DigInTraCE

Proteins recovery for wood-resin composite production v1

D5.2

DigInTraCE

A Digital value chain Integration Traceability framework for process industries for Circularity and low Emissions by waste reduction and use of secondary raw materials

Funded by
the European Union

Dissemination level	Public (PU)
Type of deliverable	R – Document, report
Work package	WP5 – Technologies for secondary materials upgrade and process optimization
Deliverable number	D5.2 Proteins recovery for wood-resin composite production v1
Status - version, date	V1, 06/2024
Deliverable leader	NTUA
Contractual date of delivery	30/06/2024
Keywords	Sunflower seed, rapeseed, protein, extraction

Quality Control

	Name	Organisation	Date
Peer review	Dimitris Moutousidis	CHIMAR	13/6/2024
Peer review	Javier GARCIA-JACA	TECNALIA	17/6/2024

Version History

Version	Date	Author	Summary of changes
01	29/5/2024	Eleni Kavetsou, Christoforos Vasileiou	First draft of document
02	12/06/2024	Eleni Gogou, Eleni Kavetsou	First draft of document sent to DIGINTRACE internal reviewers
03	21/6/2024	Eleni Gogou, Eleni Kavetsou	Final version after revision

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List of abbreviations and acronyms

Abbreviations	Meaning
MAE	Microwave Assisted Extraction
UAE	Ultrasound Assisted Extraction
PLE	Pressurized Liquid Extraction
pI	Isoelectric point
CE	conventional extraction
EH	enzymatic hydrolysis
DoE	Design of Experiments

1. Executive Summary

The present document is the first version of the reporting on Task 5.2 findings consisting ***D5.2 "Proteins recovery for wood-resin composite production v1"*** presenting the results (up to June 2024, M18 of the DIGINTRACE Project) on the extraction of proteins from different oilseed press cakes using eco-friendly extraction technologies. The experimental work will be continued in the framework of Task 5.2 with the overall results being presented in deliverable ***D5.3 "Proteins recovery for wood-resin composite production FINAL"*** planned to be delivered on M42, according to the Grant Agreement.

The objective of Task 5.2 is to efficiently recover proteins from edible oil industry by-products including rapeseed, sunflower and cotton press cakes. Among them defatted sunflower and rapeseed press cake, were used for the protein extraction applying conventional and novel, energy-efficient extraction technologies (Microwave Assisted Extraction (MAE), Ultrasound Assisted Extraction (UAE) and Pressurized Liquid Extraction (PLE)). Extracted proteins were obtained via alkaline extraction where the soluble proteins were precipitated by adjusting the pH to their isoelectric point. The extraction parameters including solid-liquid ratio (1:10, 1:25, 1:35, 1:50), microwave and ultrasound power, pressure and extraction time (5, 10, 20 and 60 min) were optimized in order to achieve high protein recovery yields. Kjeldahl method was used for the determination of protein content of the crude cakes and of the extracted proteins. The results revealed that rapeseed's and sunflower's press cake protein content was 25.2% and 72.6%, respectively, while the protein extracts recovered via the applied methods presenting protein content from 62 to 72%. Through the application of the novel and eco-friendly extraction technologies higher protein yields were achieved minimizing the extraction time, using simultaneously green solvents. In case of sunflower protein extraction, the highest protein yield was achieved for ultrasound assisted extraction reaching 53% (conventional %protein yield=27%). However, in case of rapeseed press cake the highest %protein yield was achieved through the conventional extraction method (37%). For sunflower press cake extraction, Design of Experiments was applied and the experimental results that were carried out in the suggested optimum conditions, revealed similarly %protein recovered yield with the expected values from the regression equations.

2. Introduction

Efficient use of raw materials plays a crucial role in advancing towards a sustainable bioeconomy. Specifically, the utilization of side streams (or by-products) generated in the agriculture and food supply chain for food and other valuable commodities is critical. By-products' exploitation is essential for enhancing economic efficiency, reducing environmental impact, fostering innovation, conserving energy, and supporting sustainable development. DIGINTRACE project aims to valorize by-products from the edible oil industry. Specifically, oilseed press cakes are the remaining solid material from oilseed crops after the oil extraction. Oilseed press cakes are a mixture of protein, fiber, and other nutrients and the exact composition varies depending on the type of oilseed and the extraction method used. The exploitation of these by-products aligns with principles of sustainability, economic efficiency, and innovation, offering significant benefits across environmental, economic, and social dimensions (Mirpoor et al., 2022). The exploitation, also, supports the development of a circular economy model, where waste materials are reintegrated into the production cycle. As far as environmental impact, exploiting oilseed by-products reduces waste, contributes to more sustainable agriculture and industrial practices, and maximizes the resource efficiency of crop production. Moreover, there are economic benefits including the overall increased profitability of oilseed processing operations and cost efficiency, as press cakes can be used as raw materials in various industries reducing the need for purchasing additional inputs. Nutrition aspects are, also, of utmost importance. Oilseed press cakes are a valuable source of protein. They are commonly used as a nutritious feed for livestock, providing essential amino acids (Rakita et al., 2023), or as organic fertilizers due to their nutrient content, serving as a sustainable way to recycle the by-products of oil extraction (Ancuța & Sonia, 2020).

The oilseed extraction process involves several steps to efficiently extract oil from oilseeds like soybeans, sunflower seeds, rapeseeds (canola), and others. The process can be broadly divided into two major steps: mechanical pressing and solvent extraction methods. Initially, seeds are cleaned to remove impurities such as stones, dust, and other foreign materials. They are then dehulled to remove the outer seed coat, which can be abrasive and reduces the efficiency of the extraction process. Finally, the seeds are crushed to increase the surface area, facilitating better oil extraction. During the mechanical pressing, the first step is conditioning which involves heating the crushed seeds to a temperature range between 70^{o}C to 100^{o}C to enhance oil extraction efficiency. The conditioned seeds are then fed into a mechanical press (expeller), where high pressure is applied to extract the oil. The oil flows out through small openings, while the remaining solid material (press cake) is expelled separately. The press cake, which still contains some residual oil, can be further processed through extraction methods or used as animal feed. For further extraction, the press cake is subjected to solvent extraction to recover the residual oil commonly using hexane as solvent. After mixing the cake with the solvent for a specific time, the oil-laden solvent is separated from the solid cake using filtration. Afterward, the oil is separated from the solvent using evaporation and condensation. The crude oil then undergoes refining to reach its final form, which includes degumming to remove phospholipids and impurities, neutralization to remove free fatty acids, bleaching to eliminate color pigments and impurities, deodorization to remove volatile compounds, and optionally winterization to remove waxes (Gaber et al., 2018). Among all process parameters, the temperature in the conditioning step, the pressure in the mechanical pressing step, and the time in solvent extraction are of the highest interest to ensure maximum oil recovery and maintain the quality of the extracted oil and by-products.

Protein is one of the most challenging macronutrients when it comes to sustainable production. Currently, animal-origin products are still the main protein and food ingredient sources. However, the

need to find alternative sources of protein is crucial. Oilseed press cakes contain a significant amount of protein and its recovery has been studied. Trass et al. and Wanasundara & McIntosh processed rapeseed press cakes using enzymatic treatment to enhance protein recovery. Similarly, Rozenszain & Beye (2009) applied enzymatic treatment to various oilseed press cakes, achieving comparable results. Donga et al. (2011) employed ultrasound-assisted extraction on rapeseed cake, resulting in increased protein yields with superior functional properties. Additionally, Hojilla-Evangelista et al. (2004) utilized ultrasounds for protein extraction from soybean press cake.

Sunflower seeds contain a variety of nutrients, including proteins, unsaturated fat, fibers, vitamins (mainly E), selenium, copper, zinc, folate, iron and other minerals. The oil extracted from sunflower seeds, sunflower oil, is used as a cooking oil, while the sunflower seed itself is present in the human diet as a snack (roasted or salted). In addition, it is also used as animal feed (Guo et al., 2017; Samborska et al., 2021).

Sunflower seeds have a protein content of about 20%. These proteins are rich in sulfur, which makes them ideal for many human metabolic processes, including muscle and skeletal cell growth, insulin production, and as an antioxidant. There are two main types of proteins in the sunflower seed: 11S globulins or sunflower proteins (40%) and 2S albumins or sunflower albumins (60%). In studies comparing sunflower proteins with soy proteins, it was found that the former has a better emulsifying effect while, at the same time, they are excellent under heat treatment, gelation, foam formation and stability. The sunflower seed is also a valuable source of glutamic acid, aspartic acid, arginine and cysteine and is rich in protein with a well-balanced amino acid content. It also contains essential amino acids, such as phenylalanine, tyrosine and cysteine.

Among the main extraction methods, microwave-assisted extraction, ultrasound-assisted extraction, pressurized liquid extraction and enzymatic extraction are of utmost interest as innovative and efficient methods. MAE is a technique that leverages microwave energy to rapidly heat the sample-solvent mixture, causing the disruption of cell structures and facilitating a more efficient transfer of target compounds into the solvent compared to conventional extraction methods (Eskilsson and Björklund, 2000). This process significantly enhances the extraction efficiency and reduces the time required for extraction. Several studies have demonstrated the effectiveness of MAE in various applications. For instance, Cavalluzzi et al. (2022) reported high yields of phenolic and flavonoid compounds from lentil by-products using MAE, highlighting its potential in valorizing agricultural waste. Similarly, Pavlić et al. (2023) found that MAE efficiently extracted antioxidants from olive leaves in a significantly reduced time, underscoring its efficacy and practical advantages over traditional extraction methods.

Concerning the UAE, it employs high-frequency sound waves to enhance the extraction of bioactive compounds from plant materials. The mechanism involves the generation of cavitation bubbles in the solvent, which collapse violently and create micro-jets that disrupt the cell walls of the plant material. This disruption facilitates the release of intracellular compounds into the solvent, thereby increasing the extraction efficiency and speed compared to traditional methods. UAE is particularly effective in reducing extraction time and solvent usage, making it a green and efficient extraction method (Vinatoru, 2001). Several studies have highlighted the efficacy of UAE. For example, Tiwari et al. (2009) demonstrated the successful extraction of lycopene from tomatoes using UAE, achieving higher yields and reduced extraction times compared to conventional solvent extraction. In another study, Zhang et al. (2008) optimized the extraction conditions for phenolic compounds from citrus peels, finding that UAE significantly improved the yield and antioxidant activity of the extracts.

Respectively, PLE applies elevated temperatures and pressures to enhance the extraction efficiency of bioactive compounds from solid and semi-solid samples. By maintaining the solvent in a liquid state beyond its normal boiling point, PLE increases the solubility and mass transfer of analytes, resulting in faster and more efficient extraction processes. Recent studies have demonstrated PLE's effectiveness.

For example, Barp et al. (2023) used PLE to extract contaminants from food matrices, achieving high recovery rates of pesticides, mycotoxins, and other contaminants with reduced solvent consumption and time compared to traditional methods. Another study by Ergönül and Özbek (2020) explored the extraction of glucosinolates from Camelina sativa, finding that PLE significantly enhanced the yield and purity of the extracted compounds.

Finally, enzymatic extraction involves enzymes to catalyze the breakdown of complex biomolecules into simpler ones, facilitating the efficient extraction of target compounds from raw materials. This method offers specificity, mild processing conditions, and environmental benefits compared to traditional chemical or mechanical extraction methods. Li et al. (2022) investigated the use of enzymatic hydrolysis to isolate proteins from plant sources, demonstrating that this method could efficiently produce high-quality protein extracts with preserved nutritional and functional properties.

The objective of Task 5.2 in DIGINTRACE project is the recovery of proteins from sunflower, rapeseed and cottonseed press cakes with the use of conventional and novel extraction technologies in order to achieve high yields of protein recovery with the use of green solvents and energy-efficient recovery processes. The recovery processes used and presented in the current deliverable include conventional extraction, MAE, UAE, PLE, hydrolysis, and enzymatic hydrolysis. The extracted proteins recovered in Task 5.2 will be further used in Task 6.2, in the Greek Demo Case, to partially replace the fossil-based phenol for the synthesis of adhesives used in plywood panel production.

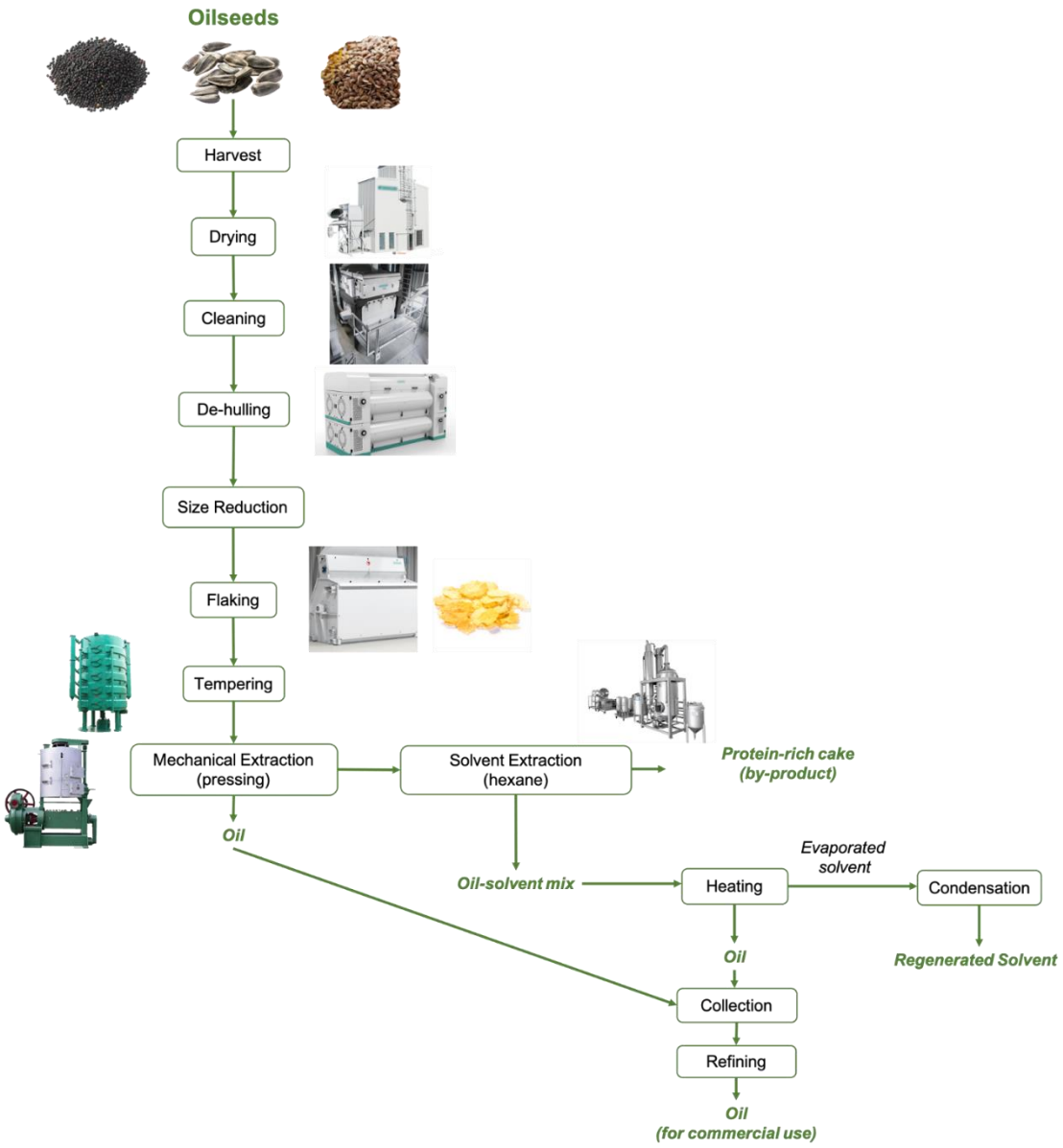


Figure 1. Oil extraction process from oilseeds.

3. Materials and Methods

Liquid sodium hydroxide (10M, NaOH) and liquid hydrochloric acid (5M, HCl) were obtained from Fisher Chemical, UK. Sunflower press cake and rapeseed press cake were provided by Agroinvest, while cotton press cake was provided by Stymon, collaborating company in Greece (Figure 2).

Figure 2. Rapeseed, sunflower and cottonseed press cake samples delivered to NTUA.

3.1 Extraction of proteins

Plant protein extraction is a solid-liquid extraction that involves several steps to isolate proteins from plant materials, such as the tested oilseed by-products. The liquid is used as solvent and should properly dilute the protein content. Depending on the nature of plant material and the targeting proteins, there have been examined water, buffers, salt solutions and organics as solvents. For the tested raw materials, aqueous buffer in alkalic pH was used. The pH adjustment was accomplished by adding NaOH solution 1M. The press cakes, as raw materials, do not need any further preparation or size reduction. The first step is the extraction method. Several methods have been tested including conventional extraction (CE), MAE and UAE (Figure 4), PLE (Figure 5) and enzymatic hydrolysis (EH). All these methods will be described in detail in the next sections. The next step is the separation of liquid extract with diluted proteins from the solid leftovers through centrifugation. After centrifugation, the liquid sample is driven to the precipitation process, in which the pH is dropped to the isoelectric point (pI) by adding HCl 1M. At pI the proteins become insoluble and are separated from other components in the extract. The pI of each protein group was determined for the three different cakes. The mixture was centrifuged and washed to remove impurities and residual solvent. The protein extract was freeze dried and stored for further characterization.

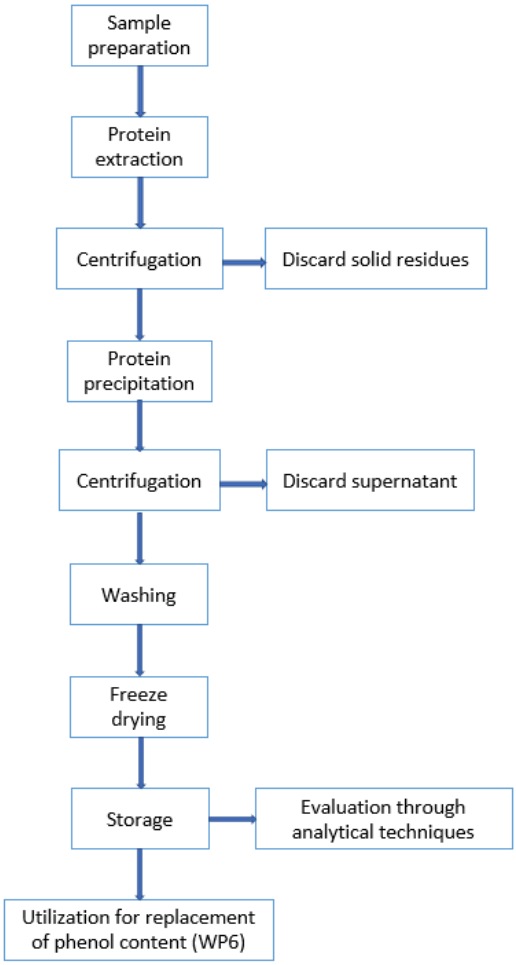


Figure 3. General experimental procedure for protein extraction.

3.2 Determination of Isoelectric point (pI)

After the alkaline extraction, the dissolved proteins are subjected to a precipitation process at a suitable pH equal to the pI of the proteins. The Isoelectric Point is the pH at which the net charge of a protein is zero. When the pH is above the pI, proteins exhibit a negative surface charge, resulting in the development of repulsive forces between negatively charged molecules. On the other hand, respectively, at pH levels below pI, proteins have a positive surface charge, which leads to repulsion between protein molecules. When the pH is set equal to the pI, the total charge is zero and attractive forces dominate, causing the proteins to complex and precipitate. The pI of each protein group differs and depends on the ionized groups on their surface.

The Isoelectric point was determined by measuring the precipitation yield applying different values of precipitation pH at constant ratio and extraction time. For each cake, the ratio was set at 1:50 and the extraction time at 60 minutes. Particularly, for the pI determination, a solid-liquid solution was prepared in a ratio of 1:50, using water as a solvent. The solutions' pH was adjusted at 11.9 using NaOH solution 1M and the alkaline extraction took place by stirring for 60 minutes. The samples were centrifuged at 3500 rpm for 6 minutes. The supernatants were subjected to acidic protein precipitation using 1M HCl solution at different pH values (3.80, 4.00, 4,20, 4.40 and 4.60 for sunflower and 3.10, 3.30, 3.40, 3.50, 3.55, 3.70 and 3.90 for rapeseed) and stored in the refrigerator for 1 day for protein

precipitation. Then, they were centrifuged at 3500 rpm for 6 minutes twice in order to wash the acid off the protein precipitates. Finally, the precipitates were freeze dried to obtain a powder product.

The precipitation yield was calculated via equation 1, while the protein recovery via equation 2:

$$\text{Precipitation yield} = \left(\frac{m1}{m2}\right) * 100 \quad \text{(Eq. 1)}$$

$$\text{Protein recovery} = \left(\frac{m1*P1}{m2*P2}\right) * 100 \quad \text{(Eq. 2)}$$

where m1 is the precipitation mass, m2 is the raw material's mass, P1 is the percentage of protein content in precipitation mass, and P2 is the percentage of protein content in raw material's mass.

3.3 Design of Experiments (DoE)

In order to minimize each method's runs for sunflower press cake while still obtaining efficient data, "Design of Experiments"-DoE was employed. Statgraphics Centurion 19 was used as statistical software. Some of each method's results were entered as initial data and the program indicated the extra runs needed. The additional results were entered and the data were statistically analyzed.

3.4 Conventional extraction

A cake-water solution was prepared in a ratio of 1:25, 1:35 and 1:50. The solutions' pH was adjusted at 8 or 11.9 using NaOH solution 1M and the alkaline extraction took place by stirring for different extraction durations (5, 10, 15, 30, 60 and 120 minutes) (Table 1 & 2). The samples were centrifuged at 3500 rpm for 6 minutes. The supernatants were subjected to acidic protein precipitation using 1M HCl solution at pH 4.39 for the sunflower cake (isoelectric point -IP) and 3.50 for the rapeseed cake and stored in the refrigerator for 1 day for protein precipitation. Then, they were centrifuged at 3500 rpm for 6 minutes twice in order to wash the acid off the protein precipitates. Finally, the precipitates were freeze dried to obtain a powder product. The conventional protein extraction from cotton press cake is underway.

Table 1. Tested parameters for the sunflower press cake.

Sunflower press cake	
Extraction Time (min)	**Solid-Liquid Ratio (w/v)**
10 15 30 60 120	1:25 1:35 1:50

Table 2. Tested parameters for the rapeseed press cake.

Rapeseed press cake	
Extraction Time (min)	**Solid-Liquid Ratio (w/v)**
5 10 15 60 120	1:10 1:25 1:35 1:50

3.5 Ultrasound assisted extraction

A cake-water solution was prepared in a ratio of 1:25, 1:35 and 1:50 (Table 3 & 4). The solutions' pH was adjusted at 8 or 11.9 using NaOH solution 1M and the alkaline extraction took place applying ultrasounds (200W, 300W, 450W, 600W) for 5, 10 and 15 minutes using Ultrasonic reaction system XO-SM50 (Nanjing Xianou Instruments Manufacture CO., LTD., China). The samples were centrifuged at 3500 rpm for 6 minutes. The supernatants were subjected to acidic protein precipitation using 1M HCl solution at pH 4.39 for the sunflower cake (IP) and 3.50 for the rapeseed cake and stored in the refrigerator for 1 day for protein precipitation. Then, they were centrifuged at 3500 rpm for 6 minutes twice in order to wash the acid off the protein precipitates. Finally, the precipitates were freeze dried to obtain a powder product.

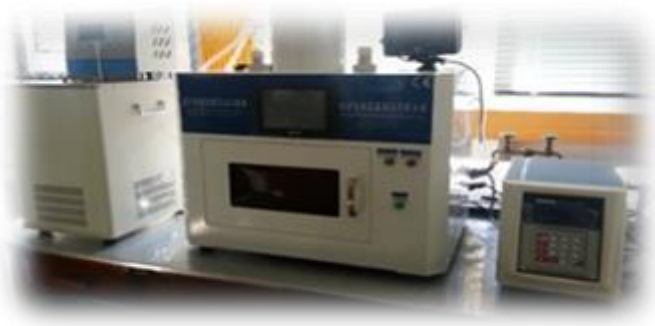


Figure 4. Microwave-Ultrasound assisted extraction machine.

Table 3. Tested parameters for the sunflower cakes.

Sunflower press cake		
Extraction Time (min)	**Solid-Liquid Ratio (w/v)**	**Ultrasound Power (Watt)**
5 10 15	1:25 1:35 1:50	200 300 450

Table 4. Tested parameters for the sunflower cakes.

Rapeseed press cake		
Extraction Time (min)	**Solid-Liquid Ratio (w/v)**	**Ultrasound Power (Watt)**
5 10 15	1:10 1:50	450

3.6 Microwave assisted extraction

A cake-water solution was prepared in a ratio of 1:25, 1:35 and 1:50. The solutions' pH was adjusted at 8 or 11.9 using NaOH solution 1M and the alkaline extraction took place applying microwave irradiation (250 W) for 5, 10 and 15 minutes using Ultrasonic reaction system XO-SM50 (Nanjing Xianou Instruments Manufacture CO., LTD., China). The samples were centrifuged at 3500 rpm for 6 minutes. The supernatants were subjected to acidic protein precipitation using 1M HCl solution at pH 4.39 for the sunflower cake (IP) and 3.50 for the rapeseed cake and stored in the refrigerator for 1 day for protein precipitation. Then, they were centrifuged at 3500 rpm for 6 minutes twice in order to wash the acid off the protein precipitates. Finally, the precipitates were freeze dried to obtain a powder product.

3.7 Combined microwave and ultrasound assisted extraction

A cake-water solution was prepared in a ratio of 1:25, 1:35 and 1:50. The solutions' pH was adjusted at 8 or 11.9 using NaOH solution 1M and the alkaline extraction took place applying combined microwave irradiation (250 W) and ultrasounds (200W, 300W, 450W) for 5, 10 and 15 minutes using Ultrasonic reaction system XO-SM50 (Nanjing Xianou Instruments Manufacture CO., LTD., China). The samples were centrifuged at 3500 rpm for 6 minutes. The supernatants were subjected to acidic protein precipitation using 1M HCl solution at pH 4.39 for the sunflower cake (IP) and 3.50 for the rapeseed cake and stored in the refrigerator for 1 day for protein precipitation. Then, they were centrifuged at 3500 rpm for 6 minutes twice in order to wash the acid off the protein precipitates. Finally, the precipitates were freeze dried to obtain a powder product.

3.8 Pressurized Liquid Extraction

Based on the existing PLE equipment (FMS PLE® Pressurized Liquid Extraction system, Fluid Managment Systems, USA), a 100 mL extraction cell with a constant volume is employed. For the extraction process, 2 grams of press cake are loaded into the cell to achieve a 1:50 solid-to-liquid ratio, and 10 grams of raw material are used to achieve a 1:10 solid-to-liquid ratio. The pH of the extraction solutions is adjusted to 8.0 or 11.9 using 1 M NaOH solution. The alkaline extraction is conducted under controlled conditions, with a constant pressure of 1750 psi and at room temperature. The extraction process is carried out for varying durations to assess the impact of extraction time on the yield and quality of the extracted proteins. The samples were centrifuged at 3500 rpm for 6 minutes. The supernatants were subjected to acidic protein precipitation using 1M HCl solution at pH 4.39 for the sunflower cake (IP) and 3.50 for the rapeseed cake and stored in the refrigerator for 1 day for protein

precipitation. Then, they were centrifuged at 3500 rpm for 6 minutes twice in order to wash the acid off the protein precipitates. Finally, the precipitates were freeze dried to obtain a powder product.

Figure 5. Pressurized Liquid Extraction (PLE) machine.

3.9 Protein Characterization-Protein content (kjeldahl method)

The determination of nitrogen, and consequently the proteins, is carried out by the Kjeldahl method in which the sample is burned in combustion bottles with an excess of sulfuric acid, in the presence of a catalyst (mercury oxide, selenium, copper sulphate or titanium dioxide) resulting in the conversion of nitrogen compounds in ammonium bisulfate. The solution is then made alkaline and the released ammonia is collected and determined volumetrically.

The combustion bottles contain:

- 0.7-1g of raw material
- 20mL of concentrated Sulfuric Acid H_2SO_4
- 10g of Potassium Sulfate K_2SO_4
- 1g of Hydrated Copper Sulfate $CuSO_4.5H_2O$
- Boiling cores

- **Combustion**

The bottle is shaken for a few minutes so that the sample is thoroughly saturated by the acid and thus intense foaming of the solution is avoided. The vacuum tube adapts to the Kjeldahl bottles, the system is mounted on the Digestor (Figure 6) and connected to the tap to create a vacuum, while the other end is closed. At first, the heating is mild (about 150°C for 45 min), so that there is no intense foaming. When sample combustion begins (first vapours are observed), the heating becomes more intense and reaches maximum intensity (420°C for 1h). When the solution has a characteristic light green colour, the boiling stops. After the combustion, the bottles are left to cool, while the vacuum is maintained for about 30 min.

Figure 6. Digestor unit of the Kjeldahl method.

- **Distillation**

The cold flasks are then attached to the special Kjeldahl steam Distillation Unit (Figure 7), which is capable of distilling one sample at a time. The unit is set to run for 5 minutes (to collect approximately 200mL of distillate) and needs the following reagents in order to perform the distillation: (i) 75mL of Sodium Hydroxide NaOH and (ii) 75mL of Deionized Water.

Figure 7. Distillation unit of the Kjeldahl method.

- **Titration**

At the output of the distillation unit, there is a conical flask, containing 50mL of a standard solution of 0.5 N sulfuric acid, in which the distillate is collected.

Finally, the excess acid in the conical flask is titrated with a standard 0.5N sodium hydroxide solution and methyl red - methylene blue indicator (approximately 1 mL), which results from the dissolution of 0.125g of methyl red and 0.08g of methylene blue in 100mL of ethanol (Figure 8). Under the same conditions, a blind determination is also required.

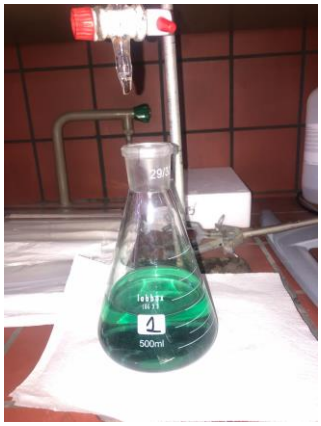


Figure 8. Titration during the Kjeldahl method.

The nitrogen content is calculated by equation 3:

$$\text{Nitrogen}\% = 1.4007 * [\text{V1} - \text{V2}] * \left(\frac{N}{b}\right) \quad \text{(Eq. 3)}$$

where:

V1 is the consumed volume of the sodium hydroxide standard solution during the main determination [mL], V2 is the consumed volume of the sodium hydroxide standard solution during the blank determination [mL], N is the normality of standard sodium hydroxide solution (0.5N), B is the raw material's weight [g].

The protein content is calculated indirectly through the equation 4, multiplying the nitrogen content with the suitable factor (5.30 for oilseed press cake):

$$\text{Protein}\% = \text{Nitrogen}\% * 5.3 \quad \text{(Eq. 4)}$$

The Kjeldahl method was applied to determine the protein content of sunflower and rapeseed press cake and the final protein isolates.

4. Results and Discussion

The various pH values that were evaluated in order to identify the pI of sunflower and rapeseed cakes were incorporated into Tables 5 and 6. Moreover, the diagrams (1 &2) of the pH related to the precipitation yield were also illustrated.

4.1 Sunflower press cake pI

Table 5. Determination of sunflower oil seed cake isoelectric point.

Results		
# Sample	**Precipitation pH**	**Precipitation Yield (%)**

1	3.81	13.2
2	4.01	14.6
3	4.18	15.4
4	**4.40**	**20.9**
5	4.58	16.3

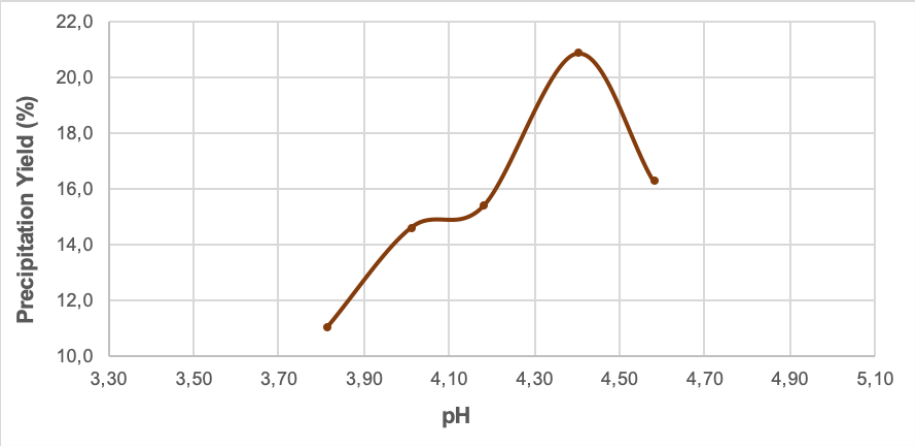


Diagram 1. The influence of the pH in the precipitation of sunflower oil seed cake protein.

Gheysasuddin et al. (1970) discovered that the minimal protein extractability between pH 4 and 6 corresponds to the isoelectric point of sunflower proteins. Subasi et al. (2020) found the isoelectric point of sunflower at 4.40 ± 0.1. The value of the optimum pI found in this study, 4.40, is in complete accordance with the literature data.

4.2 Rapeseed press cake pI

Table 6. Determination of rapeseed cake isoelectric point.

Results		
# Sample	**Precipitation pH**	**Precipitation Yield (%)**
1	3.09	12.34
2	3.30	13.21
3	3.41	13.78
4	**3.51**	13.85
5	3.55	14.20
6	3.72	13.52
7	3.87	12.71

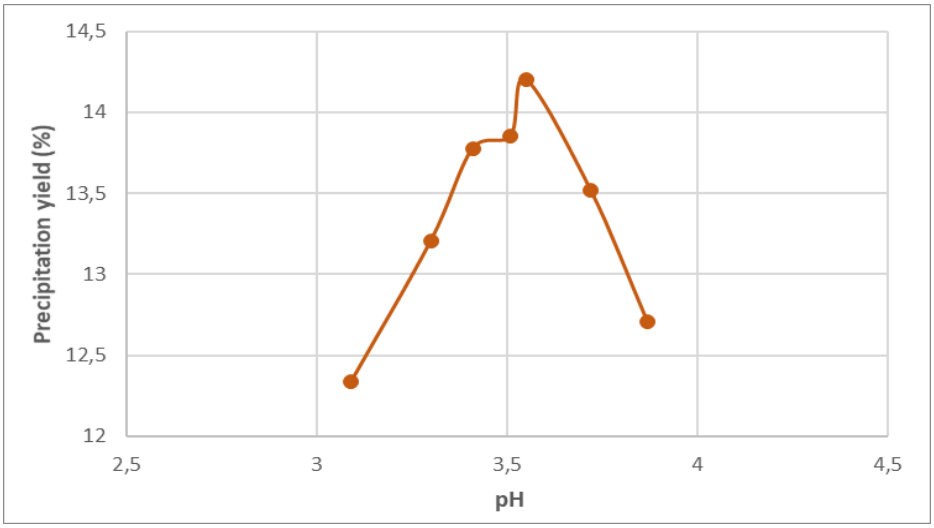


Diagram 2. The influence of the pH in the precipitation of rapeseed oil seed cake protein.

The value of the optimum pH for rapeseed cake protein precipitation was found at 3.55 using HCl. This value agrees with corresponding researches that have been done. Ahlström et al. and Klockeman, Toledo & Sims adjusted pH at value of 3.50 for optimum precipitation using citric acid.

For sunflower cake the Extraction pH was determined by measuring the precipitation yield applying different values of extraction pH (11.9 and 8.0) at constant ratio and extraction time.

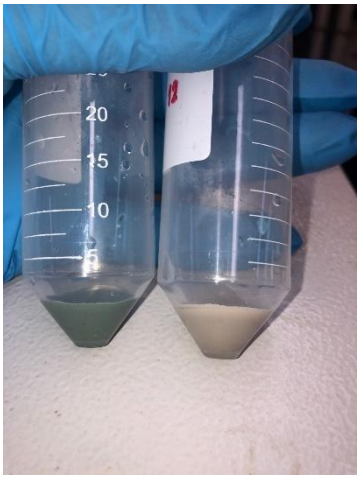


Figure 9. Comparison of different values of extraction pH; 11.9 (left tube) and 8.0 (right tube).

The dark green colour that appears at extraction pH 11.9 is the result of protein denaturation because of protein-phenolic interactions (Figure 9). Extreme alkaline conditions affect proteins by reducing their digestibility and damaging several amino acids (like lysine and cysteine) according to Subaşı et al. So, the extraction pH was selected equal to 8.0.

4.3 Protein content

The protein content of the crude press cake as well as of the isolated proteins from the different cakes and methods was calculated through the Kjeldahl Method (N factor 5.3) (Table 7). The results of %protein yield for all the applied extraction methods were calculated using an average value obtained from random samples. The highest protein content was obtained for sunflower press cake and isolated samples.

Table 7. Protein content determination using the Kjeldahl method.

Raw material	weight (g)	V NaoH (mL)	nitrogen content (%)	protein content (%)
Sunflower press cake	0.8353	40.2	13.7	72.6
Rapeseed press cake	0.9004	43.4	4.8	25.2
Sunflower protein isolate	0.9061	30.8	14.5	72.4
Rapeseed protein isolate	0.9042	34.5	11.7	61.9

4.4 Conventional extraction

4.4.1 Sunflower press cake

The steps that were followed for conventional protein extraction from sunflower cake are illustrated in Figure 10. The experiments that were conducted for the sunflower protein conventional extraction are presented in Table 8.

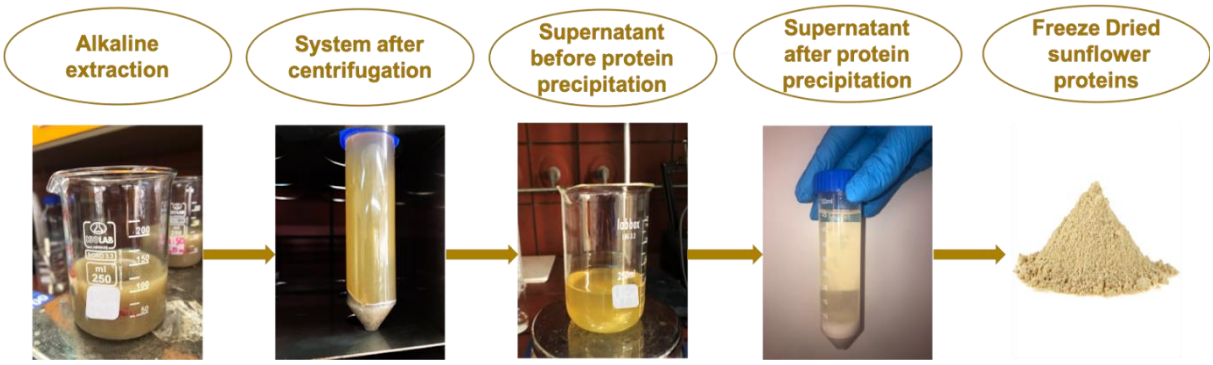
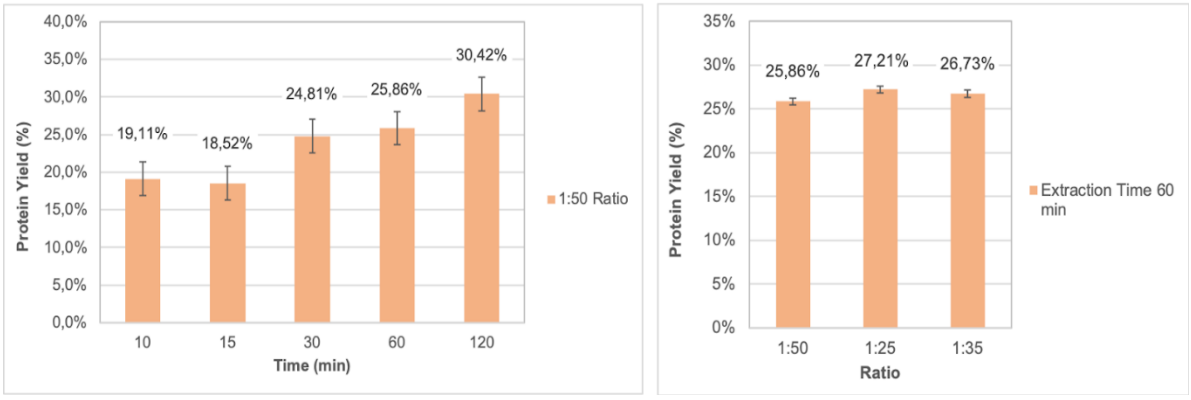


Figure 10. Conventional extraction - Alkaline precipitation for sunflower press cake.

Table 8. Conventional extraction results for sunflower press cake.

Conventional Extraction			
Ratio	Extraction Time (min)	Precipitation Yield (%)	Protein Recovery (%)
1:50	10	9,7%	19,1%
1:50	15	9,4%	18,5%
1:50	30	12,6%	24,8%
1:50	60	13,1%	25,9%
1:50	120	15,4%	30,4%
1:25	**60**	**13,8%**	**27,2%**
1:35	60	13,6%	26,7%



(a) **(b)**

Diagram 3. Sunflower's conventional extraction results at constant ratio (a) and constant extraction time (b).

The first chart (Diagram 3-a) illustrates the Protein Yield as a function of extraction time at constant ratio 1:50. Overall, there is an upward trajectory as the extraction time increases. Given that 120 minutes of extraction time correspond to 30% protein yield while 60 minutes (half time) correspond to 26%, it is safe to accept that 60 minutes is the optimum extraction time at 1:50 ratio.

The second chart (Diagram 3-b) illustrates the Protein Yield as a function of ratio (1:25, 1:35, 1:50) at constant extraction time 60 minutes. The graph shows insignificant differences between the three ratios, with 1:25 being the optimum one.

Ivanova et. al (2012) calculated the protein yield equal to 43% at extraction pH 8 and 32% at extraction pH 10. However, the initial protein content of the sunflower meal was greater in their study (43%), while the final protein content of the isolate is not provided.

Design of Experiments - DoE

Two factors (extraction time and ratio were used to optimize the conventional extraction using Response Surface Design. A Central Composite Design and, more specifically, Center-Faced Design, was employed to generate a total of 12 experiments that were carried out in random order. The experimental (independent) variables were:

- extraction time: 10, 20, 30, 60, 120 minutes
- ratio: 1:25, 1:35, 1:50

The only response was the Protein Yield (%) and the 12 experiments are presented in Table 9.

Table 9. Experimental runs for Conventional Extraction using Response Surface Design (sunflower cake).

Conventional Extraction				
Standard order	**Run order**	**Extraction Time (min)**	**Ratio**	**Protein Yield (%)**
1	5	10	1:50	19,1
2	4	60	1:50	25,9
3	6	10	1:25	22,8
4	1	60	1:25	27,2
5	9	35	1:35	25,0
6	2	35	1:35	25,1
7	7	10	1:35	21,0
8	12	60	1:35	26,7
9	10	35	1:50	24,8
10	11	35	1:25	26,0
11	8	35	1:35	25,2
12	3	35	1:35	25,1

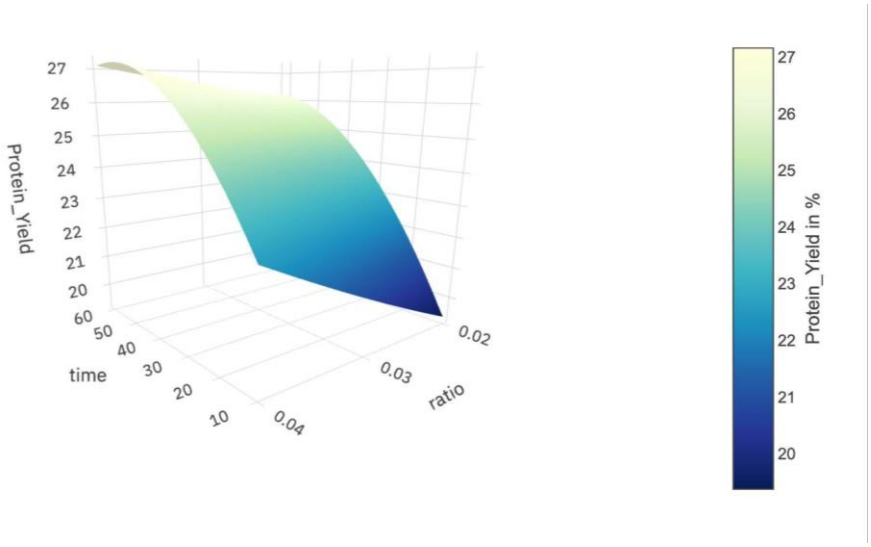


Diagram 4. Response Surface Model for the Conventional Extraction (sunflower cake).

In order to statistically analyze the data in Table 9, ANalysis Of VAriance (ANOVA test) and Multiple Regression were performed. The ANOVA table and the Regression Equation are shown below (Table 10).

Table 10. ANOVA table for the Conventional Extraction (sunflower cake).

Factors	Sum Of Squares	DF	F-Value	p-Value	significant
time	15.16	1.0	122.742	0.0	*
ratio	0.017	1.0	0.135	0.726	
time:ratio	1.381	1.0	11.178	0.016	*
I(ratio ** 2)	0.023	1.0	0.19	0.678	
I(time ** 2)	5.578	1.0	45.158	0.001	*
Residual	0.741	6.0	-	-	

The contribution of each factor is measured having removed the effects of all other factors. The p-values test the statistical significance of each factor. Since the p-values of extraction time, ratio:time interaction and quadratic extraction time are less than 0,05 (0.000, 0.016 and 0.001 respectively), these factors have a statistically significant effect on Protein Recovery at the 95,0% confidence level.

The Regression Equation shows the result of fitting a multiple non-linear regression model to describe the relationship between Protein Recovery and 2 independent variables (extraction time and ratio). The equation of the fitted model, taking into consideration the two factors, the quadratic factors and their interaction, is:

$$\textbf{Protein yield} = 13.65335 + 0.3450133 * \textbf{extraction time} + 130 * \textbf{ratio} - 2.35 * \textbf{time} * \textbf{ratio} + 937.5 * \boldsymbol{ratio^2} - 0.002314 * \boldsymbol{time^2} \quad \text{(Eq. 5)}$$

The R-Squared indicates that the model as fitted explains 99% of the variability in Protein Recovery. The adjusted R-squared statistic, which is more suitable for comparing models with different numbers of independent variables, is 98%.

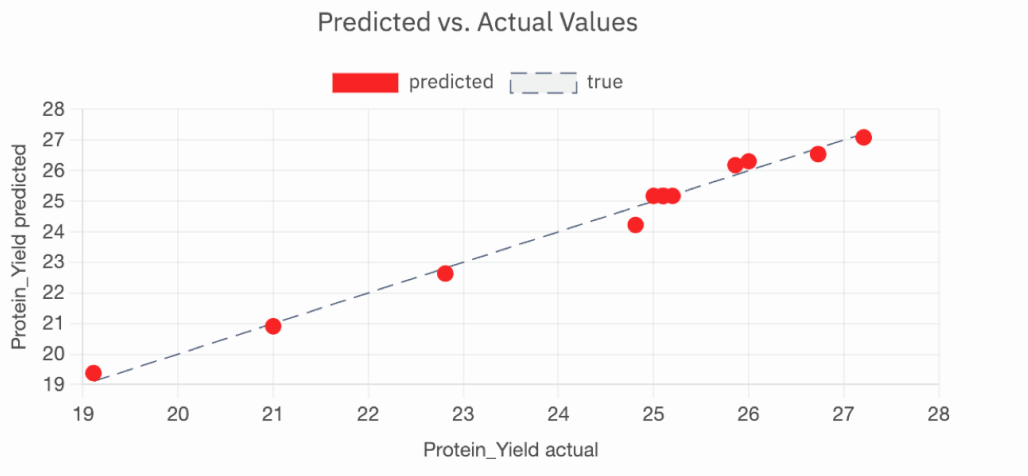


Diagram 5. Predicted by the regression model vs Actual values of Protein Yield (sunflower cake).

Model Optimization

Based on the Regression Equation for Conventional Extraction, the optimum conditions are:

- Extraction Time → 52.8571 minutes (~ 60 minutes)
- Ratio → 1:50
- Expected Protein Yield = 27.2%

4.4.2 Rapeseed press cake

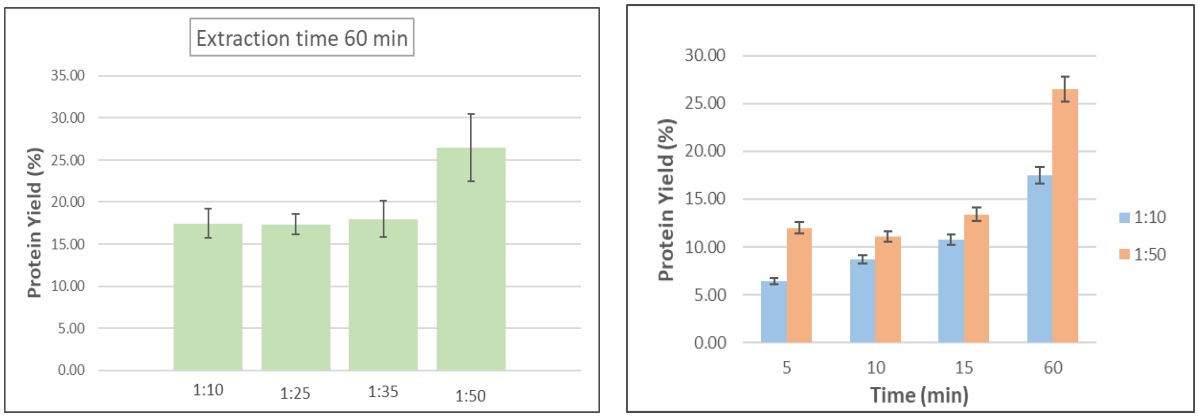
In Table 11 the results from conventional rapeseed extraction were presented. The results revealed the differences in %protein recovery when different ratio and extraction time were applied.

Table 11. Precipitation yield and protein recovery for different times and ratios through conventional extraction methods.

Ratio (solid:liquid)	Extraction time (min)	Precipitation yield (%)	Protein recovery (%)
1:10	5	2,63	9,01
1:10	10	2,53	8,68
1:10	15	4,40	15,08
1:10	60	7,16	24,54
1:50	5	4,91	16,81
1:50	10	4,55	15,58
1:50	15	10,60	36,33
1:50	**60**	**10,85**	**37,19**

Table 12. Precipitation yield and protein recovery for different ratios through conventional extraction methods for extraction duration of 60 minutes.

Ratio (solid:liquid)	Extraction time (min)	Precipitation yield (%)	Protein recovery (%)
1:10	60	7.16	17.47
1:25	60	7.10	17.32
1:35	60	7.36	17.96
1:50	**60**	**10.85**	**26.47**



(a) **(b)**

Diagram 6. Conventional extraction results at constant ratio (a) and constant extraction time (b).

The first chart (Diagram 6-a) shows how protein yield varies with extraction time at constant ratios of 1:10 and 1:50. Overall, protein yield increases as extraction time extends. The optimum extraction time for both ratios are 60 min, while the samples with the ratio of 1:50 revealed increased yields.

The second chart (Diagram 6-b) displays the protein yield across different ratios (1:10, 1:25, 1:35, 1:50) while maintaining a constant extraction time of 60 minutes. The graph indicates minimal variation in protein yield among the four ratios, except for 1:50 which is the most effective. Gerzhova et al. (2015) found 31.18% protein yield using conventional alkaline extraction at pH 10 for extraction duration of 60 minutes and for a ratio approximately of 1:6.

From Diagrams 6a-6b, it is assumed that an increased ratio correlates with enhanced precipitation yields and protein recoveries when employing the conventional method. The same trend was observed over time for both ratios. The maximum recovery is found for the ratio of 1:50 and extraction duration of 120 min.

4.5 Ultrasound assisted extraction

Sunflower press cake

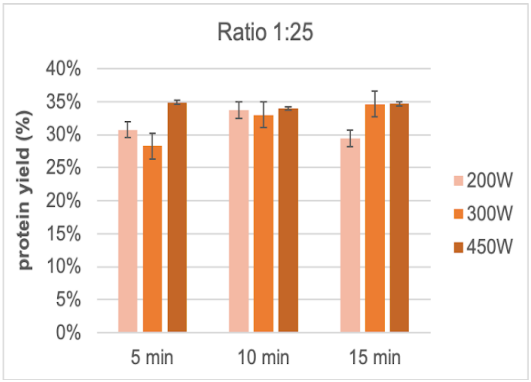
Ultrasound assisted extraction was applied for sunflower protein extraction as an eco-friendly method (Table 12). The first two charts that conclude the obtained results (Diagrams 7a, 7b), illustrate the Protein Yield as a function of extraction time and ultrasound power at constant ratio 1:25. The graphs show that higher values of power result in greater protein yield with 450 Watt being the optimum power. Moreover, extraction time does not affect the yield significantly. The optimum conditions are 5 minutes of extraction time and 450 Watt.

The other two charts (Diagrams 7c, 7d) illustrate the Protein Yield as a function of extraction time and ultrasound power at constant ratio 1:50. It is evident that 300 and 450 Watt do not differ at constant ratio and time, 200 Watt power results in the lowest values of protein yield. The optimum conditions are 10 minutes of extraction time and 450 Watt.

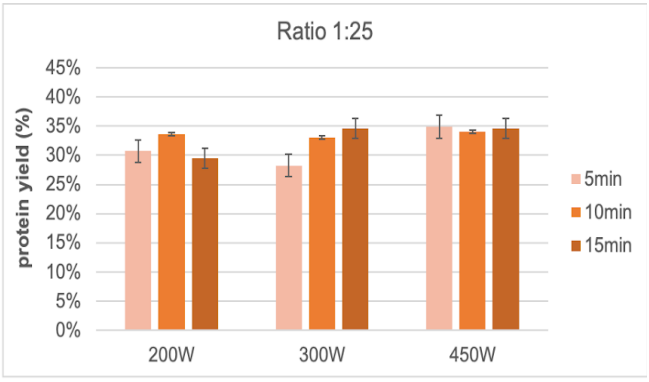
Table 12. Ultrasound-Assisted Extraction results (sunflower cake).

Ultrasound Power: 200W			Ultrasound Power: 300W			Ultrasound Power: 450W		
Ratio	Extraction Time (min)	Precipitation Yield (%) / Protein Recovery (%)	Ratio	Extraction Time (min)	Precipitation Yield / Protein Recovery (%)	Ratio	Extraction Time (min)	Precipitation Yield / Protein Recovery (%)
1:25	5	15,6% / **30,8%**	1:25	5	14,3% / **28,3%**	1:25	5	17,7% / **34,9%**
1:25	10	17,1% / **33,7%**	1:25	10	16,8% / **33,0%**	1:25	10	17,3% / **34,0%**
1:25	15	15,0% / **29,5%**	1:25	15	17,6% / **34,6%**	1:25	15	17,6% / **34,7%**
1:50	5	17,0%	1:50	5	18,9%	1:50	5	19,1%

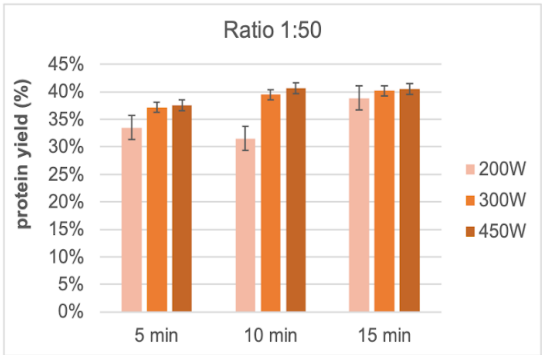
		33,5%			**37,2%**		**37,6%**
1:50	10	16,0%	1:50	10	20,1%	1:50	10	20,6%
		31,5%			**39,5%**			**40,6%**
1:50	15	19,7%	1:50	15	20,4%	1:50	15	20,6%
		38,9%			**40,2%**			**40,5%**



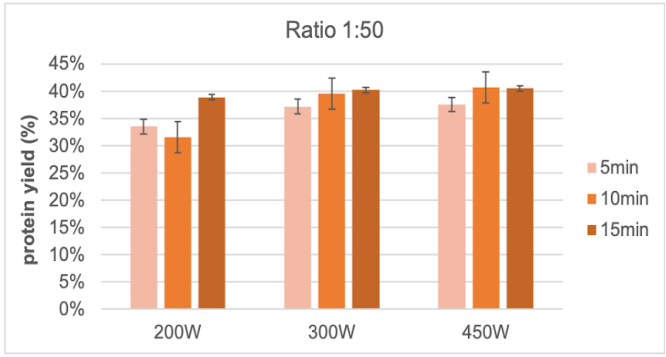
(a)



(b)



(c)



(d)

Diagram 7. Ultrasound-Assisted Extraction results at different values of ratio, extraction time and ultrasound power (sunflower cake).

Design of Experiments - DoE

Three factors (extraction time, ratio and ultrasound power) were used to optimize the UAE using Response Surface Design. A Central Composite Design and, more specifically, Center-Faced Design, was

employed to generate a total of 20 experiments that were carried out in random order. The experimental (independent) variables were:

- extraction time: 5, 10, 15 minutes
- ratio: 1:25, 1:35, 1:50
- ultrasound power: 200, 300, 450, 600 Watt

The only response was the Protein Yield (%) and the 20 experiments are presented in Table 13.

Table 13. Experimental runs for UAE using Response Surface Design (sunflower cake).

UAE					
Standard order	Run order	Extraction Time (min)	Ratio	Ultrasound Power (Watt)	Protein Yield (%)
1	20	5	0,02	200	33,5
2	9	15	0,02	200	38,9
3	21	5	0,04	200	30,8
4	12	15	0,04	200	29,5
5	14	5	0,02	600	53,1
6	1	15	0,02	600	40,3
7	15	5	0,04	600	38,3
8	4	15	0,04	600	37,5
9	19	10	0,03	400	40,0
10	16	10	0,03	400	40,0
11	11	5	0,03	400	37,0
12	6	15	0,03	400	41,0
13	8	10	0,02	400	40,6
14	7	10	0,04	400	34,0
15	3	10	0,03	200	37,0
16	5	10	0,03	600	41,2
17	13	10	0,03	400	40,1
18	2	10	0,03	400	40,2
19	17	10	0,03	400	41,3
20	10	10	0,03	400	40,4

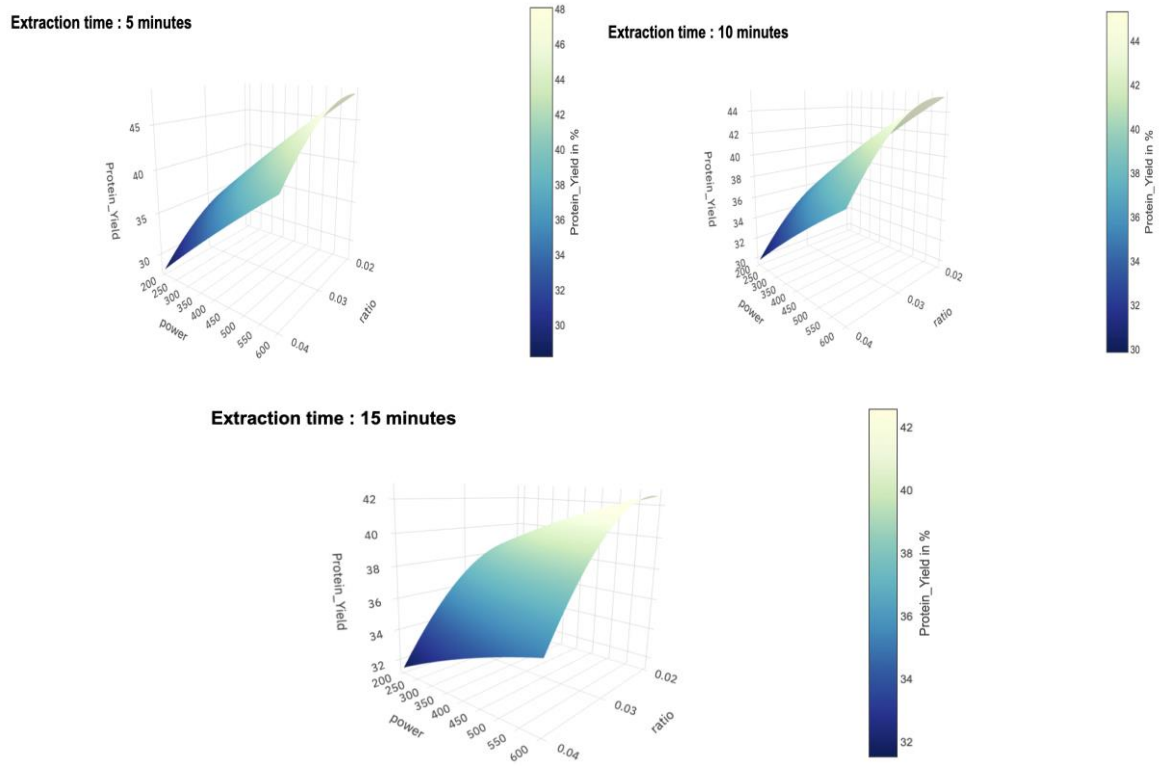


Diagram 8. Response Surface Model for the UAE at different values of extraction time (sunflower cake).

In order to statistically analyze the data in Table 13, ANalysis Of VAriance (ANOVA test) and Multiple Regression were performed. The ANOVA table and the Regression Equation are shown below (Table 14).

Table 14. ANOVA table for UAE (sunflower cake).

Factors	Sum Of Squares	DF	F-Value	p-Value	significant
time	3.036	1.0	0.515	0.485	
ratio	6.697	1.0	1.137	0.304	
power	5.3	1.0	0.9	0.359	
time:power	39.029	1.0	6.624	0.022	*
I(power ** 2)	0.236	1.0	0.04	0.844	
I(ratio ** 2)	13.599	1.0	2.308	0.151	
Residual	82.489	14.0	-	-	

The contribution of each factor is measured having removed the effects of all other factors. The p-values test the statistical significance of each factor. Since the p-values of time:power interaction is less than 0,05 (0.022), this factor has a statistically significant effect on Protein Recovery at the 95,0% confidence level.

The Regression Equation shows the result of fitting a multiple non-linear regression model to describe the relationship between Protein Recovery and three independent variables (extraction time, ratio and ultrasound power). The equation of the fitted model, taking into consideration the three factors, the quadratic factors and their interaction, is:

$$\textbf{Protein yield} = 14.8859 + 0.7733 * \textbf{extraction time} + 967.08182 * \textbf{ratio} + 0.04255425 * \textbf{power} - 0.002209 * \textbf{time} * \textbf{ratio} - 22166.363637 * \textbf{ratio}^2 \quad \text{(Eq. 6)}$$

The R-Squared indicates that the model as fitted explains 82% of the variability in Protein Recovery. The adjusted R-squared statistic, which is more suitable for comparing models with different numbers of independent variables, is 75%.

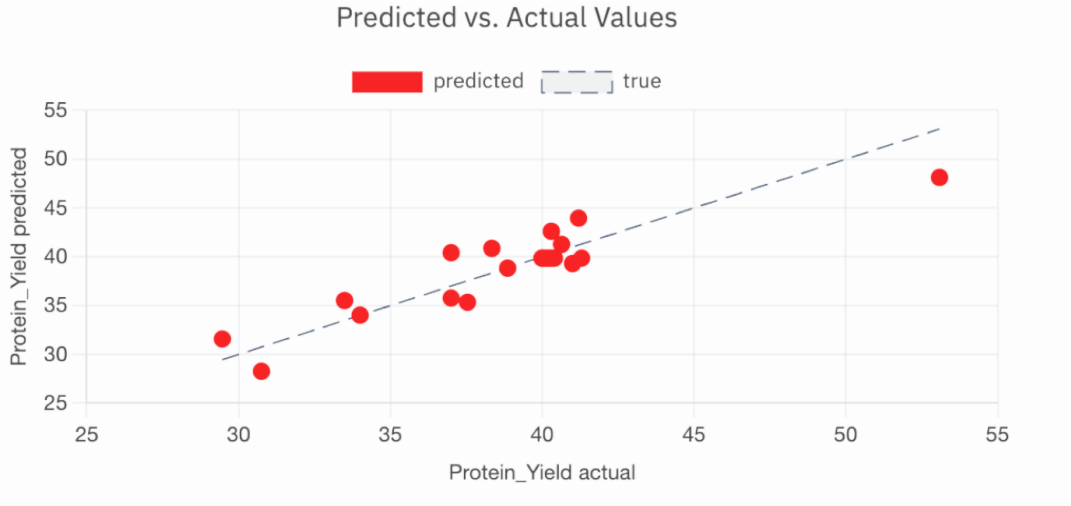


Diagram 9. Predicted by the regression model vs Actual values of Protein Yield for UAE (sunflower cake).

Model Optimization

Based on the Regression Equation for UAE, the optimum conditions are:

- Extraction Time → 5 minutes
- Ratio → 1:50
- Ultrasound Power → 600 Watt
- Expected protein yield = 48.2%

Rapeseed cake

Ultrasound Assisted extraction was applied as an eco-friendly method for the extraction of rapeseed protein and the results are included in Table 15. Based on Diagram 10, distinct ratios exhibit varying trends in ultrasound-assisted extraction method for rapeseed protein extraction. Concerning the ratio of 1:50, both precipitation yield and protein recovery increase over time. Conversely, these values

remain stable over the examined time periods for the ratio of 1:10. The maximum recovery is found for the ratio of 1:50 and extraction duration of 15 min. According to Diagram 10, rapeseed's protein yield increases over time for both ratios examined. The highest recovery was observed at an extraction time of 15 minutes and a ratio of 1:10.

Table 15. Precipitation yield and protein recovery for different times and ratios through UAE method.

UAE			
Ratio (solid:liquid)	**Extraction Time (min)**	**Precipitation yield (%)**	**Protein recovery (%)**
1:10	5	6.99	17.06
1:10	10	8.20	20.01
1:10	**15**	**10.89**	**26.57**
1:50	5	8.03	19.58
1:50	10	9.45	12.73
1:50	15	9.72	23.72

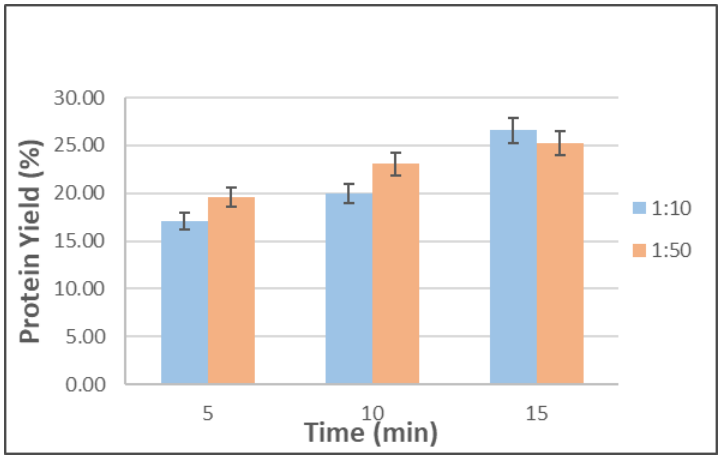


Diagram 10. Results of protein yields for different times and ratios through UAE method.

4.6 Microwave assisted extraction

The chart below (Diagram 11) describes the Protein Yield from sunflower cake as a function of extraction time and ratio at constant microwave power 250 Watt. Overall, at 5 minutes of extraction time, there is an upward trajectory as the ratio decreases. At 10 and 15 minutes there is not a specific pattern. The optimum conditions are observed at 5 minutes of extraction time and 1:50 ratio. The results from rapeseed protein microwave extraction are not concluded in this deliverable.

Table 16. Microwave-Assisted Extraction results.

Microwave Power: 250W			
Ratio	**Extraction Time (min)**	**Precipitation Yield (%)**	**Protein Recovery (%)**
1:25	5	9,8%	19,3%
1:25	10	10,9%	21,5%
1:25	15	14,5%	28,6%
1:35	5	12,8%	25,1%
1:35	10	12,8%	25,3%
1:35	15	10,0%	19,6%
1:50	**5**	**15,0%**	**29,6%**
1:50	10	8,4%	16,6%
1:50	15	11,0%	21,6%

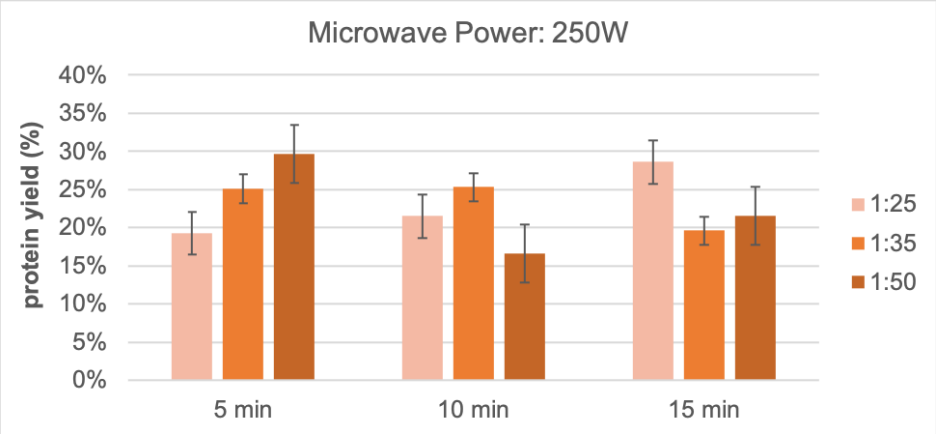


Diagram 11. Microwave-Assisted Extraction results at different values of microwave power, extraction time and ratio.

Design of Experiments - DoE

Two factors (extraction time and ratio) were used to optimize the MAE using Response Surface Design. A Central Composite Design and, more specifically, Center-Faced Design, was employed to generate a total of 12 experiments that were carried out in random order. The experimental (independent) variables were:

- extraction time: 5, 10, 15 minutes
- ratio: 1:25, 1:35, 1:50

The only response was the Protein Yield (%) and the 12 experiments are presented in Table 17.

Table 17. Experimental runs for MAE using Response Surface Design (sunflower cake).

MAE				
Standard order	**Run order**	**Extraction Time (min)**	**Ratio**	**Protein Yield (%)**
1	6	5	0:28	29,6
2	4	15	0:28	21,6
3	10	5	0:57	19,3
4	2	15	0:57	28,6
5	7	10	0:43	25,3
6	9	10	0:43	25,3
7	11	5	0:43	25,1
8	8	15	0:43	23,6
9	1	10	0:28	20,6
10	3	10	0:57	21,5
11	5	10	0:43	25,3
12	12	10	0:43	25,3

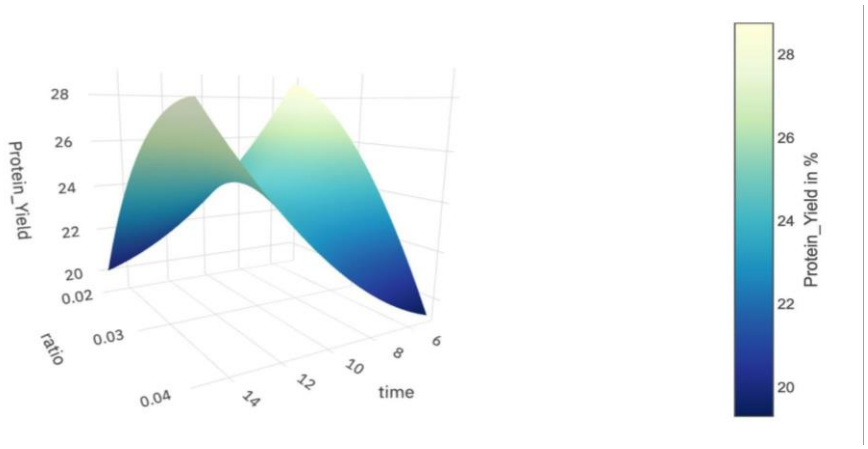


Diagram 12. Response Surface Model for the MAE (sunflower cake).

In order to statistically analyze the data in Table 17, ANalysis Of VAriance (ANOVA test) and Multiple Regression were performed. The ANOVA table and the Regression Equation are shown below (Table18).

Table 18. ANOVA table for MAE (sunflower cake).

Factors	Sum Of Squares	DF	F-Value	p-Value	significant
time	5.055	1.0	1.659	0.245	
ratio	8.975	1.0	2.946	0.137	
time:ratio	74.823	1.0	24.56	0.003	*
I(time ** 2)	5.134	1.0	1.685	0.242	
I(ratio ** 2)	9.754	1.0	3.202	0.124	
Residual	18.279	6.0	-	-	

The contribution of each factor is measured having removed the effects of all other factors. The p-values test the statistical significance of each factor. Since the p-value of time:ratio interaction is less than 0,05 (0.003), this factor has a statistically significant effect on Protein Recovery at the 95,0% confidence level.

The Regression Equation shows the result of fitting a multiple non-linear regression model to describe the relationship between Protein Recovery and two independent variables (extraction time and ratio). The equation of the fitted model, taking into consideration the three factors, the quadratic factors and their interaction, is:

$$\textbf{Protein yield} = 40.075 - 3.711667 * \textbf{extraction time} + 242.5 * \textbf{ratio} - 86.5 * \textbf{time} * \textbf{ratio} - 19125 * \boldsymbol{ratio^2} + 0.0555 * \boldsymbol{time^2} \quad \text{(Eq. 7)}$$

The R-Squared indicates that the model as fitted explains 83% of the variability in Protein Recovery. The adjusted R-squared statistic, which is more suitable for comparing models with different numbers of independent variables, is 68%.

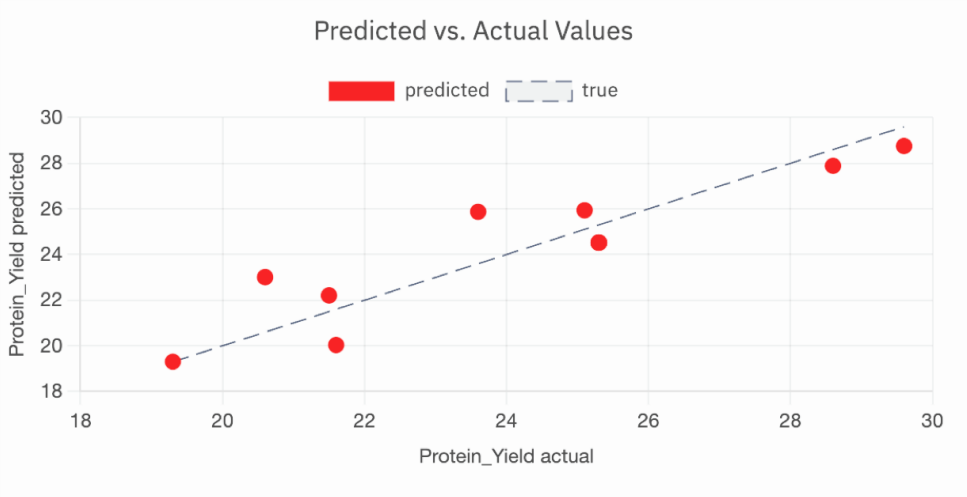


Diagram 13. Predicted by the regression model vs Actual values of Protein Yield for MAE (sunflower cake).

Model Optimization

Based on the Regression Equation for MAE, the optimum conditions are:

- Extraction Time → 5 minutes
- Ratio → 1:50
- Expected Protein Yield = 28.8%

Optimization - Summary

According to the Design of experiments and the optimization results, the optimum conditions for the three extraction methods regarding the sunflower protein extraction are presented below:

CE
Time 60 min
Ratio 1:25

UAE
Time 5 min
Ratio 1:50
Ultrasound Power 600 Watt

MAE
Time 5 min
Ratio 1:50
Microwave Power 250 Watt

Comparison between the optimum results obtained from the Conventional Extraction (CE), the Ultrasound-Assisted Extraction (UAE) and the Microwave Assisted Extraction (MAE) is shown below:

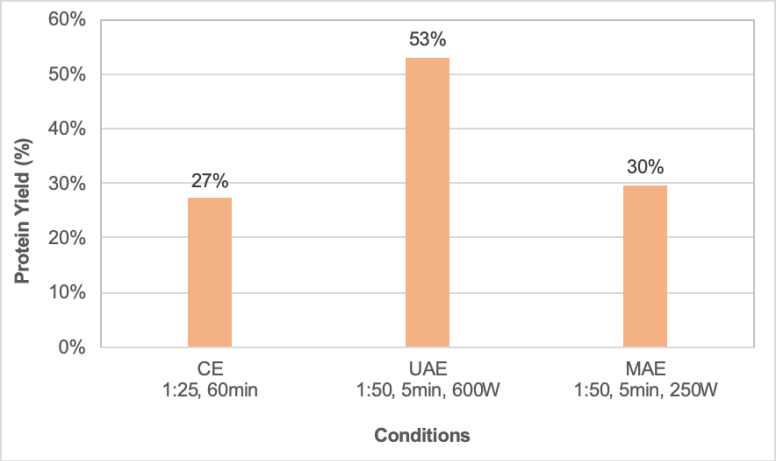


Diagram 14. Comparison between the optimum conditions of CE, UAE and MAE for sunflower protein extraction.

As Diagram 14 indicates, the protein yields obtained after the application of the optimum conditions of each method are in accordance with the expected yields derived from the Regression Models. UAE is the best extraction method of the three studied. A combination of microwaves and ultrasounds seems a promising approach for higher protein extraction.

4.7 Pressure Liquid Extraction

Preliminary experiments were conducted for the extraction of protein from both rapeseed and sunflower cakes using pressure liquid extraction. In Table 19, the data of rapeseed protein PL extraction are summarized. Data from sunflower cake PL extraction are not shown. Regarding the parameters, the pressure was set stable at 1750 psi and the temperature remained near the room temperature.

Table 19. Precipitation yield and protein recovery for different times and ratio of 1:50 through PLE method.

Pressurized Liquid Extraction			
Ratio (solid:liquid)	**Extraction Time (min)**	**Precipitation yield (%)**	**Protein recovery (%)**
1:50	5	2.15	5.31
1:50	10	2.08	5.12

According to the results from the preliminary experiments, it seems that the PLE method is not as effective as the corresponding method of conventional and ultrasound-assisted extraction. The protein yield is much lower for the same extraction times and ratios. Therefore, further study of the method is recommended.

5.Conclusions

The objective of Task 5.2 is to evaluate the efficient recovery of proteins from edible oil industry by-products, namely, rapeseed, sunflower and cotton press cakes, and the results (up to June 2024) are reported in the present Deliverable (D5.2), comprising the first version of the reporting focused on "***Proteins recovery for wood-resin composite production***". Specifically, defatted sunflower and rapeseed press cake (protein extraction from cotton press cake is underway), were used for the extraction of protein via the implementation of conventional and novel, energy-efficient extraction technologies, including Microwave Assisted Extraction, Ultrasound Assisted Extraction and Pressurized Liquid Extraction. Following the alkaline extraction of the sunflower and rapeseed press cakes, the soluble proteins were precipitated by adjusting the pH to their isoelectric point and the residues were freeze-dried to obtain the protein isolates. The extraction parameters including solid-liquid ratio (1:10, 1:25, 1:35, 1:50), microwave and ultrasound power, pressure and extraction time (5, 10, 20 and 60 min) were optimized in order to achieve high protein recovery yields. The protein content of the crude cakes and the extracts was evaluated using Kjeldahl method. According to the results, rapeseed's and sunflower's press cake protein content was calculated 25.2% and 72.6%, respectively. Protein extracts from rapeseed and sunflower press cakes can be effectively recovered via the studied methods presenting protein content 62 to 72%. The novel technologies resulted in higher protein yields decreasing the extraction time using green solvents and leading to the production of high-standards proteins. Especially, for sunflower protein extraction the highest protein yield was calculated for ultrasound assisted extraction reaching 53%. However, in case of rapeseed the %protein yield achieved the highest value applying conventional methodology (37%). For sunflower press cake extraction design of experiments was used and the experimental results that were conducted in the optimum conditions revealed similarly %protein recovered yield with the expected values from the regression equations.

In conclusion, D. 5.2 "Proteins recovery for wood-resin composite production v1" is the first version of the reporting presenting the results on Task 5.2, focusing on the extraction of proteins from different oilseed press cakes using eco-friendly extraction technologies, accomplished during the period up to Jine 2024 (M18). The experimental work in Task 5.2 will be continued with the overall results being presented in deliverable D5.3 "Proteins recovery for wood-resin composite production FINAL" according to the Grant Agreement.

Disclaimer of Warranties

6.References

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