described by BEUCHAT (1977). 300 mg of meal was weighed into weighed plastic tubes with a volume of 5 ml of deion water and the mixture was shaken thoroughly. After 30 minutes, the mixture was centrifuged (4500 rpm, 20 ° C, 15 min). The supernatant was thoroughly discarded, and the tubes were weighed. WHC represented by the percentage difference of the obtained sample to the weight of the original batch. After the samples went through lyophilization process and were weighted again. Water solubility represented by the percentage difference of dry pellets after lyophilization to original batch. Fat absorption capacity (FAC) was determined in the same way, but rapeseed oil was used instead of water.

4.2.3. Crude protein content

The determination of nitrogen and nitrogenous substances was performed using a modified Dumas method on a Rapid N Cube apparatus (Elementar, Germany) (Figure 9). The advantage of the method in combination with the used instrumentation is easy use and fully automated process. Compared to the Kjeldahl method, it is significantly fast. Homogenized hemp compacts were weighed in a weight of 25 mg (± 5%) into prepared tin capsules and placed in an autosampler, which automatically dispenses capsules for individual measurements (Figures 10, 11). The samples were burned at high temperatures

(above 900° C) in an oxygen-rich atmosphere. Carbon dioxide, water and nitrogen oxides are released. In the gas chromatography mode, the gas release passes through several columns where carbon dioxide and water are sorbed. Nitrogen oxides are gradually reduced to nitrogen, which is detected by a thermal conductivity detector. From the determined nitrogen content, the content of nitrogenous substances in the given sample was also determined using the standardly used coefficient of 6.25. Prior to the analysis of the above samples, the so-called "N factor (daily factor)" was determined by measuring the standard in the form of aspartic acid in five replicates.

Figure 9. Rapid N Cube (www.elementar.de)

Figure 10: Preparation of samples (Author)

Figure 11: Prepared molds for samples (www.elementar.co.uk)

4.2.4. Determination of fat content

The residual fat content was extracted with petroleum ether on an XT10 (ANKOM, USA). 1 g of sample was weighed into special XT4 filter bags, which was first weighed, then sealed with a pulse welder. The samples thus prepared were dried at 105 ° C for 3 hours. After cooling in a desiccator, the bags were weighed and placed in an extractor. Extraction was followed by drying at 105 ° C for 30 minutes, and after cooling in a desiccator, they were weighed again.

4.2.5. Determination of ash content

The ash is determined by weight as the remainder of the mass after incineration of the sample at 550 ° C to constant weight under the prescribed conditions. 1 g of the sample was weighed into pre-weighed porcelain crucibles to the nearest 0.001 g - the sample in the crucible was then fired in a muffle furnace for 5 hours at 550 ° C - allowed to cool in a desiccator and weighed with accuracy about 0.001 g.

4.2.6. Polyphenol content

The content of total polyphenols was determined spectrophotometrically using a modified method using Folin-Cicalteau reagents (LACHMAN et al., 2006). It also called the Gallic Acid Equivalence method (GAE). Method is used to determine the total polyphenol content using a solution containing oxides of tungsten and molybdenum. The reagent is reduced with polyphenols to give yellow, green to blue products, depending on the polyphenol content (Figure 12). The Folin-Cicalteau reagent is non-specific and reacts with all phenolic groups contained in the samples.

Prior to the measurement, samples of hemp meal were weighed into microcentrifuge tubes at 100 ± 1% mg and extracted for 24 h in 1 ml of 80% aqueous methanol with constant shaking. Extraction was followed by centrifugation at 10.000 rpm for 15 min at 20 ° C. Subsequently, the supernatant with the extract was collected.

During the measurement, 20 µl of samples were diluted with 1980 µl of distilled water and mixed. To the diluted sample was added 100 µl of Folin-Cicalteau solution and 300 µl of sodium carbonate. The resulting reaction was mixed and incubated at room temperature for 2 h. After incubation, the sample was measured spectrophotometrically at 765 nm against a blank. Absorbance was measured at 765 nm. Gallic acid was used as a standard; a calibration curve was generated and an equation for calculating the polyphenol content was obtained. An 80% aqueous methanol solution was used as a blank. The result was expressed as gallic acid equivalent (GAE mg/g of dry matter).

Figure 12. Polyphenol content reaction, changing the color from yellow to blue due phenolic groups contained in the samples (Author)

4.2.7. Antioxidant activity

The determination of antioxidant activity was performed according to the method of ŠULC et al. (2007). The activity is measured by mg of ascorbic acid equivalent (AAE) on 1g of original matter. The principle of the method using ABTS (2,2-azinobis (3-ethyl-2,3-dihydrobenzothiazole-6-sulfonate) also called TEAC (Trolox equivalent antioxidant capacity) is in the inactivation of the radical cation ABTS ++ by means of antioxidants, which is formed by the oxidation of the original ABTS, in this work by means of manganese dioxide. Due to chemical quenching (by changing the color reaction) there is a decrease in absorbance at wavelength 734, which is measurable spectrophotometrically. 54.9 mg of ABTS was dissolved in 5 mM phosphate buffer (pH 7.0) and activated to the radical cation with 1 g of MnO 2 with stirring for 30 minutes. The solution was then centrifuged for 5 minutes at 7000 rpm. It was then filtered through a syringe filter (0.25 μm) and diluted with phosphate buffer to an absorbance of 0.500 ± 0.01. To measure the antioxidant activity of hemp meals, samples prepared by extraction were originally used to measure the content of polyphenols, the samples were diluted in a ratio of 1: 9 (sample: water). Solutions of protein fractions at a concentration of 1 mg / ml were added in an amount of 100 μl to 900 μl of treated radical. Absorbance was measured at 734 nm.

4.2.8. Electrophoresis

The spectrum of contained proteins was determined by electrophoretic method in denaturation system according to method of LAEMMLI (1970). Therefore, protein extraction from hemp flour was performed first. 40 mg of hemp meal was weighed into microcentrifuge tubes. After weighing the samples, extraction was performed with SDS-extraction buffer containing 0.0625 M Tris-HCl + 2% (w / v) sodium dodecyl sulfate. 400 μl of extraction buffer was transferred to microcentrifuge tubes, which was mixed with weighed hemp meal. The extraction was performed on ice for 4 hours. At the end of the extraction time, the samples were centrifuged with a ROTINA 420 R centrifuge (Hettich, Germany) for 10 minutes at 12,000 rpm and 4 ° C. Subsequently, the supernatant was collected by pipette and frozen for subsequent SDS-PAGE analysis.

SDS-PAGE is an electrophoretic method for the separation and identification of proteins. It is a vertical electrophoresis on a polyacrylamide gel. An Hoefer SE 600 electrophoretic system (Hoefer, USA) was used for SDS-PAGE. A 12% separation gel and a 3.75% focusing gel were used for separation. Samples were loaded onto the gel in an amount of 10 µl, formed by mixing 40 µl of the extracted sample and 10 µl of loading buffer 0.0625 M Tris-HCl, pH 6.8; 25% (v / v) glycerol; 2% (w / v) SDS; 0.01% (w / v) bromophenol blue. Just before mixing the sample with the loading buffer, 170 2l of 2-mercaptoethanol was added to 500 µl of loading buffer. The sample containing loading buffer was heated in a thermostat at 99 ° C for 3 min, then cooled and applied to a gel on a gel in an amount of 10 µl. Blue Protein ladder 3.5-245 kDa (Central European Biosystems, Czech Republic) was used as a mass marker, which was applied undiluted to the gel in an amount of 5 µl.

The actual electrophoresis was performed under conditions of 150 V for the first 30 minutes and then at 200 V for 4-6 hours. 0.025 M Tris - 0.192 M glycine buffer containing 0.1% (w/v) SDS was used as the lower and upper baths. After separation, the gels were rinsed in water and stained overnight with a solution composed of 0.1% (w/v) Coomassie Brilliant Blue R-250, 50% (v / v) methanol, 10% (v/v) 99.8 % acetic acid and 40% (v/v) distilled water. For subsequent destaining of the gels was used the solution composed of 25% (v/v) ethanol, 10% (v/v) 99.8% acetic acid, 65% (v/v) and distilled water. The gels were destained for approximately 6 h and then with distilled water under very low intensity horizontal agitation conditions, in Figure 13. After destaining, the gels were evaluated using a Gel Doc XR + photodocumentation device (Bio-Rad, USA).

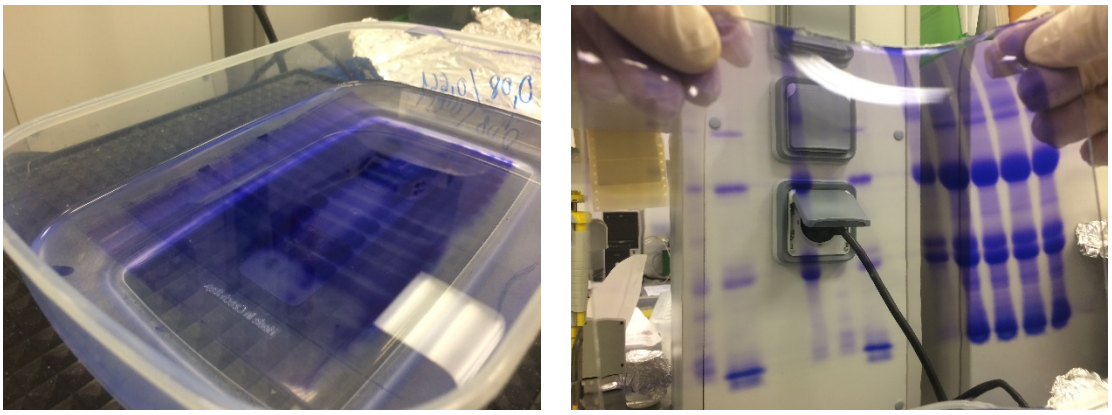


Figure 13. Polyacrylamide gel with samples of hemp proteins (Author)

4.2.9. Statistical analysis

The results were analyzed by three-ways analysis of variance with Fisher LSD test, which was performed by Statistica 12.0 (Statsoft, Tulsa, OK). The significance of differences among the mean values was indicated at the 95% confidence level.

5. Results and Discussion

5.1. Basic chemical composition of hemp meal fractions

Within frameworks of this study about hemp meals were tested two different varieties of *Cannabis sativa*: USO-31 and Finola. Obtained results of the analysis did not reveal considerable differences between varieties but in other factors as size fractioning and extracting process. Nevertheless, the difference between the varieties was proved by measuring of dry matter content in grounded meals vice versa of moisture content. The results are shown in Figure 14 below. The letter-code is used for illustration of statistically significant similarity. As can be seen, variety Finola had approximately 8% of moisture content, then USO 31 had 7% and less. It can be interpreted as different genetic composing, furthermore, it can be accounted for different field conditions during cultivation or post-harvesting storage condition.

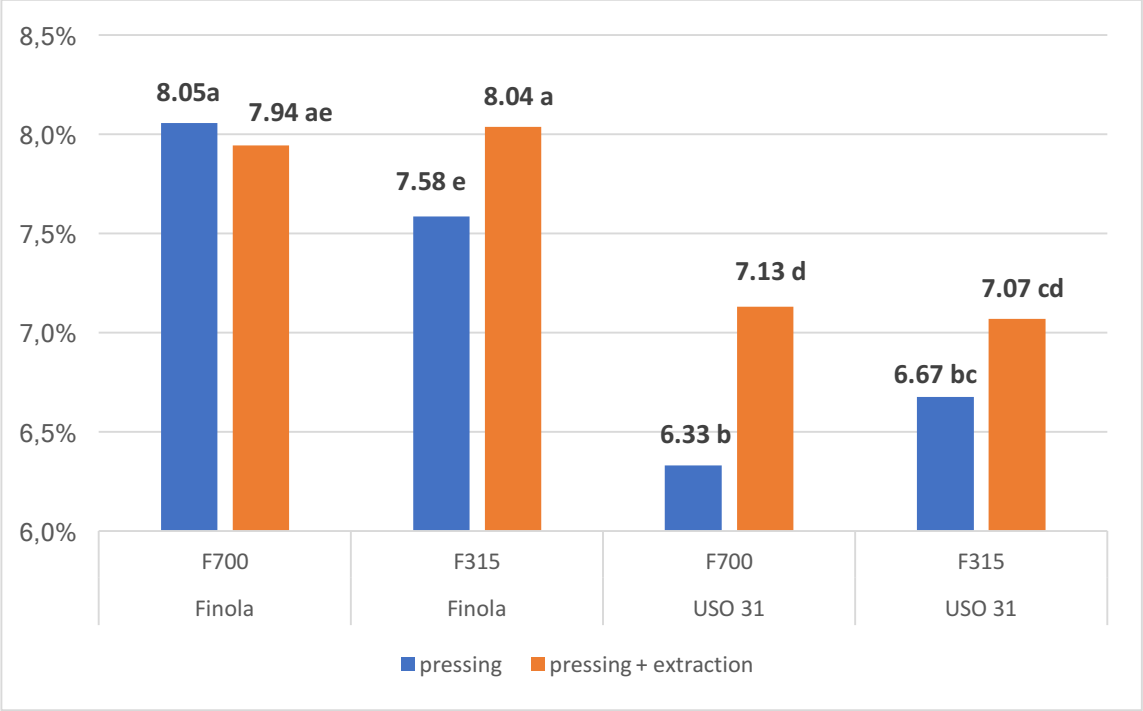


Figure 14. Moisture content of hemp meals (% of original matter)

(F700 for fraction size of 700-1000 µm and F315 for the fraction of <315µm;
Letter code indicate significant difference at significance level α = 0.05 (ANOVA, Fisher LSD test))

The fractioning of hemp meal has shown the significant impact on the crude protein content after defatting process by cold-pressing extraction (Figure 15). With regard to the content of residues in samples (Figure 17) it can be proven, that main protein content is locating in the kernel of hemp seed, so the fractioning process brought the possibility for increasing the protein yield from hemp for 50%. Due to followed defatting process by using petroleum ether extraction was recovered for approx. 5% more of crude protein from each sample (Figure 15). Furthermore, as shown by the level of crude fat content (Figure 16), the extraction by petroleum ether can increase oil yield for approx. 10% for the fraction of 700-1000 µm and 15% for the fraction of <315µm. And by letter code in Figure 16 can be noted that there was some relation between samples defatted by petroleum ether. In total, can be said that the variety Finola provides bigger amount of crude protein but the difference is not very significant for manufactory production.

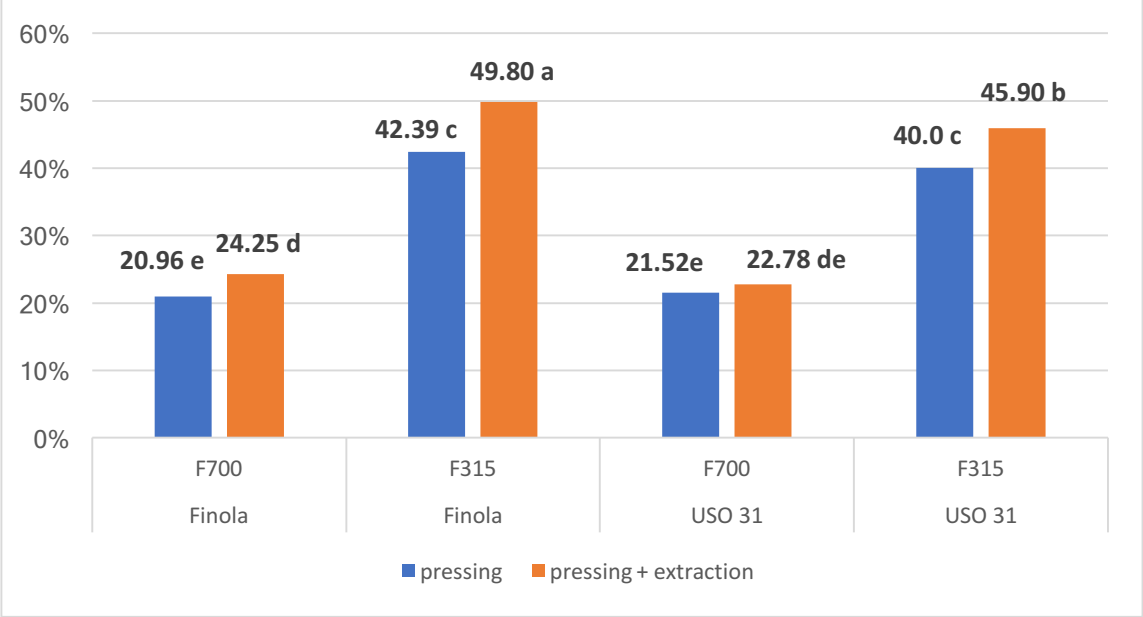


Figure 15. Crude protein content of hemp meal (Nx6,25) (% of original matter)

(F700 for fraction size of 700-1000 µm and F315 for the fraction of <315µm;
Letter code indicate significant difference at significance level α = 0.05 (ANOVA, Fisher LSD test))

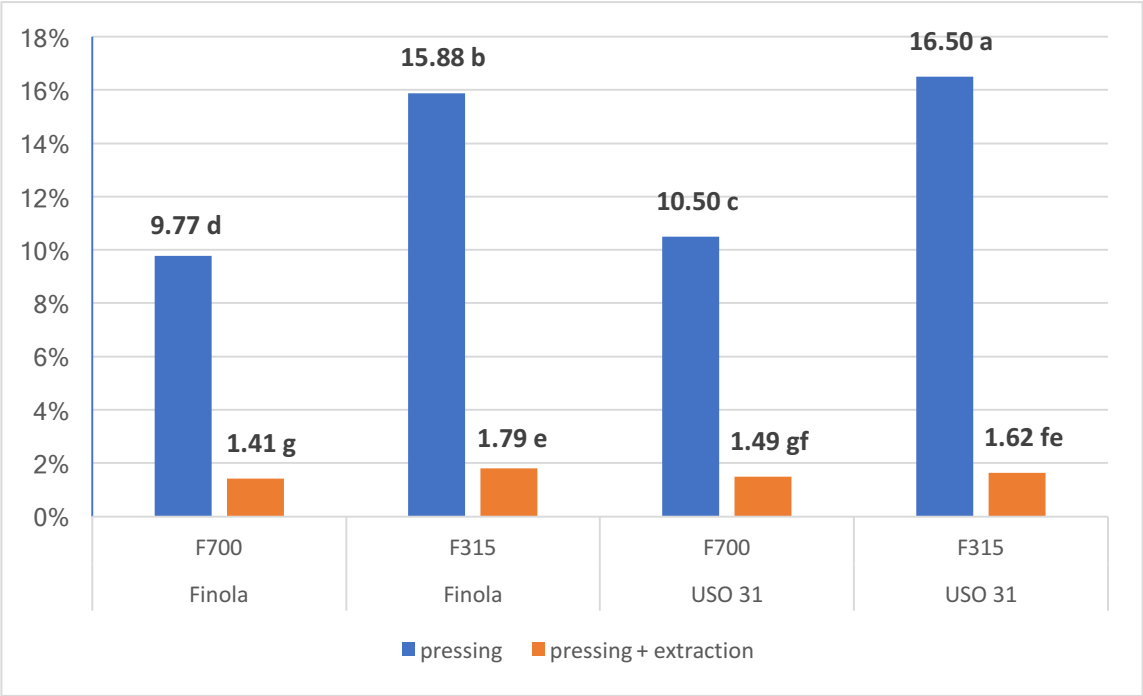


Figure 16. Crude fat content of hemp meal (% of original matter)

(F700 for fraction size of 700-1000 µm and F315 for the fraction of <315µm;
Letter code indicate significant difference at significance level α = 0.05 (ANOVA, Fisher LSD test))

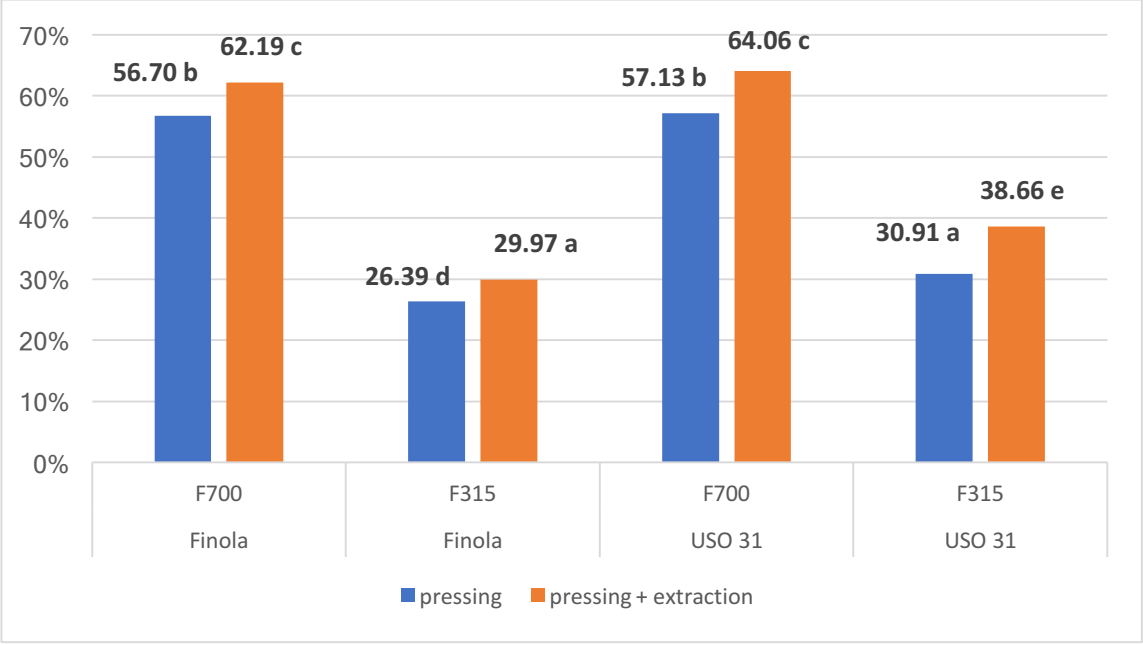


Figure 17. Residue content of hemp meals (% of original matter)

(F700 for fraction size of 700-1000 µm and F315 for the fraction of <315µm;
Letter code indicate significant difference at significance level α = 0.05 (ANOVA, Fisher LSD test))

After firing samples in a muffle furnace at 550 ° C the content of ash was counted, but no correlation between samples was provided (Figure 18). It only defined that hemp seed coats contain two times more particles of ash than kernel.

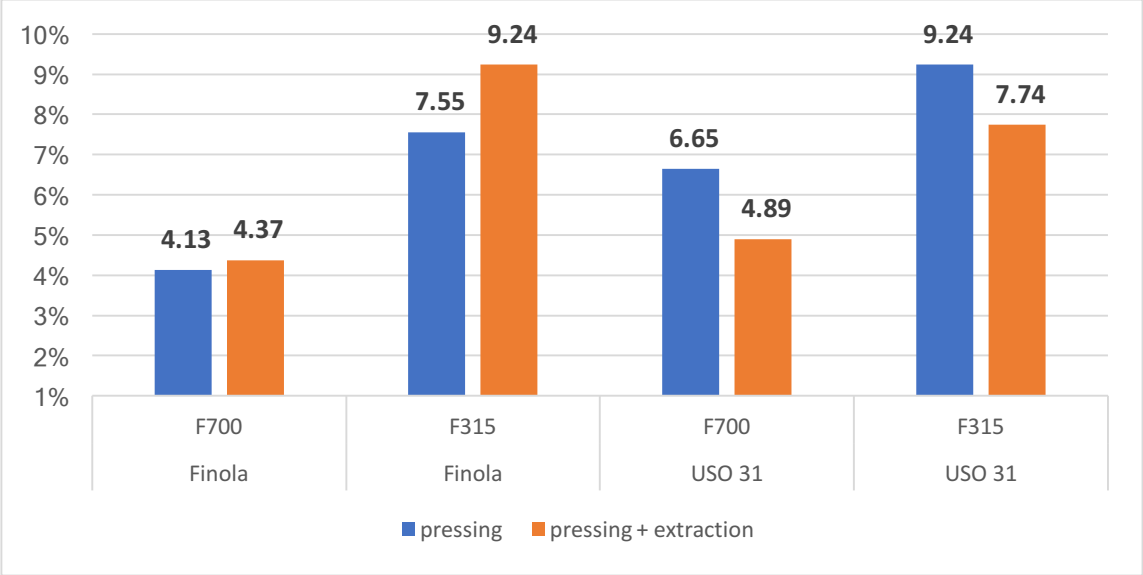


Figure 18. Ash content of hemp meals (% of original matter)

(F700 for fraction size of 700-1000 µm and F315 for the fraction of <315µm)

In study of POTIN et al. (2019) the meal had a dry matter content of 95.9% (what gives 4.1% of moisture in original matter), and protein, lipid, and ash contents of 30.0%, 8.7%, and 6.9%. The characteristics of the hemp press-cake were similar to those found by CALLAWAY (2004). This author reported protein, oil, and ash contents of 35.5%, 11.8%, and 7.6%, respectively, and 5.6% of moisture content. The results were also consistent with those of POJIC et al. (2014), who recorded values of 30.3%, 12.8%, 7.3%, and 6.7% for protein, fat, ash, and moisture contents, respectively. House et al. (2010) obtained similar lipid, ash, and moisture contents of 10.7%, 7.0%, and 4.9%, respectively.

The scanned by Gel Doc XR+ (Bio-Rad, USA) gels of SDS-PAGE electrophoresis shown on Figure 19. All the samples are represented in two repetitions and each pare is noted by variety, defatting process and fraction size; on the left side, the control scale is placed with indicated molecular weight (kDa) of proteins. From scans can be detected homogeneous acidic subunits of edestin (below 35 kDa) and two subunits of basic subunits of edestin (about 17 kDa and 15 kDa). Between them are placed disulfide bonds. Under edestin placed albumin.

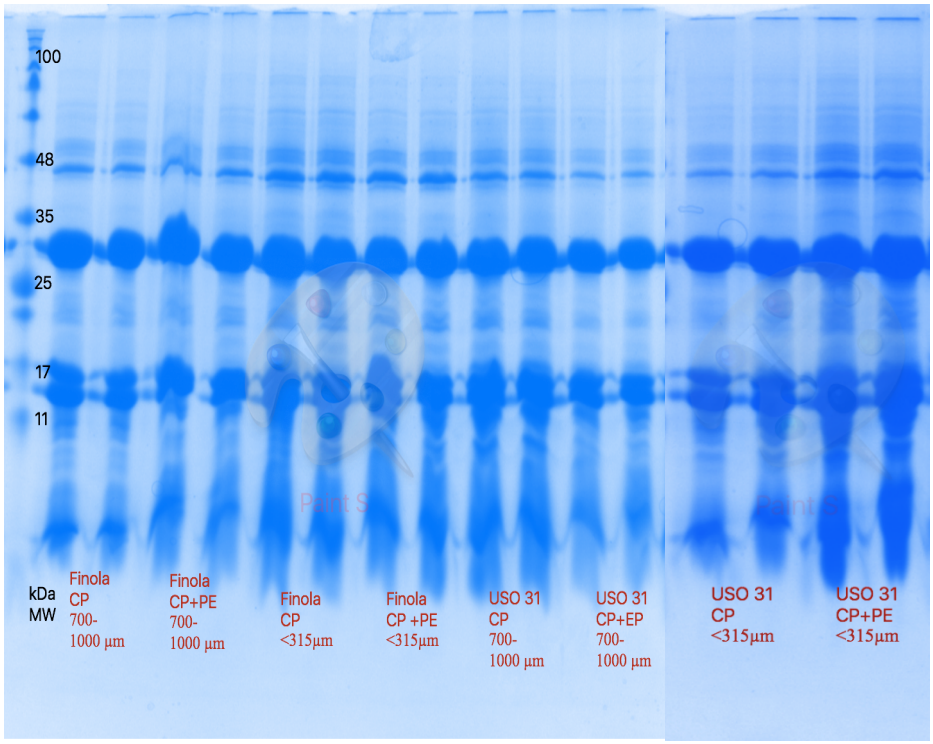


Figure 19. SDS-PAGE gels evaluated by Gel Doc XR+ (Bio-Rad, USA)

(All the samples are represented in two repetitions and each pare is noted by variety, defatting process (CP for cold-pressing or CP+PE for cold-pressing and secondary petroleum ether extraction) and fraction size; on the left side placed control scale with indicated molecular weight (kDa) of proteins)

5.2. Selected functional properties of hemp meal fractions

As was said before, the variety Finola was breed for seed harvesting and it provide higher amount of oil and protein content than the variety USO 31. But it is possible to assume that the technological parameters are similar for both varieties and therefore should not show significant varietal differences in the possible use of hemp meals as a filler for food products.

Protein solubility, a manifestation and result of strong interaction of polar groups with water molecules, is a prerequisite to many other functional properties of proteins. It has a great influence on the colloidal structure development, such as gelation, foaming, and emulsification, hence, plays a crucial role in the specific applications in fabricated foods (WANG, XIONG, 2019).

The level of water solubility has shown the interconnection within samples of same fractions and process of defatting, it shown by letter code (Figure 20). Samples with doubled extraction by non-polar solvent has shown raising of solubility for 1%. But fractioning meals for <315μm showed beneficial effect for solubility level and increase it from 13% to 20%.

At neutral pH, hemp proteins generally have poor solubility, and the value ranges from 8% to 38% (HADNACEV et al., 2018). However, at pH greater than 8.0, the protein solubility can increase to 65% to 90% suggesting that hemp protein in a strict sense is a type of alkali-soluble protein. The underlying mechanism of solubilization at alkaline pH (especially at pH > 10.0) could be related to the dissociation of edestin, much like the alkaline effect on soy glycinin or β-conglycinin. In comparison with soy protein, hemp protein has a higher solubility at pH values less than 8.0. At pH above 8.0, the solubility of the two types of proteins was similar. The difference in protein solubility in the acidic pH range may be attributed to differences of protein constituents and aggregation extent of hexamers (glycinin or edestin). The high content of cysteine residues in edestin may

predispose it to intermolecular disulfide bond formation, and thereby increasing the extent of aggregation (WANG, XIONG, 2019).

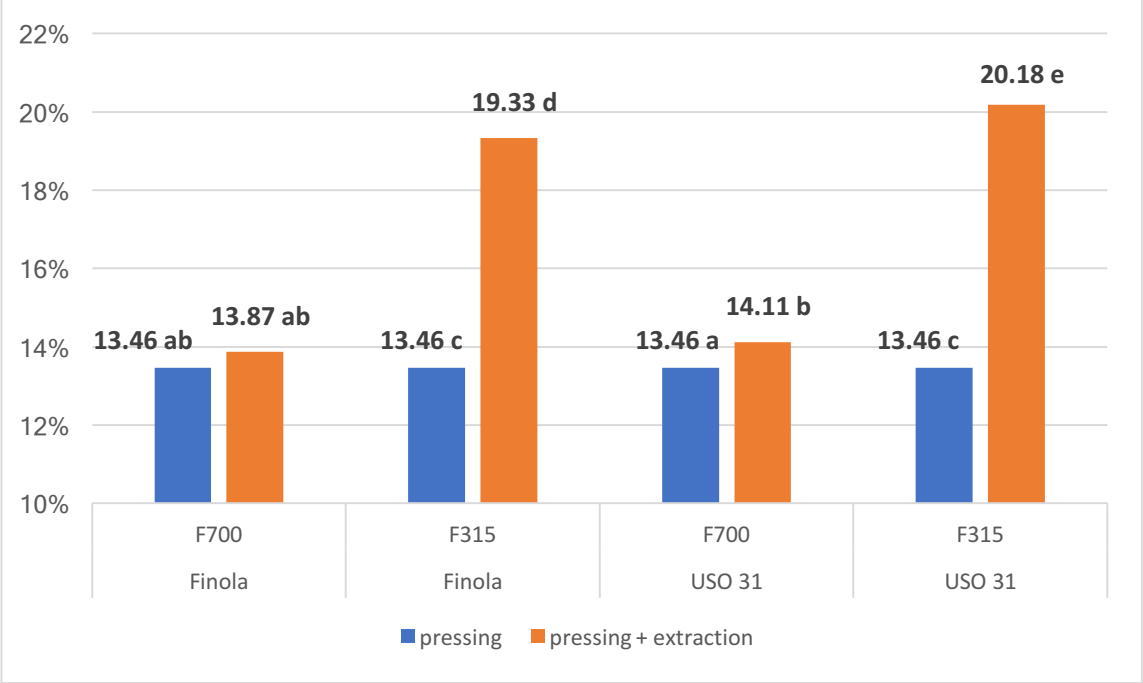


Figure 20. Water solubility level of hemp meals (% of original matter)

(F700 for fraction size of 700-1000 µm and F315 for the fraction of <315µm;
Letter code indicate significant difference at significance level α = 0.05 (ANOVA, Fisher LSD test))

The result of water holding capacity (WHC) represents the amount of water which can be entrapped in the structural matrix. The ability to interact and entrap water voluminously is extremely important for the application of proteins, fiber and other substances in food products. It is an important property of flours which to a large extent determines their applicability as food ingredients. Hence flours with high WHC are widely used in meat products, custards and soups to enhance body thickening and viscosity, and in baked products to improve freshness and handling characteristics. WHC is related to many intrinsic and extrinsic factors, for example, the amino acid profile, protein concentration, conformation, hydrophobicity, pH, temperature, and ionic strength (RAIKOS et al., 2014).

The data indicated that petroleum ether extraction had higher ability to prevent water loss from hemp meals. Due to it higher level of defatting of meal it can absorb and hold bigger amount of water. It was recovered the same for the fat absorption capacity. In case that water or fat is held by protein matrix the using of smaller size fraction with lower level of residues show significant effect for binding substances as water or fat (Figure 21, 22).

Water holding capacity in average 1.5 g water/g meal and fat absorption capacity in average about 1g/g showed a good result in compare with other types of flours. For example, in study of TRAYNHAM et al. (2007) the WHC for wheat flour had results of 0.63 to 0.87 g water/g flour.

In the study reported by RAIKOS et al. (2014) were observed significant differences between the water holding capacity of the different types of flours and followed the order lupin>hemp>fava bean>buckwheat>green pea>wheat.

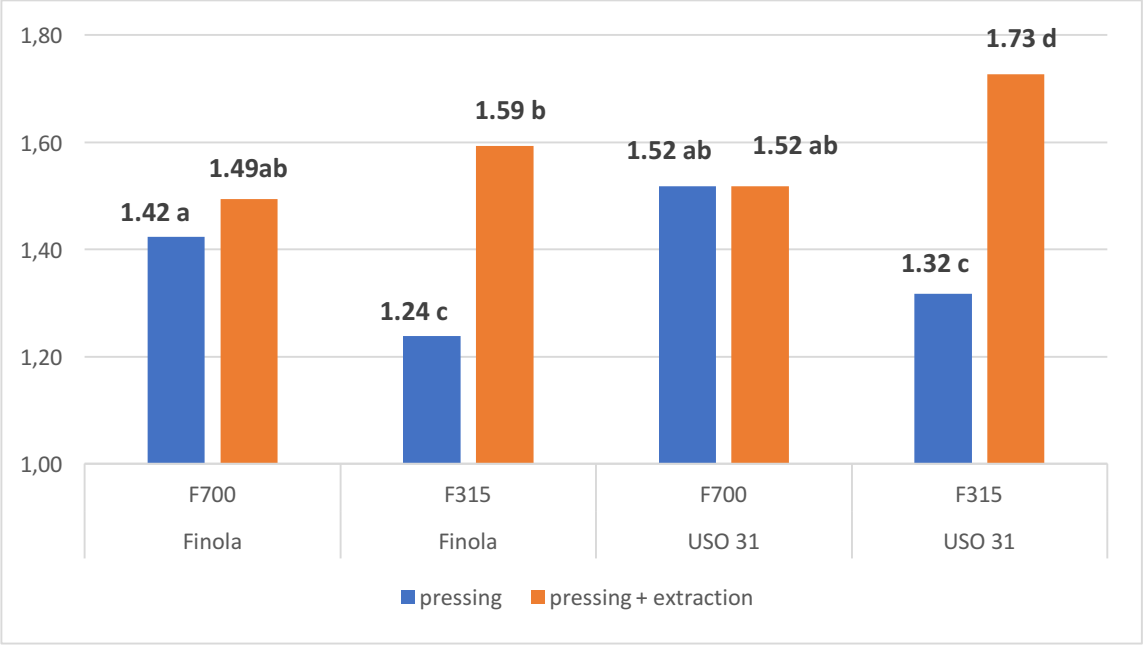


Figure 21. Water holding capacity of hemp meals (g/g)

(F700 for fraction size of 700-1000 µm and F315 for the fraction of <315µm;
Letter code indicate significant difference at significance level α = 0.05 (ANOVA, Fisher LSD test))

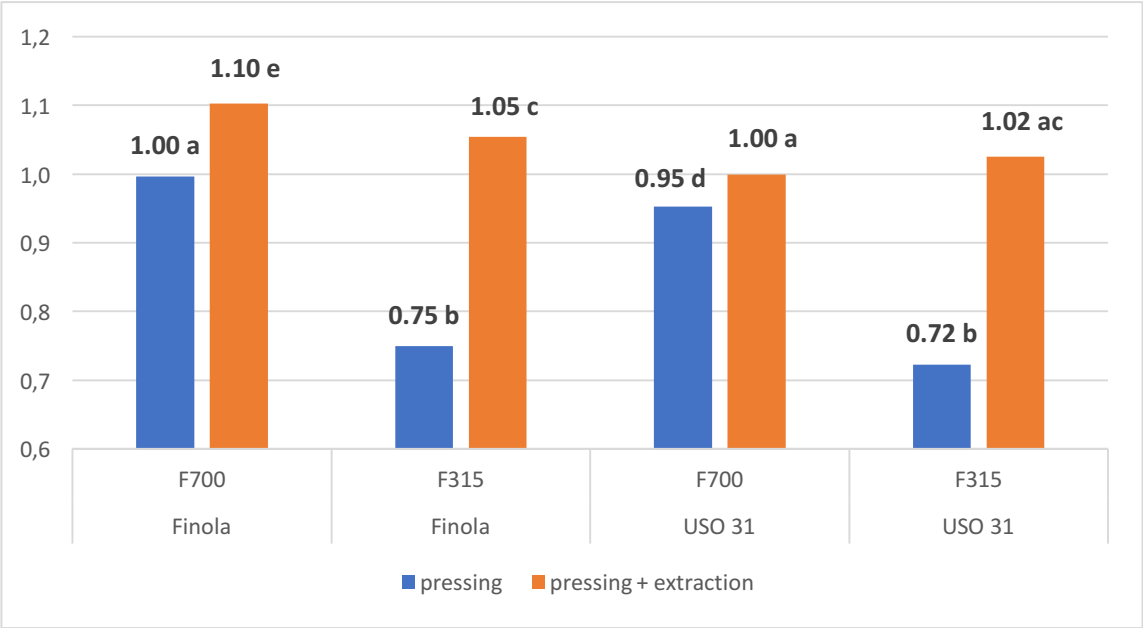


Figure 22. Fat absorption capacity of hemp meals (g/g)

(F700 for fraction size of 700-1000 µm and F315 for the fraction of <315µm;
Letter code indicate significant difference at significance level α = 0.05 (ANOVA, Fisher LSD test))

5.3. Total polyphenols and antioxidant activity

Polyphenols, chlorophyll, fiber and other content substances contribute to the antioxidant activity. On the Figure 24 shown that the total polyphenol content was almost 5 times higher for fractions 700-1000 µm of meal and provided higher level of antioxidant activity (Figure 23). That proved that most of polyphenols are located in husk and coats of the seed and by grinding and sifting process that particles were excluded from meals of fractions <315 µm which provided lower level of antioxidant activity for it. As it shown on Figures 23, 24 the defatting by petroleum ether had positive effect on releasing polyphenol content from meals and provided better antioxidant activity as well.

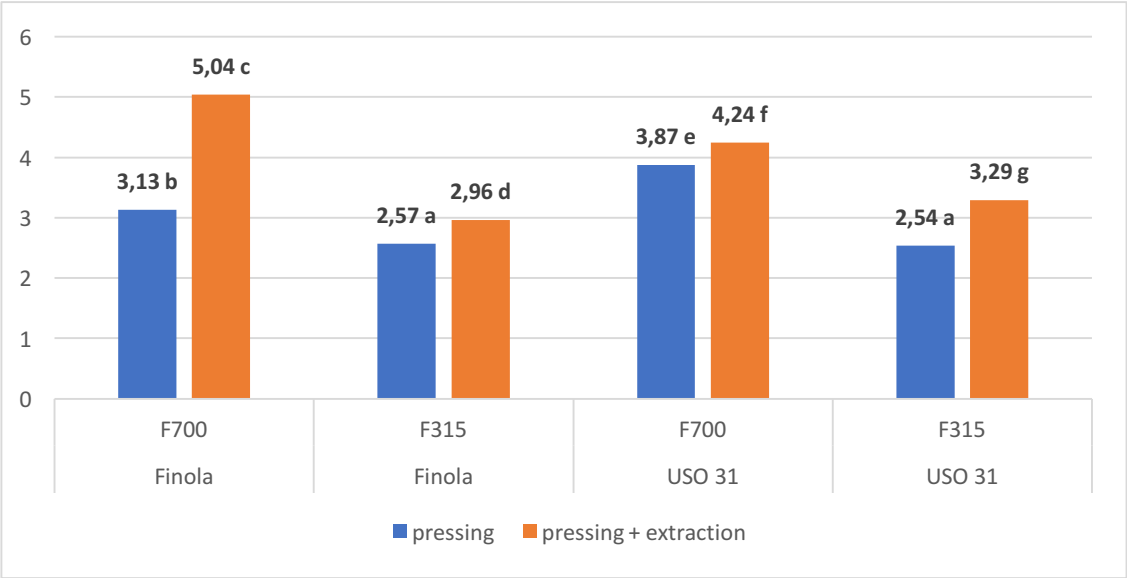


Figure 23. Antioxidant activity of hemp meals (AAE mg/g)

(F700 for fraction size of 700-1000 µm and F315 for the fraction of <315µm;
Letter code indicate significant difference at significance level α = 0.05 (ANOVA, Fisher LSD test))

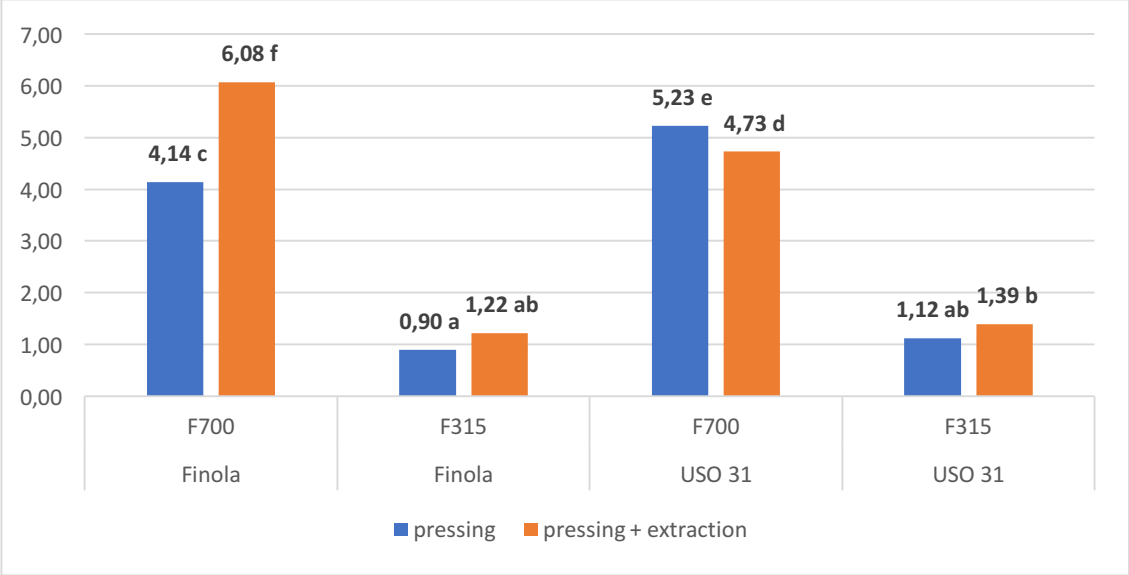


Figure 24. Total polyphenol content (mg GAE/g)

(F700 for fraction size of 700-1000 µm and F315 for the fraction of <315µm;
Letter code indicate significant difference at significance level α = 0.05 (ANOVA, Fisher LSD test))

The obtained results could be compared with the study of HADNACEV (2018). In that study was used Isoelectric Precipitation (HPI) and Micellization (HMI) (salt extraction followed by membrane filtration) processes for protein isolation on fractions of <250 mm. Total polyphenol content (mg GAE/g) was 1.0 for HMI and 1.3 for HPI, what can be compared with samples <350 mm of this study regard to fractioning size of meals. The traditional method of protein isolation involves isoelectric precipitation, which damages protein functionality and reduces performance as an ingredient below the required level for high quality food products manufacture. (MALOMO, ALUKO, 2015)

The results obtained during the present study show that hemp flour and its fractions have technologically interesting properties with the potential use in various types of food applications. For example, in the study of POJIC et al. (2015) were tested effects of adding hemp flour on wheat bread baking. The obtained results indicated that hemp flour affected water absorption and dough development time, and consequently bread volume, color and structural and textural properties of bread crumb regardless of the level of substitution. However, the presence of hemp flour in formulation, as being non-gluten flour, did not necessarily distort all rheological properties. In this context, dough stability and dough strength were not significantly affected by the addition of hemp flour up to 10 %, whereas substitution level of 20 % resulted in the decrease of these parameters. Bread supplemented with hemp flour had higher nutritional value and provided elevated intake of important nutrients such as proteins and macro- and microelements. The consumption of 300 g of bread supplemented with 20 % of hemp flour can theoretically satisfy the recommended daily intake of iron. However, substitution level of 10 % of hemp flour should fulfill requirements in terms of providing the balance between bread quality and nutritional properties.

Hemp-based food products are considered less allergenic than those from other edible seeds. In vitro protein digestibility was determined using a static model of gastrointestinal digestion, which included a final step with purified brush border membrane enzyme preparations. Hemp flour and hemp protein isolate showed a high degree of digestibility in study of MAMONE et al. (2019).

6. Conclusion

Hemp seed oil remains the most valuable product of hemp seed industry. But due to the modern knowledges about food composing and dietary, demand for new products and no-waste manufacturing it opens new possibility for using hemp pressed-cake after cold-pressing process. This study was focused on the composition determination of the hemp meals reminded after defatting process by different way of extraction. It studied properties of compounds and effect of extraction by petroleum ether on two different fraction size. This study proved the beneficial effect of using non-polar solvent as petroleum ether for defatting of hemp meal and raising the level of protein and functional residues yield. It showed significant effect on functional parameters as solubility, WHC and FAC in case of using fractioning size <315 μm. Otherwise, in case of using fractioning size 700-1000μm, it showed a positive effect for polyphenol content and measured antioxidant activity.

Due to distinctly different functional properties of kernel of hemp seed and its coats it could be expected that the two parts, resp. different fractioning of meals could be used in different food systems. Smaller sized fraction with higher amount of protein make it more suitable for incorporation into high-protein food products, such as the food intended for sport active people. In addition of results for solubility of smaller fraction with defatting by non-polar solvent it makes that meal be very profitable for utilizing it for sportive protein cocktails or shakes. On the contrary, the bigger fractioning size of hemp meal can be beneficial used as food supplement in different food products. Due to extraction by non-polar solvent the higher product recoveries and ability to retain higher amount of water or fat, make hemp meals desirable for industrial food and non-food applications as thickening and texturizing agent.

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