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Review article

RAPESEED AS THE SOURCE OF PROTEINS: A REVIEW

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Abstract: Rapeseed proteins can be isolated as high-value components from residual materials of oilseed processing. This review provides an overview of rapeseed protein isolate production, with an emphasis on conventional alkaline extraction and alternative methods. Special attention is given to antinutrient compounds found in rapeseed (glucosinolates, phenolic compounds, phytic acid, and others) and the strategies to mitigate them. Techniques that are effective in not only removing antinutrients but also increasing protein yield and reducing extraction time are discussed, including ultrasound, microwave, and enzymatic pretreatments. Enzymatic hydrolysis for obtaining rapeseed protein hydrolysates is also discussed, along with novel extraction methods for protein isolate production, particularly the use of natural deep eutectic solvents (NADES).

Key words: *rapeseed, protein isolates, antinutrients, extraction*

INTRODUCTION

Rapeseed (*Brassica napus* L.) is an herbaceous, annual plant belonging to the *Brassicaceae* family (Chmielewska et al., 2021). Its oil-rich seeds contain approximately 40–45% oil, characterized by a favorable ratio of omega-3 to omega-6 fatty acids (Banaś, Piwowar & Harasym, 2023). As a result, rapeseed holds a prominent position in the global oilseed industry, ranking after palm and soybean oil in term of production volume (FEDIOL, 2022). After industrial de-oiling using hexane, a protein-rich rapeseed meal is obtained as a by-product, containing more than 40% protein with a well-balanced amino acid composition (Aider & Barbana, 2011; Chmielewska et al., 2021). Additionally, rapeseed meal includes carbohydrates, crude fibres, minerals, and secondary plant metabolites (Von Der Haar, Müller, Bader-Mittermaier & Eisner, 2014). During cold-pressed rapeseed oil production, rapeseed cake is generated as another protein-rich by-product. Both of these by-products are traditionally used as animal feed but are increasingly recognised as valuable raw materials for nutrient recovery and sustainable protein production (Borrello, Caracciolo, Lombardi, Pascucci & Cembalo, 2017).

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The protein profile of rapeseed by-products, characterised by a balanced amino acid composition and desirable functional properties, makes rapeseed a promising alternative in the plant-based protein market, which is currently dominated by soy protein (Chmielewska et al., 2021). Additionally, rapeseed's adaptability to diverse agricultural conditions and its substantial global production reinforces its potential as a sustainable source of proteins with significant nutritional value.

Despite its advantages, the utilization of rapeseed proteins is limited by the presence of antinutritional factors such as phytates, glucosinolates, and polyphenols (Zhang et al., 2020; 2024). These compounds reduce nutrient bioavailability, necessitating the development of effective mitigation strategies during protein extraction and processing (Fetzer, Müller, Schmid & Eisner, 2020).

Rapeseed proteins primarily consist of three major fractions – cruciferin, napin, and oleosins, among approximately 40 identified protein fractions (Von Der Haar et al., 2014).

Cruciferin and napin, the predominant storage proteins, exhibit diverse functional properties, including emulsification, gelling, and foaming, making them suitable for various food applications such as meat substitutes, confectionery, and beverages (Aider & Barbana, 2011; Wanasundara, 2011).

Additionally, the growing demand for sustainable protein sources has sparked interest in non-food purposes of rapeseed proteins, including bio-adhesives, bio-foams, and bioplastics, highlighting their versatility in industrial applications (Wanasundara, 2011).

This review aims to comprehensively evaluate conventional and novel methods for rapeseed protein extraction, discuss strategies for reducing antinutritional factors, and explore the potential of rapeseed protein isolates in both food and industrial sectors. By synthesising current research findings and identifying future research directions, this review seeks to contribute to the advancement of sustainable protein utilisation from rapeseed.

RAPESEED PROTEINS

Rapeseed is a rich source of various components, including oil, protein, carbohydrates (such as cellulose, polysaccharides, and free sugars), glucosinolates, phenolic compounds, and phytic acid. The primary product derived from rapeseed is oil, while the remaining meal, after oil extraction, is notably high in protein, typically containing around 34–40% protein (Wanasundara, Tan, Alashi, Pudel & Blanchard, 2017). Due to its well-balanced amino acid composition and functional properties, rapeseed protein is gaining attention as a promising alternative for plant-based proteins. With the presence of essential amino acids at levels exceeding 400 mg/g of protein, and sulphur-containig amino acids (methionine and cysteine) ranging from 3.0–4.0% (40–49 mg/g of protein), it is perfectly suited to be used as a functional food ingredient and to meet human requirements for both essential and non-essential amino acids (Chmielewska et al., 2021).

Around 90% of the proteins found in rapeseed are storage proteins, with the predominant types being 11/12S cruciferin and 2S napin. These proteins have molecular weights of approximately 300–350 kDa and 12–16 kDa, respectively (Wanasundara, McIntosh, Perera, Withana-Gamage & Mitra, 2016). Additionally, other minor proteins, such as oil body proteins and lipid transfer proteins, have also been identified (Wanasundara et al., 2017). These proteins, besides having nutritional benefits, exhibit diverse functional properties, including good solubility, emulsification, foaming, and gelation, making them valuable functional food components (Chmielewska et al., 2021).

Cruciferin

Cruciferin, classified as an 11/12S globulin, belongs to the cupin superfamily. It has a well-defined primary, secondary, tertiary, and quaternary structure. The protein is composed of six subunits or protomers, organised into two trimers, with each subunit containing two polypeptides. The polypeptide α (approximately 30 kDa) consists of 254–296 amino acids, while polypeptide β (approximately 20 kDa) consists of 189–191 amino acids (Wanasundara et al., 2017). A single disulphide bond links these polypeptide chains and trimers are primarily stabilised by non-covalent bonds such as hydrophobic, electrostatic, hydrogen, van der Walls, and hydrogen-bonded salt bridges (Wanasundara et al., 2016). The secondary structure of cruciferin comprises 27 β strands and 7 heli-

ces, which are folded into domains including two β-barrel and two extended helices. The mature subunit of cruciferin has a molecular mass ranging from 48 to 56 kDa and an isoelectric point at pH 7.25 (Wanasundara et al., 2017).

Cruciferin is predominantly insoluble at lower pH levels but becomes highly soluble above pH 5.5. It possesses moderate emulsifying properties; however, its globular conformation limits surface activity, leading to formation of emulsions with relatively large droplet sizes. Stability of these emulsions is influenced by pH and protein purity. Additionally, cruciferin exhibits satisfactory foaming ability, though its foam stability is lower than that of napin (Wanasundara et al., 2016). Cruciferin also exhibits strong gelation properties, forming elastic gels, particularly under high-temperature and alkaline conditions (pH 9–11). Its ability to form structured gel networks makes it potential gelling agent in food applications including soups, sauces and processed meat products (Chmielewska et al., 2021).

Napine

Napine, classified as a 2S albumin, is a member of the prolamin superfamily. Its mature structure consists of two polypeptide chains, a short one (4 kDa) and a long one (9 kDa), linked together by two inter-chain disulphide bonds. The primary structure is composed of 111–180 amino acid residues (Wanasundara et al., 2017). Additionally, the long chain contains two intra-chain disulphide bonds, stabilising the molecule through the development of four disulphide bridges (Wanasundara et al., 2016). Several isoforms of napin have been identified, including Napin-1, Napin-2, Napin-3, Nap1, Napin-1A, and Napin-B (Wanasundara et al., 2016). The secondary structure of napin is characterised by 38% β-sheet and 25% α-helix composition (Wanasundara, 2011). Napin exhibits an isoelectric point above pH 10 (Von Der Haar et al., 2014) and remains stable up to 75 ºC (Wanasundara et al., 2017).

Unlike cruciferin, napin exhibits high solubility across a broad pH range (2–10) and in water, enhancing its versatility in various food applications. Its superior emulsifying properties enable formation of more stable and finely dispersed emulsion compared to cruciferin. Additionally, napin demonstrates exceptional foaming ability, particularly in its purified form, contributing to the stability of aerated food systems. These unique functional attributes make napin a valuable ingredient in a range of applications, including beverages, confectionery, and baked goods (Wanasundara et al., 2016). Beyond its nutritional and functional roles in food products, napin-like proteins have been associated with various biological activities, such as antimicrobial properties. Furthermore, Wanasundara (2011) identified bioactive peptide sequences embedded within napin, exhibiting potential antioxidative, antihypertensive, anorectic properties, highlighting multifunctional potential of napin.

Oil body proteins (OBP)

Oil bodies are intracellular organelles primarily composed of triacylglycerols and sterol esters, surrounded by phospholipids and integral proteins. These proteins play a crucial role in stabilizing oil bodies, preventing coalescence, and regulating lipid storage and mobilisation. They possess both hydrophilic and hydrophobic domains, with the hydrophilic domain exposed to the aqueous phase, and the hydrophobic domain interacting with the lipid phase (Chmielewska et al., 2021).

Showing good oil-water interface stabilisation ability makes oil-body proteins relevant for food and industrial applications (Wanasundara et al., 2017).

Three types of oil body proteins have been identified: oleosins, caleosins, and steroleosins. Oleosins, with a molecular mass ranging from 18 to 25 kDa, are the predominant proteins found in rapeseed oil bodies (90%). Because of their unique structure, resembling surfactants with hydrophilic heads and hydrophobic chains, they exhibit high interfacial activity and emulsifying properties, making them valuable in various food emulsions (Chmielewska et al., 2021). Caleosins and steroleosins, although less abundant, have important functional roles. Caleosins, with molecular mass of approximately 30 kDa, possess ability to bind calcium and stabilize oil bodies, while steroleosins, with molecular mass of approximately 40 kDa, are much less investigated. With their sterol-binding sites, steroleosins are thought to play a role in sterol signal transduction and membrane dynamics (Tzen, 2012). Due to their sterol-binding sites and amphipathic structure, both proteins could contribute to

emulsifying capacity of rapeseed protein fractions.

CONVENTIONAL PROTEIN EXTRACTION METHODS

The extraction of rapeseed proteins from seeds or defatted meals involves various isolation techniques, resulting in different ratios and purity levels of cruciferin and napin depending on the method used (Fetzer et al., 2020).

Aqueous extraction, which utilises water as the extraction medium alone or combined with acidic, basic, or saline agents or solvents, is a widely used method. Traditionally, aqueous extraction can be either acidic or alkaline (Arrutia, Binner, Williams & Waldron, 2020). Proteins isolated using aqueous extraction are further purified using techniques such as precipitation or ultrafiltration (Fetzer et al., 2020).

Aqueous alkaline extraction, patented by Louis and Morton in 1957, is the prevailing conventional method for extracting protein-enriched fractions from oilseed press cakes and meals. This method dissolves the proteins in an alkaline solution, eliminates insoluble material via centrifugation, and precipitates the protein by adjusting the pH to their isoelectric point, followed by of centrifugation for separation. Numerous alternative improved versions of this method have emerged (Arrutia et al., 2020).

The solubility of proteins in an aqueous medium is influenced by the balance between electrostatic and hydrophobic interactions among the protein molecules. Stronger electrostatic forces increase solubility, with proteins exhibiting negative (or positive) charges at pH levels above (or below) their isoelectric point (pI), leading to an increase in solubility. Given the importance of protein solubility for extraction, rapeseed protein extractions are typically performed at alkaline pH levels (Table 1). Alkalis such as sodium hydroxide (NaOH) are commonly employed to achieve high alkaline pH values. pH levels between 10–12 are typically required to achieve optimal extraction yields and efficiencies from rape-seed meals (Wanasundara et al., 2017).

Variations in the isoelectric points affect solubility, enabling selective precipitation. In the case of rapeseed, lowering the pH to around pH 3.5–4.5, predominantly precipitates cruciferin proteins (Fetzer et al., 2020). Acidic solutions such as HCl or acetic acid are commonly utilised in laboratory settings for protein recovery. Ultrafiltration, a membrane separation technique, serves as an alternative to pH adjustments for recovering proteins from alkaline extracts, while simultaneously removing non-protein compounds such as phytates, gluco-sinolates, and polyphenolic compounds (Wanasundara et al., 2017). Ultrafiltration allows for selective protein fractionation and is often used after precipitation or directly after

Table 1. Extraction procedures, protein yields, and protein contents of rapeseed protein isolates

Material	Process conditions	Protein yield (% w/w)	Protein content (N x 6.25) (% dry basis)	References
Defatted canola meal	1) ethanol pretreatment EtOH: H_2O = 7:3, s:l = 1:10 2) pH 10.0 (1 M NaOH), s:l = 1:9, 300 rpm, 60 °C, 15 min 3) 3300 rpm, 4 °C, 10 min 4) pH to 5.0 (acetic acid) or ultrafiltration for protein purification 5) 3300 rpm, 4 °C, 10 min (for acidic precipitation) 6) lyophilisation	i) alkaline extraction only: AP: 9.54 UF: 4.88 ii) ethanol pretreatment + alkaline extraction: AP: 18.5 UF: 20.6	i) alkaline extraction only: AP: 85.2 UF: 88.1 ii) ethanol pretreatment + alkaline extraction: AP: 88.2 UF: 89.7	Cháirez-Jiménez, Castro-López, Serna-Saldívar & Chuck-Hernández (2023)
Dehulled and defatted rapeseed meal	1) 0.2 M NaCl, s:l = 1:8, pH to 8.0 (1 M NaOH), 25 °C, 1 h 2) 8000 rpm, 10 min 3) ultrafiltration and dialysis 4) freeze-drying	36.0	94.4	Jia et al. (2021)

Table 1. continued

Dehulled and defatted canola flour	1) ethanol pretreatment EtOH: H_2O = 7:3, s:l = 1:10 2) pH 11.0 (2 M NaOH), s:l = 1:10, 2 h, RT 3) 8000 rpm, 30 min 4) pH to 4.5 (1 M HCl) 5) 5000 rpm, 20 min, washed H_2O 6) lyophilization	n/a	n/a	Jiang, Nie, Sun & Xiong (2021)
i) Cold-pressed rapeseed press cake **ii) Hot-pressed rapeseed meal** **iii) Solvent-extracted rapeseed meal**	1) pH 10.5 (2 M NaOH), s:l = 1:10, 4 °C, 3 h 2) 5000 rpm, 4 °C, 30 min 3) pH 5.0 (citric acid) 4) heating: 80 °C, 4 min 5) cooling to 25 °C in a cold water bath (5.5 min) 6) 5000 rpm, 4 °C, 30 min 7) no drying *steps 3)–5) optional	i) 45; heated: 34 ii) 26; heated: 23 iii) 5; heated: 8	i) 59.3; heated: 40.1 ii) 72.0; heated: 55.6 iii) 65.7; heated: 80.3	Östbring, Malmqvist, Nilsson, Roselind & Rayner (2020)
Cold-pressed rapeseed press cake	1) pH 10.5, s:l = 1:10, 3 h, 4 °C 2) 5000 rpm, 4 °C, 30 min 3) pH 5.0 (citric acid) 4) heating: 80 °C, 4 min 5) cooling to RT in a cold water bath 6) 5000 rpm, 4 °C, 30 min 7) no drying *steps 3)–5) optional	29–41; heated: 26–41	52.0–63.8; heated: 37.4–49.2	Östbring, Tullberg, Burri, Malmqvist & Rayner (2019)
Industrial defatted rapeseed meal	1) washed EtOH:H_2O = 3:1, (4 x s:l = 1:3, 30 min, RT) 2) pH 12.0, 75 min, 40 °C 3) separation n/a 4) pH to 4.5 (HCl) 5) 1800 rpm, 15 min, washed H_2O pH 4.5 (3 x 6 volumes) 6) no ultrafiltration, supernatant, and all washing liquids combined 7) freeze-drying	n/a	89.9 supernatant: 28.9	Ivanova, Kalaydzihev, Rustad, Silva & Chalova (2017)
Hexane-defatted commercial canola meal	1) acidic washing at pH 4.0 2) pH 12.5, s:l = 1:10, 1 h 3) filtering 4) pH to 4.0 and 4.5 (0.1M HCl) 5) 15,000 rpm, 5 °C, 20 min, washed H_2O pH 4.0 (3 x 150 mL) 6) 15,000 rpm, 5 °C, 20 min 7) freeze-drying	pH 4: 38.6 pH 4.5: 35.0	91.0 85.7	Akbari & Wu (2015)

s:l – solid:liquid; AP – acidic precipitation; UF – ultrafiltration; RT – room temperature

after extraction to obtain mixed protein preparation (Fetzer et al., 2020; Singh et al., 2022). Although alkaline extraction yields high protein levels and disrupts the cell wall structure, it is also associated with significant drawbacks (Arrutia et al., 2020). One major concern is the potential loss of protein functionality due to hydrolysis and denaturation during extrac-

tion. The protein content of isolates prepared by alkaline extraction and acid precipitation typically falls within the range of 60–90% (Wanasundara et al., 2017). Nevertheless, despite its efficacy, this method comes with notable drawbacks (Arrutia et al., 2020).

The primary concern associated with alkaline extraction is the potential loss of protein functionality. The use of strong chemicals during extraction can result in partial hydrolysis and denaturation of proteins. Protein functionality, including solubility, gelation, emulsification, and viscosity, is highly sensitive to pH. Moreover, at pH values exceeding 12, irreversible denaturation of proteins occurs, leading to a decline in their nutritive value. Severe alkaline extraction methods may also trigger various adverse effects on proteins, ultimately impacting their quality and nutritional value. These effects include racemisation of L-amino acids to their D-analogues, reduced protein digestibility due to formation of cross-linked amino acids such as lysoalanine, and damage to specific amino acids like threonine, lysine, and cysteine. Such damage to essential amino acid residues can compromise the overall nutritional quality of proteins, which are vital for human and animal health (Arrutia et al., 2020). Hence, the extraction procedure of rapeseed proteins should be conducted under mild conditions to preserve the native structure and functional properties of the proteins (Fetzer, Herfellner & Eisner, 2019).

In addition to the potential loss of protein functionality, alkaline extraction poses another significant challenge, the risk of introducing antinutrients into the end products due to enhanced extraction of non-protein components. These antinutrients may co-precipitate with the proteins during extraction, resulting in reduced product quality. This issue is particularly critical in industries where high purity is crucial, such as the food industry. Furthermore, alkaline extraction can induce the oxidation of polyphenols and initiate Maillard reactions, leading to darkening of product colour and adverse effects on taste and aroma. In general, it compromises functional, nutritional, and sensory attributes of the final product (Arrutia et al., 2020).

It is clear that despite high protein yield, the alkaline extraction method is deemed unsuitable for producing rapeseed-derived protein products intended for food application. In efforts to enhance product quality, experimentation with diverse reagents and varied conditions has been undertaken, including incorporation of salts, adjustments in extraction duration and temperature, and exploration of different meal-to-solvent ratios. However, efforts to improve extraction methods continue. Recent studies have explored alternative techniques such as membrane separation, protein micellization, electro-activated technologies, and enzymatic treatments (Chmielewska et al., 2021).

An exciting alternative is weakly acidic salt extraction, which has shown promising results. Zhang et al. (2024) demonstrated that weakly acidic salt extraction (pH 6.5, 150 mmol/L $MgCl_2$) produced rapeseed proteins, with minimal presence of antinutritional factors and superior amino acid profiles and digestibility compared to alkali extraction. Moreover, proteins extracted this method, followed by ultrafiltration displayed notable characteristics including lighter colour and odour, alongside improved solubility, emulsion activity, foaming properties, and water/oil holding capacity suggesting that this technique could be a viable option for producing high-quality, consumable rapeseed proteins. Conventional extraction techniques have long been criticised for being time-consuming, energy-intensive, and environmentally unfriendly due to their reliance on organic solvents, alkalis, and acids. However, many industries still depend on conventional methods due to their economic sustainability (Kumar et al., 2021).

Due to their good functional properties and nutritional value, rapeseed proteins are increasingly being explored for use in food applications, such as plant-based meat alternatives, dairy substitutes, protein-enriched products, etc.

Also, rapeseed meals are widely used as animal feed, but their high levels of antinutrients can reduce nutrient bioavailability. Beyond food and feed, rapeseed proteins are explored for biodegradable films, adhesives, bio-based plastics, and superabsorbent hydrogels.

Additionally, bioactive peptides derived from rapeseed proteins show potential in pharmaceutical and cosmetic industries due to their antioxidant and antimicrobial properties (Chmielewska et al., 2021).

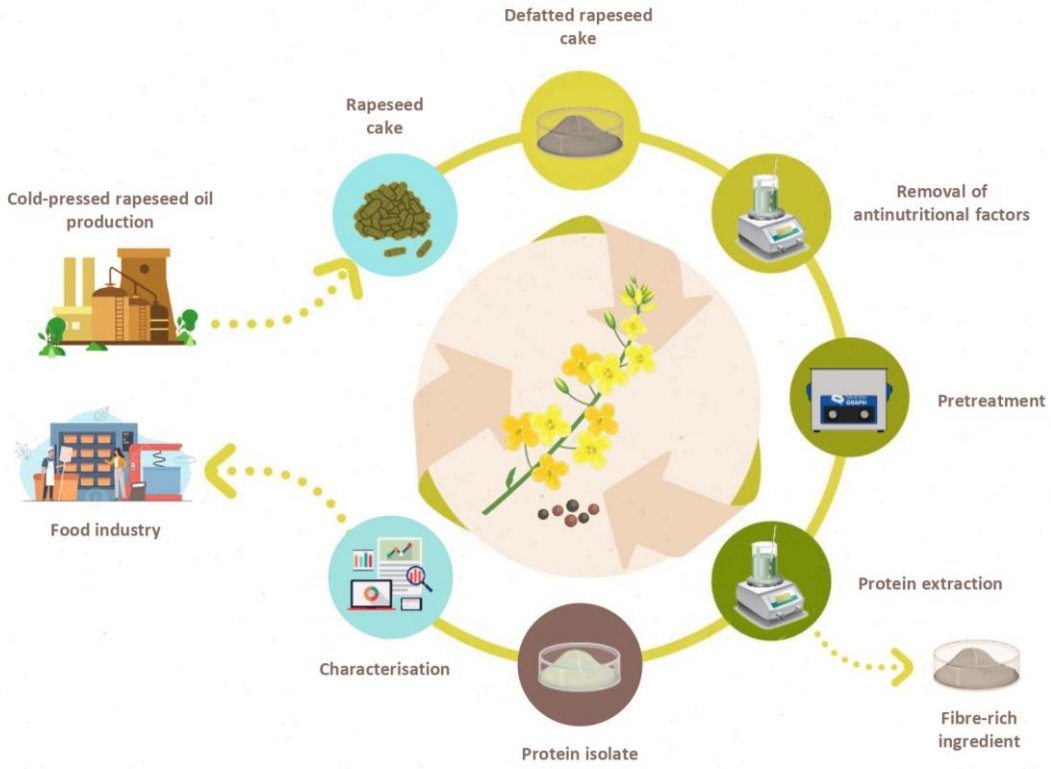

Figure 1. Schematic representation of the rapeseed protein extraction process, illustrating key steps from cold pressing and meal production to pretreatment, protein extraction, and final applications

Since gaining attention across various industries, optimizing extraction methods for rapeseed proteins is crucial for enhancing their applicability while addressing challenges related to product quality, functionality and sustainability (Chmielewska et al., 2021). In recent years, there has been a collaborative effort to explore novel and advanced extraction technologies, along with the use of eco-friendly solvents. Recent advancements also focus on eco-friendly methods, such as ultrasonic, microwave, enzyme-assisted, natural deep eutectic solvents, pulsed electric field, and subcritical water extraction, which have shown promise in reducing environ-mental impact while improving extraction efficiency and protein functionality (Singh et al., 2022). This shift towards greener extraction methods marks a significant evolution in protein extraction techniques and continued innovation is likely to drive improvements in both efficiency and sustainability from oilseed cakes and meals (Kumar et al., 2021).

To facilitate understanding of the extraction process and its industrial relevance, Fig. 1. presents a schematic representation of rapeseed meal processing, including the removal of antinutritional factors and subsequent protein isolation.

ANTINUTRITIENTS AND STRATEGIES FOR THEIR MITIGATION

As previously discussed, the presence of antinutrients in rapeseed presents a significant challenge during the alkaline extraction processes designed for rapeseed protein isolation. These antinutrients, including glucosinolates, phenolic compounds, phytic acid, erucic acid, and fibre, need to be removed as their presence severely limits the development of nutritionnally and sensorially acceptable rapeseed proteins.

Antinutritional factors in rapeseed meal

Antinutritional compounds are secondary metabolites produced during various metabolic processes in plants, serving roles such as protection, antimicrobial properties, or storage. Despite their potential health benefits, these compounds are viewed as the primary drawback to using rapeseed protein in food applications. Thus, they often need to be reduced or

eliminated to obtain protein products suitable for human consumption (Chmielewska et al., 2021).

Rapeseed contains glucosinolates, with concentrations ranging from 10 to 20 μmol/kg. These sulphur and nitrogen-containing glycosides are characterised by a common structure, consisting of β-D-thioglucose, sulphonated oxime, and a lateral chain derived from methionine, phenylalanine, or tryptophan (Arrutia et al., 2020; Chmielewska et al., 2021). Glucosinolates are susceptible to hydrolysis by myrosinases, resulting in the formation of potentially toxic products that can interfere with thyroid function and decrease food palatability (Arrutia et al., 2020). Historically, glucosinolates have been a concern in rapeseed utilisation, as they can adversely affect animal performance and thyroid activity in the case of using rapeseed-containing feedstuffs, and contribute to their bitter taste (Chmielewska et al., 2021). Rapeseed meal has relatively high glucosinolate levels of 18 to 30 μmol/g (Sorensen, 1990). Therefore, reducing glucosinolate levels during rapeseed protein extraction is essential to ensure that proteins extracted from rapeseed meals are suitable for human consumption (Tan, Mailer, Blanchard & Agboola, 2011).

Rapeseed also contains notably high levels of phenolic compounds, approximately ten times more abundant (1–3%) than those found in soybeans (Chew, 2020). These compounds have affect the properties of rapeseed-derived products, including alterations in solubility, digestibility, and sensory characteristics such as bitterness and dark colouration (Arrutia et al., 2020). While phenolic compounds have antinutritive effects, they also exhibit beneficial properties, including anti-allergic, anti-inflammatory, and cardioprotective effects (Chmielewska et al., 2021).

Sinapic acid and sinapine are abundant among the phenolic acids in rapeseed, contributing to the undesirable taste and colour of rapeseed meal and protein products (Chmielewska et al., 2021). Additionally, condensed tannins, present in crucifer seeds, contribute to the dark colour and bitter taste of rapeseed-derived products (Wanasundara, 2011).

Interaction between phenolic compounds and proteins can result in irreversible changes to rapeseed proteins' physicochemical and functional properties (Wanasundara, 2011). In alkaline environments with a pH range of 7.5 to 12.5, phenolics are easily extracted, intensifying their adverse effect on protein products.

Therefore, strategies to mitigate the impact of phenolic compounds are essential for enhance their quality and acceptability of rapeseed-derived products for human consumption (Wanasundara, 2011).

Rapeseed contains phytic acid, also known as myo-inositol hexaphosphate or phytate, at concentrations of 1–2% (Wnukowski, Smolders & Veerman, 2013). Phytic acid serves as the primary phosphorus and myo-inositol reserve in cereals and oilseeds but also acts as a potent chelating agent. It forms insoluble complexes with minerals such as zinc, iron, calcium, and magnesium, inhibiting their availability for metabolism and potentially leading to deficiencies with prolonged consumption. Additionally, phytic acid binds with proteins to form insoluble complexes, reducing the bioavailability of essential amino acids and hindering enzymatic degradation (Albe-Slabi, Defaix, Beaubier, Galet & Kapel, 2022). It also inhibits enzymes such as α-amylase, trypsin, tyrosinase, and pepsin, affecting nutrient absorption and digestion (Arrutia et al., 2020).

Levels of phytic acid levels in rapeseed products can vary depending on the extraction method, with reported levels ranging from 2.0% to 5.0% in defatted meals and up to 9.8% in protein isolates and concentrates (Tan et al., 2011). The pH of the extraction medium significantly influences the extent of phytic acid extraction and its complexation with proteins. Strategies such as ultrafiltration/diafiltration and counter-current extraction have been effective in reducing phytic acid levels in rapeseed protein isolates, although they may impact protein extraction yield and product colour (Wanasundara, 2011). Overall, mitigating the effects of phytic acid is essential for enhancing the nutritional quality and safety of rapeseed-derived products.

Erucic acid, the predominant fatty acid found in rapeseed, can pose health risks to both animals and humans when consumed in high concentrations. Excessive intake of erucic acid is linked to adverse effects on the heart, primarily manifested as myocardial lipidosis (Arrutia et al., 2020; Knutsen, et al., 2016). To safeguard public health, the European Council

has established a maximum allowable level of 20 g/kg for erucic acid content within the total fatty acids present in oils, including its bound form in fats (European Union, 2023).

Rapeseed seeds contain a diverse carbohydrate and fibre profile, including both soluble and insoluble dietary fibres. In rapeseed protein products, low molecular weight carbohydrates are prevalent, with ribose, arabinose, and glucose being prominent sugars (Wanasundara, 2011). Insoluble dietary fibre (IDF) is mostly comprised of cellulose and lignins, while soluble dietary fibre (SDF) includes hemicellulose, pectins, gums, mixed β-glucans, and mucilage. These fibre components, primarily situated in the seed hulls, play a crucial role in the seed's overall nutritional composition. However, excessive fibre content may reduce the nutritional value of rapeseed products by impeding digestibility (Chmielewska et al., 2021). A comprehensive understanding of the distribution and impact of carbohydrates and fibre in rapeseed is essential for optimizing its nutritional profile and utilisation across diverse applications (Wanasundara, 2011).

Removal of antinutritional factors

Various treatments are available to reduce antinutritional factors in rapeseed meals, including physical, chemical, biological, and crop breeding methods (Nega, 2018). Physical treatments involve mechanical processes like dehulling, fractioning, and grinding, as well as thermal methods like heating, steaming, and toasting, which can reduce glucosinolates and other antinutrient. Removing the hull before oil extraction is one such method that can mitigate the negative effects of fibre, as the hull contains over 70% of the total fibre content. However, this process can be technically challenging and affects oil quality, increasing production costs (Gołębiewska, Fraś & Gołębiewski, 2022). Another mechanical approach is seed fractionation, where seeds are sorted by size (sieving) or density (air classification) to reduce fibre content and improve meal quality (Cheng et al., 2022).

Thermal treatments, such as steam heating and toasting, are effective in reducing specific antinutritional compounds in rapeseed meals. Steam heating removes a significant portion of glucosinolates, while toasting also decreases glucosinolate content and deactivates enzymes like myrosinase. However, a non-toasted canola meal possesses higher levels of digestible amino acids, like lysine, compared to a toasted meal (Gołębiewska et al., 2022). Despite their effectiveness, excessive heating treatments can decrease protein solubility and amino acid availability due to the Maillard reaction (Mykola et al., 2023). Similarly to alkali treatment, excessive heating leads to racemisation and formation of cross-linked amino acid side chains such as lysoalanine (Friedman, Gumbmann & Masters, 1984).

Chemical treatments, including acid and alkaline degradation, metal salts degradation, and solvent extractions, have shown efficacy in reducing glucosinolates and phytic acid (Bhatty & Sosulski, 1972; Das & Singhal, 2005; Das Purkayastha et al., 2014). However, these methods frequently require heat, which can result in high costs and pose pollution risks, and potential damage to the protein structure. Solvent extraction, using ethanol, methanol, or acetone, is widely used, while aqueous solutions of acids, alkalis, or salts can also remove toxic compounds (Mykola et al., 2023). Techniques such as acidic washing (Akbari & Wu, 2015) and ethanol washing (Jia, Rodriguez-Alonso, Bianeis, Keppler & van der Goot, 2021) target specific antinutrients, such as phytic acid and phenolic compounds.

Despite their effectiveness, chemical methods can disrupt protein structure, reducing bioavailability and limiting the meal's use in food and feed. Additionally, they often fail to fully detoxify rapeseed meal and may negatively impact its sensory properties while contributing to environmental pollution (Yang, Huang & Cao, 2022).

Biological detoxification, including microbial fermentation and enzymatic hydrolysis, is a promising alternative due to its sustainability and minimal environmental impact (Yang et al., 2022). Microbial fermentation, using strains such as *Bacillus subtilis*, *Saccharomyces cerevisiae*, *Aspergillus niger*, and *Lactobacillus delbrueckii*, reduce glucosinolates, phytic acid, and crude fibre while increasing protein content (Gołębiewska et al., 2022). Multi-strain fermentation is more effective than single-strain approaches, often incorporating aerobic and anaerobic phases with enzyme supplementation enhance degradation (Yang et al., 2022).

Enzymatic extraction utilises the unique properties of enzymes, exhibiting high specificity, regional selectivity, and operation under mild conditions, thus preserving the potency of biological compounds (Yang et al., 2022). The application of dietary enzymes, including phytase, proteases, and carbohydrases, has been extensively studied. Phytase, for instance, enhances phosphate and mineral uptake in animals unable to metabolise phytate (Cheng et al., 2022). Research conducted by Rodrigues, Carvalho, and Rocha (2016) suggests that pretreating rapeseed meals with phytase alongside alkaline extraction leads to improved protein extraction compared to a simple extraction process. Chen et al. (2024) also improved the flavour of rapeseed protein isolate using phytase/ethanol treatment.

Breeding programs have also made progress in developing varieties of rapeseed with lower levels of antinutrients. The development of 'double-low' rapeseed varieties in the 1980s, which feature low erucic acid and glucosinolate content, marked a significant breakthrough (Nega, 2018). These varieties were carefully bred to have low levels of two key antinutrients: erucic acid (less than 2%) and glucosinolates (less than 30 μmol/g). The genetically improved rapeseed varieties are known as 'Canola'.

In conclusion, physical, chemical, and biological approaches offer promising strategies for mitigating levels of antinutrients in rapeseed meals, each with distinct advantages and challenges. Moving forward, future research should focus on optimising these methods and exploring innovative approaches, such as high-pressure processing, cold plasma processing, microwave frequency, and ultrasound. Additionally, advances in rapeseed genetics, breeding, and biotechnology will further enhance the efficacy of antinutrient mitigation techniques to the development of sustainable, high-quality rapeseed products with improved nutritional profiles and broader applications in food, feed, and industrial sectors (Faizal, Ahmad, Yaacob, Halim-Lim & Abd Rahim, 2023; Yang et al., 2022).

PRETREATMENTS IN PROTEIN EXTRACTION

Another approach that has proven effective, not only in removing antinutrients but also in increasing protein yield and reducing extraction time, is the application of non-thermal pretreatments, which include ultrasound, microwave, and enzymatic treatments, each operating on distinct principles and offering specific advantages (Li, Shi, Scanlon, Xue & Lu, 2021).

Ultrasound pretreatment

Ultrasound-assisted extraction (UAE) has emerged as a highly effective method for improving protein extraction efficiency. By utilising acoustic cavitation, UAE facilitates cell wall disruption, enhances mass transfer, and accelerates the release of target compounds. This technique offers several advantages, including a significant reduction in extraction time – often to just a few minutes – while ensuring high repeatability, minimising solvent consumption, and lowering overall energy requirements compared to conventional extraction methods. Additionally, UAE eliminates the need for subsequent wastewater treatment, contributing to more sustainable processing (Chemat et al., 2017).

The primary mechanism underlying ultrasound-assisted extraction is cavitation, a phenomenon that involves the formation, growth, and collapse of gas bubbles in a liquid medium. This process generates localised mechanical energy, effectively disrupting cell structures and leading to enhanced extraction yields (Perera & Alzahrani, 2021). Given its ability to extract bioactive components from challenging food matrices, UAE is increasingly recognised as a valuable tool in plant protein extraction (Ampofo & Ngadi, 2022).

To enhance the competitiveness and long-term sustainability of the plant protein industry, it is crucial to optimize the conditions for UAE and other novel extraction techniques (Ampofo & Ngadi, 2022). Since ultrasound is a mechanical wave, key parameters such as frequency, amplitude, and wavelength significantly influence cavitation dynamics, directly impacting extraction efficiency (Chemat et al., 2017). Furthermore, factors such as temperature, extraction time, solvent-to-sample ratio, and the specific characteristics of the sonoreactor and sonotrode play an essential role in determining overall effectiveness (Ampofo & Ngadi, 2022).

Several studies have demonstrated the effectiveness of UAE for protein extraction. Yagoub, Ma, and Zhou (2017) identified

optimal conditions for protein extraction from rapeseed meal, including a pH of 11.71, an ultrasonic treatment time of 41.48 minutes, ultrasound power of 40%, and a power intensity of 0.228 W/cm². These conditions resulted in a 43.3% increase in protein yield and an 18.13% improvement in nitrogen solubility compared to conventional extraction.

In another study, Dong et al. (2011) observed an 8.31% higher protein yield using ultrasound at 450 W, with an ultrasonic treatment time of 84 minutes, a solid-to-liquid ratio of 1:24, a pH of 11.5, and a temperature of 35 °C, compared to traditional methods. Similarly, Zhang et al. (2024) conducted ultrasound-assisted extraction at 25 kHz, 300 W, and 25 °C for 30 minutes, achieving the highest protein extraction yield (52.6 ± 1.6%) among the five extraction methods tested. Compared to the initial protein content in defatted rapeseed meal (36.8%), this represents a significant increase in extraction efficiency. Additionally, Boukroufa et al. (2017) demonstrated that combining ultrasound-assisted extraction with conventional methods resulted in the highest total protein yield (9.81 g/100 g DM).

Optimizing these parameters is crucial for achieving maximum protein recovery, making UAE a promising strategy for improving plant protein extraction efficiency while maintaining sustainability and minimizing resource consumption.

Microwave pretreatment

Microwave heating enhances protein extraction efficiency through the combined effects of dipole rotation and ionic conduction, leading to the disruption of hydrogen bonds within plant cell walls. This process increases cell wall porosity, allowing for improved solvent infiltration and the effective release of intracellular compounds into the extraction medium (Kumar et al., 2021).

Microwave irradiation has been widely recognised as an effective pretreatment method for protein extraction from various plant-based raw materials. Additionally, it serves as a suitable approach for reducing antinutritional factors in rapeseed. Niu, Jiang, Wan, Yang and Hu (2013) reported a 21.0% decrease in sinapine content in rapeseed meal following a seven-minute microwave treatment. Similarly, Li et al. (2010) demonstrated that microwave irradiation at 500 W and 46 °C for seven minutes significantly reduced hydrolysis time from four hours to just seven minutes. These findings highlight the potential of microwave pretreatment to enhance protein hydrolysis efficiency, thereby improving extraction yields and processing sustainability.

Enzymatic pretreatment

The enzymatic release of proteins from plant materials relies on the selective activity of carbohydrases and pectinases, which degrade plant cell wall components, while proteases hydrolyse high-molecular-weight proteins into more soluble forms. Enzymes also reduce protein complexation with other cellular components, improving overall extractability. Additionally, the use of proteases allows for processing at lower pH values, minimising the risk of protein denaturation (Baker & Charlton, 2020).

Rommi et al. (2014) emphasised the critical role of pectin hydrolysis in facilitating protein extraction from rapeseed meal, demonstrating a 1.7-fold increase in extraction efficiency using pectinolytic and xylanolytic enzymes. Further studies by Sari, Bruins and Sanders (2013) showed that enzymatic treatment increased protein extraction yields from 15–30% for alkaline extraction to 50–80%, reinforcing the benefits of enzymatic hydrolysis in protein recovery.

Rodrigues et al. (2016) investigated the combined effects of phytase pretreatment and alkaline extraction, achieving a protein yield of 72.1% under optimized conditions (pH 12.5, 75 °C, 60 minutes, and a solid-to-liquid ratio of 1:10). In contrast, alkaline extraction alone yielded only 51.3%. Additionally, the resulting protein concentrates exhibited lower phytic acid content (approximately 1 g/kg), improving their nutritional profile and commercial potential for protein-enriched food applications.

Beyond enhancing protein extraction efficiency, enzymatic pretreatment effectively reduces antinutritional factors. Xiong et al. (2022) evaluated the impact of phytase pretreatment combined with ethanol extraction on rapeseed meal composition. Using an enzyme-to-substrate ratio of 1:5 mg/g for 1.5 hours, followed by double ethanol extraction, the resulting protein isolate contained 88.26%

protein, with significantly reduced levels of fat (0.57%), ash (2.72%), crude fibre (0.77%), and moisture (1.90%). Moreover, this process improved protein whiteness and minimised the presence of antinutritional compounds compared to commercial protein isolates obtained via salt extraction.

Tian et al. (2022) further investigated the effects of enzymatic pretreatment, incorporating protease and carbohydrase in rapeseed protein extraction. Their study revealed that enzymatic hydrolysis significantly increased protein solubility while reducing phytic acid, glucosinolates, and phenolic alkaloids. However, protein yield remained lower than that achieved through alkaline extraction (40–82 g/100 g dry matter compared to 91 g/100 g dry matter).

Interestingly, the combination of protease and carbohydrase did not result in higher yields compared to protease alone, a trend observed in multiple studies (Sari, Mulder, Sanders & Bruins, 2015).

Extensive research has also focused on the direct production of protein hydrolysates from rapeseed meal using proteases, given their widespread application in improving the techno-functional and bioactive properties of protein isolates (Tian et al., 2022). These findings highlight the versatility of enzymatic pretreatment in protein extraction while emphasising the need for process optimization to maximise yields and functional quality.

ENZYMATIC HYDROLYSIS

Beyond improving protein extraction, enzymatic treatments also influence the structural and functional properties of proteins. One of the key challenges with rapeseed proteins is their limited solubility in water at neutral pH, which restricts their application in food processing. To address this, enzymatic hydrolysis has been widely explored as a method to enhance solubility and to improve techno-functional properties such as water absorption, gelling, and emulsification (Alashi, Blanchard, Mailer & Agboola, 2013).

Protein hydrolysis refers to the cleavage of peptide bonds and can be achieved through enzymatic and chemical processes. Enzymatic hydrolysis is preferred because it can be carried out under mild conditions (pH 6–8, temperatures of 40–60 °C), avoiding the extreme conditions required by chemical processes. Additionally, enzymes exhibit substrate specificity, allowing the development of protein hydrolysates with precisely defined chemical and nutritional characteristics (Tavano, 2013). Enzymatic hydrolysis not only improves the nutritional value of proteins but also enables the production of bioactive peptides with potential health benefits. Vioque, Sánchez-Vioque, Clemente, Pedroche and Millán (2000) reported improvements in water absorption, increased oil retention capacity, and greater foaming capacity, although with reduced foam stability due to decreased peptide size as a result of enzymatic hydrolysis of rapeseed. These findings highlight the trade-off between enhanced functional properties and the stability of certain functionalities, such as foam. The most studied bioactive properties of canola protein hydrolysates include their angiotensin-converting enzyme (ACE) inhibitory effects and antioxidant activities. Other potential bioactivities, such as antimicrobial and anticancer effects, have been explored to a lesser extent (Alashi et al., 2013).

The degree of hydrolysis (DH) plays a critical role in determining the properties of protein hydrolysates. Therefore, by adjusting variables such as enzyme specificity and hydrolysis conditions, it is possible to develop a range of hydrolysates with different functional properties suitable for various food applications (Alashi et al., 2013). For example, a moderate degree of hydrolysis (up to 5% DH) enhances emulsifying and foaming properties. However, further hydrolysis does not provide additional improvements and may even have adverse effects. This phenomenon is attributed to the release of hydrophobic residues during moderate hydrolysis, which increases interfacial adsorption capacity. As hydrolysis progresses, the peptide size becomes too small to form stable interfacial networks, leading to reduced emulsion and foam stability (Fetzer et al., 2020).

Protein hydrolysates with low degrees of hydrolysis (DH < 10%) are widely used as food ingredients, such as flavourings in soups, sauces, and meat products. On the other hand, extensive protein hydrolysates (DH > 10%) are used as protein supplements in nutrition or special medical diets, such as the production of hypoallergenic foods (Vioque et al., 2000).

However, enzymatic hydrolysis alone may not provide an adequate product without additional modifications after hydrolysis. Several post-hydrolysis processes have been successfully introduced to control the molecular size and reduce bitterness in the resulting hydrolysates (Clemente, 2000).

Despite this limitation, complete protein hydrolysis by a single protease is often unattainable, as minor changes in protein structure can significantly impair enzyme function, hinder cofactor formation, or induce autolysis (Tavano, 2013).

The release of bioactive peptides from rapeseed is still in the early stages of development for food industry applications. One promising approach for enhancing bioactive peptide production is the application of various pretreatments before enzymolysis (Wali et al., 2017b). Among these, ultrasonic technology has gained attention in recent years for its potential to improve the efficiency of rapeseed protein hydrolysis. Numerous studies have demonstrated that ultrasound can significantly accelerate the degree of hydrolysis, although the precise mechanism remains unclear. Some researchers suggest that this improvement results from the modification of the spatial structure of substrate proteins, making them more flexible and exposing previously hidden active sites. Others propose that ultrasound alters the structure of the protease itself.

Additionally, it has been reported that short-term ultrasound exposure can enhance enzyme activity, whereas prolonged exposure at high intensity may inhibit catalytic efficiency (Wang et al., 2016).

Jin et al. (2016) reported that ultrasonic pretreatment significantly improved enzymolysis by reducing the Michaelis constant (K_M) by 32.8% and altering the thermodynamic parameters E_a (activation energy), ΔH (enthalpy change of the reaction), and ΔS (entropy change of the reaction) by 16.6%, 17.7%, and 9.2%, respectively. These changes suggest that ultrasonic treatment facilitates the unfolding of protein molecules, exposing more hydrophobic groups and increasing the surface area for enzyme interaction. Ultrasonic pretreatment also improved the functional properties of protein hydrolysates. Wali et al. (2017b) reported a 130.76% increase in surface hydrophobicity and 34.22% increase in solubility index compared to the control group. Furthermore, stability tests showed that ACE inhibitory activity of the hydrolysates remained stable even after heat treatments. In another study, Wali et al. (2017a) reported a significant increase in ACE inhibitory activity, which rose by 19.12% and 35.08% compared to the control group, further highlighting the potential of ultrasonic pretreatment to enhance the bioactive properties of protein hydro-lysates.

NOVEL ISOLATION METHODS

As the demand for protein separation and purification grows across various research fields, the development of efficient and cost-effective protein extraction technologies has become a critical focus. It is well known that conventional extraction agents, primarily organic solvents, are volatile, flammable, and hazardous to both human health and the environment (Zhang et al., 2016). In contrast, green technologies are gaining attention because they minimise the use of toxic solvents, use environmentally friendly solubilisation techniques, and develop novel solvents that are safer and more sustainable. (Dai, van Spronsen, Witkamp, Verpoorte & Choi, 2013).

Among the various green solvents, ionic liquids (ILs) have drawn significant attention due to their negligible vapour pressure, as well as their tunable polarity and selectivity (Dai et al., 2013). However, ionic liquids also have several drawbacks, including poor biodegradability, biocompatibility, and sustainability. Moreover, they are expensive and toxic, making them less optimal for replacing traditional organic solvents in large-scale applications (Liu, Li & Row, 2022). While the efficiency of ionic liquids in protein extraction has been confirmed in studies on various plant species, rapeseed has not been included in these studies, leaving a gap in research regarding the extraction of its proteins using this method.

Another class of solvents that has gained significant interest is deep eutectic solvents (DESs) (Dai et al., 2013). Like ILs, DESs exhibit properties such as high thermal stability, low volatility, low vapour pressure, and tunable polarity, which make them suitable for various extraction processes (Hansen et al., 2021). However, DESs have the advantage of being partially or entirely composed of non-ionic compounds, which contributes to their cost-effectiveness, environmental friendliness, and

biodegradability to ILs (Bowen et al., 2022). Furthermore, DESs are prepared by simply mixing hydrogen bond donors and hydrogen bond acceptors in appropriate molar ratios, followed by mild heating (50–100 °C) to facilitate mixing (Kist, Zhao, Mitchell-Koch & Baker, 2021). If the compounds forming a DES are primary metabolites, such as amino acids, sugars, or choline derivatives, the resulting DES is classified as a natural deep eutectic solvent (NADES). Both DESs and NADESs are formed through the complexation of a hydrogen bond acceptor and a hydrogen bond donor (Paiva et al., 2014). The extensive hydrogen bonding network between these components results in a mixture that remains in a liquid state, with a melting point significantly lower than that of its individual constituents (Karimi, Bhowmik, Yang, Samaranayaka & Chen, 2024).

To date, more than 150 different NADES combinations have been reported (Vanda, Dai, Wilson, Verpoorte & Choi, 2018), with the most commonly used ones based on choline chloride (ChCl) and various hydrogen bond donors such as carboxylic acids, urea, citric acid, and glycerol (Paiva et al., 2014). In a study by Karimi, Bhowmik, Yang, Samaranayaka and Chen (2024), NADESs were compared to alkaline extraction at pH 9 and pH 12 for rapeseed protein extraction. The results showed that NADES isolates exhibited higher extraction efficiency and similar functionality to those obtained at pH 9, while also displaying improved colour and better preserved native protein structures compared to both alkaline treatments. Regarding antinutritional components, alkaline extraction resulted in a higher content of phenolic compounds, whereas NADES isolates contained higher levels of phytic acid (Karimi et al., 2024).

Grudniewska et al. (2018) also demonstrated the potential of deep eutectic solvents for improving rapeseed protein extraction. Using a glycerol-ChCl-based DES, they achieved a protein yield of 40–50% in the isolate, representing an increase of up to 20% compared to the starting material.

In addition to being used as pretreatment methods, ultrasound, microwaves, and enzymes can serve as standalone extraction techniques, eliminating the need for additional processing steps. Although not yet applied to rapeseed, green technologies such as pressurised liquid extraction, supercritical fluid extraction, cold plasma-assisted extraction, and electrically driven extraction have shown promise as sustainable alternatives to conventional extraction methods for rapeseed protein isolation (Picot-Allain, Mahomoodally, Ak & Zengin, 2021).

AUTHOR CONTRIBUTIONS

Conceptualization, D.M.T., J.S.S., B.B.Đ., and A.Z.M; Writing-original draft preparation, D.M.T., J.S.S., and B.Đ.R; Writing-review and editing, M.B.S., P.T.J., and B.M.Š; Supervision, A.Z.M., B.M.Š., M.B.S, and P.T.J.

DATA AVAILABILITY STATEMENT

Data contained within the article.

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CONFLICT OF INTEREST

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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ULJANA REPICA KAO IZVOR PROTEINA

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Sažetak: Visokovredni proteini uljane repice mogu se izolovati iz sporednih proizvoda dobijenih u procesu proizvodnje ulja od uljane repice. Ovaj pregledni rad pruža uvid u proizvodnju proteinskih izolata uljane repice, s naglaskom na konvencionalnu alkalnu ekstrakciju i alternativne metode. Posebna pažnja posvećena je antinutrijentima koji se nalaze u uljanoj repici (glukozinolati, fenolna jedinjenja, fitinska kiselina i drugi) i strategijama za smanjenje njihovog sadržaja. Razmatrane su tehnike koje su efikasne ne samo u uklanjanju antinutrijenata, već i u povećanju prinosa proteina i smanjenju vremena ekstrakcije, uključujući primenu ultrazvuka, mikrotalasni tretman i enzimske pretretmane. Razmatrana je, takođe, enzimska hidroliza za dobijanje hidrolizata proteina uljane repice, kao i noviji postupci ekstrakcije za proizvodnju proteinskih izolata, naročito upotreba prirodnih dubokih eutektičkih rastvarača (natural deep eutectic solvents – NADES).

Ključne reči: *uljana repica, proteinski izolati, antinutrijenti, ekstrakcija*

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