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# Combining colloid milling and twin screw pressing for oleosome extraction

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ABSTRACT

Oleosomes are oil droplets abundant in oilseeds that can be extracted together with proteins for use in emulsion-like foods and can be used as natural, ready emulsions as alternatives to dairy and fat-based products. To make optimal use of the raw material, we look at simultaneous extraction of oleosomes and proteins from rapeseed. The combined approach of colloid milling and twin screw pressing resulted in a higher yield of oleosomes (70 % w/w) when compared to twin screw press alone (54 % w/w), which may be due to extensive cell wall disruption caused by colloid mill processing, as evidenced by SEM imaging. The protein yield remained constant irrespective of the degree of cell wall disruption (50 % w/w). In conclusion, combining the colloid mill and twin screw press is a straightforward way for the extraction of oleosome and proteins from rapeseeds for food application.

## 1. Introduction

Oleosomes are the oil-storage vesicles in all oilseeds, consisting of triglycerides surrounded by a monolayer membrane. The membrane surface is mainly comprised of polar phospholipids heads and hydrophilic membrane-bound proteins, such as oleosins, caleosins, and steroleosins (Nikiforidis, 2019). Due to the hydrophilic nature of the oleosome surface membrane, the extraction of intact oleosomes is performed after soaking oilseed in an aqueous environment, which also favors the co-extraction of storage proteins (Romero-Guzmán et al., 2020a). This is different from the conventional oil extraction using mechanical pressing or organic solvents, which breaks the oleosome membrane and releases oil from dry oilseeds. To obtain good quality oil for human consumption, additional and extensive refining steps are needed to remove the undesired components, such as oxidation compounds (Gharby, 2022). Thus, the aqueous extraction of oleosomes is preferred as a green extraction method which avoids the use of organic solvents while preserving the integrity of oleosomes. Furthermore, the extracted oleosomes are a natural emulsion which can be formulated into food products (Bibat et al., 2022; Xu et al., 2023), such as, but not limited to, white cheese (Sheikh et al., 2023), yogurt (Mantzouridou et al., 2019), and edible films (Matsakidou et al., 2019).

The aqueous extraction of oleosomes is achieved by soaking oilseeds, soaking is a necessary step before the milling to soften the cell wall of oilseeds (Romero-Guzmán et al., 2020d). Next, the softened oilseeds can be disrupted by the milling, which allows the release of the oleosome into the aqueous solution. Finally, the oleosomes are separated from the insoluble solids by centrifugation. The extent of the cell wall disruption directly relates to the exposure of the oleosomes to the aqueous environment, and therefore determines the extraction yield. The common milling method in oleosome extraction is known as twin screw press or blending (Ntone et al., 2020; Romero-Guzmán et al., 2020a; Yang et al., 2023). However, a twin screw press alone or blending alone gives a low extraction yield of approximately 60 % w/w (Romero-Guzmán et al., 2020a). Thus, the extraction yield should still be further improved for the large-scale production and food applications (Nikiforidis et al., 2014). Besides these two milling methods, cell wall disruption can also be achieved by colloid mill (Dickey et al., 2008; Phongthai et al., 2017; Xia et al., 2012; Zhao and Dong, 2016), which is known for fine grinding of legumes, such as chickpea (Rawson et al., 2021; Samofalova and Donskaya, 2021). The extent of the cell wall disruption can be controlled by the gap size of the stator and rotor in colloid mill (King and Keswani, 1994). However, this promising alternative method has not yet been applied for oleosome extraction. Thus, in this study we introduce a combination extraction method by combining the colloid mill with the twin screw press to achieve a higher extraction yield. We hypothesized

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that the extent of the cell wall disruption by the combination of colloid mill and twin screw press will be enhanced compared to twin screw press alone.

The aim of this study is to enhance cell wall disruption and increase oleosome and protein extraction, we here propose to pretreat the rapeseed with a colloid mill, to ensure extensive cell wall opening, before pressing. The oleosome-protein mixtures obtained were analyzed for their composition, particle size, surface charge and morphology. To further understand the effect of colloid mill on the disruption of the rapeseed cell wall matrix, the remaining residues after the extraction process were collected and visualized using Scanning Electron Microscopy (SEM). To further improve the total yield, the remaining residues were passed once more through the colloid mill and twin screw press. Our results may be helpful in the design of simple large-scale extraction processes for oleosome and proteins from oilseeds and promote their uses in food products.

## 2. Materials and methods

### 2.1. Materials

Untreated rapeseeds (*Alizze*) were purchased from a local seed producer. All chemicals were in analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA). Deionized water was prepared using a Merck Milli-Q system (Darmstadt, Germany).

### 2.2. Continuous extraction of oleosomes

Extraction of rapeseed oleosomes was conducted according to the twin screw press method described by Romero-Guzmán et al. (2020b) with some modifications. Briefly, 100 g of rapeseed was soaked in Milli-Q water (pH 7) in a 1 L Erlenmeyer flask. The optimal water ratio for the colloid mill is 1:5 w/v, which comes from the preliminary experimental optimization (Supplementary Figs. S1 and S2). Therefore, this ratio was applied and seed water dispersions were kept for 5 h at room temperature (20 °C) under Magnetic Stirrer (EUROSTAR 60 digital, IKA, Staufen, Germany). The aqueous extraction process was divided into 4 steps after soaking. First, the steeped rapeseed dispersions were pressed through a unique and multi-functional small-scale laboratory colloid mill machine named magic LAB® (MK module, IKA, Staufen, Germany). The colloid mill consists of a rotor and stator with a conical gap which can be adjusted to control the size of the milled grit that can pass the device. In preliminary tests, the optimal gap size of colloid mill is 0.557 mm (Supplementary Figs. S1 and S2), thus the conical gap of 0.557 mm was applied here. Second, the milled rapeseed dispersions were passed through a twin screw press (Angelia 8000, Angel Co., Ltd., Busan, Korea) which separated the coarse insoluble residues from a fine aqueous dispersion and remained in the twin screw press. Third, the aqueous solution contained a considerable amount of finer insoluble components which were centrifuged out at 4000 g for 20 min (Sorval Lynx 4000 Centrifuge, Thermo Scientific USA). The insoluble sediment was removed and the remaining oleosome-protein dispersion was collected. Fourth, the collected oleosome-protein dispersion was mixed enough and named as an oleosome-protein mixture (OPM).

### 2.3. Extraction yield and concentration of oleosome protein mixture

The oil content of the OPM was determined using a Soxtherm automatic oil extractor (Sox 416 Macro, Gerhard, Germany), using petroleum ether as solvent and 3 h extraction time. The oleosome extraction yields were calculated by the methods of Yang et al. (2023) according to equation (1). The protein content of OPM was determined by using a Nitrogen Analyzer (Flash EA 1112 Series, Thermo scientific, Netherlands), with 6.25 as the conversion coefficient. The protein extraction yields were calculated using equation (2).

$$\text{Oleosome extraction yield} = \left(\frac{W_{\text{oil in OPM}}}{W_{\text{oil in initial sample}}}\right) \quad (1)$$

$$\text{Protein extraction yield} = \left(\frac{W_{\text{protein in OPM}}}{W_{\text{protein in initial sample}}}\right) \quad (2)$$

The oleosome and protein concentration were then calculated using equations (3) and (4).

$$\text{Oleosome concentration} = \left(\frac{W_{\text{Oil in OPM}}}{W_{OPM}}\right) \quad (3)$$

$$\text{Protein concentration} = \left(\frac{W_{\text{protein in OPM}}}{W_{OPM}}\right) \quad (4)$$

### 2.4. Particle size distribution

Particle size distributions of the OPM were determined according to the previously reported method (Nikiforidis and Kiosseoglou, 2011) using static light scattering (Malvern Mastersizer 3000, Malvern Instruments Ltd, UK). Specifically, the oleosome-protein mixture was diluted at a ratio of 1:100 (w/v), and then the dispersion was added into a cup with a stirrer at 1400 rpm to break up any loose aggregates, with an assumed refractive index of the dispersed phase of 1.47. An average of 5 measurements was taken.

### 2.5. ζ-potential measurements

The ζ-potential of the OPM was measured according to the previously reported approach (Romero-Guzmán et al., 2020c) with several adaptations using Zetasizer analyzer (Malvern Zetasizer Nano90, UK). First of all, the dispersions were diluted at a ratio of 1:1000 (w/v). Then, the diluted dispersions were added into a U cell to measure the ζ-potential. The equilibration time was set to 120 s and the temperature to 25 °C. The refractive indice used was 1.47 for the dispersed phase and 1.33 for the continuous phase. In total, three measurements were performed per sample, and the average value was calculated.

### 2.6. SDS-PAGE

The protein molecular weight in the OPM was characterized using SDS-PAGE, using the technique of Ntone et al. (2020) in reducing conditions with some modification. Mini-protein gels (low range PROTEAN TGXTM. 30 µl, Bio-Rad, Netherlands) were used. First of all, the samples were diluted in Milli-Q water to reach a concentration of 2 mg/mL. Afterward, the samples were added to Tris buffer containing 2 % w/w SDS, 10 % glycerol, 0.5 % w/v bromophenol blue, and 5% β-mercaptoethanol. The sample was vortexed and heated at 95 °C for 15 min, then the sample was centrifuged at 10,000 rpm for 1 min. The supernatant was used for the SDS-PAGE, and electrophoresis was performed at 200 V for approximately 35 min in a Mini-Protean II electrophoresis cell (Bio-Rad, Veenendaal, Netherlands). The SDS-PAGE marker of 5 µL and 20 µL of the sample was loaded onto the gel wheels. The gels obtained after electrophoresis were stained using Bio-safe Coomassie Blue (Bio--Rad, Netherlands). After 1 h, the gels were washed with Millipore water and scanned using a Gel scanner (Bio-Rad GS900, Netherlands).

### 2.7. Confocal light scanning microscopy (CLSM)

The oleosome-protein dispersions were visualized by CLSM (Ti-2 microscope, Nikon Microsystems, Japan) using the approach described by Ding et al. (2020). One milliliter of oleosome emulsion was mixed with 10 µL of Nile Red (0.01 % w/v) to stain the lipids (red fluorescence). He/Ne lasers were operated at excitation wavelengths of 561 nm.

### 2.8. Scanning electron microscope (SEM)

For SEM analysis, residues by twin screw press only and by the combination of twin screw press and colloid mill were freeze-dried and fixated on an aluminum sample holder using carbon adhesive tabs. Petroleum ether was used to remove the fat residue. Before imaging, the samples were coated with gold by a sputter-coater (Joel Smart-Coater). SEM images were taken at 5 kV using a JEOL JCOLLOID MILL-7000 (Wetterauw et al., 2023).

### 2.9. Statistical analysis

All experiments were carried out at least in triplicate with mean ± standard deviation error bars. One-way analysis of variance (ANOVA) and Duncan's test were used to determine statistical differences between three or more groups, while the independent sample *t*-test was used to determine statistical differences between two groups. Differences were significant when $P < 0.05$. The analyses were performed with SPSS Version 25.0 software (IBM software, Chicago, USA).

## 3. Results and discussion

### 3.1. Release of oleosome and protein from rapeseeds

The rapeseeds contained oil (37.21 ± 0.68%), protein (21.21 ± 0.34%), and moisture (7.79 ± 0.01%). To efficiently extract oleosome and proteins from rapeseeds, we followed a previously suggested extraction process (Romero-Guzmán et al., 2020a) and added a milling step using a colloid mill. After milling, the insoluble components were removed and the oleosome and protein were collected in an aqueous solution for the analysis of the composition and physical properties. This procedure gave an oleosome extraction yield of 54 % w/w (Fig. 2). During the oleosome extraction process, 49 % w/w of the proteins were also co-extracted (Fig. 2), thus almost 46 % w/w of the initial oleosomes and 51 % w/w of the initial proteins remained in the residues when only twin screw pressing (Residue A) and centrifugation (Residue B) was used.

When the colloid mill was applied before the twin screw press, the oleosome extraction yield increased to 70 % w/w (Fig. 2), this observation highlights the significance of the milling step in improving the efficiency of oleosome extraction from rapeseeds. In terms of protein extraction yield, the study revealed a consistent outcome, with the yield remaining constant at 50 % w/w (Fig. 2). The similar protein extraction yield might be explained by the extraction pH being pH 6.0 when deionized water was used. This is close to the isoelectric point of the suspension, which is between 4 and 6 (Romero-Guzmán et al., 2020a), and therefore did not allow more extensive protein solubilization and extraction (Tan et al., 2011; Sari et al., 2015).

With the two-stage extraction process (twin screw press and the combination of colloid and twin screw press), we found an oleosome concentration of 6.3 % w/w, while this was only 4.8 % w/w with the twin screw press only (Fig. 2). The higher oleosome concentration is probably due to the colloid mill destroying the cell wall more effectively, allowing more oleosomes to be released into the solution.

The extracted protein concentration remained the same at around 2.5 % w/w, just as the protein extraction yield remained the same. SEM was used to assess the matrix structure after both extraction processes (Fig. 2 B and C) corresponding to the residue A (Fig. 1). The press extraction does not cause complete cell wall disruption (Fig. 2 B), while the matrix obtained after milling and pressing was disrupted more extensively (Fig. 2 C). While SEM imaging cannot give statistically firm conclusions, the observations confirm our hypothesis that this extra step leads to greater cell wall disruption increasing oleosome extraction yield (Fig. 2 A).

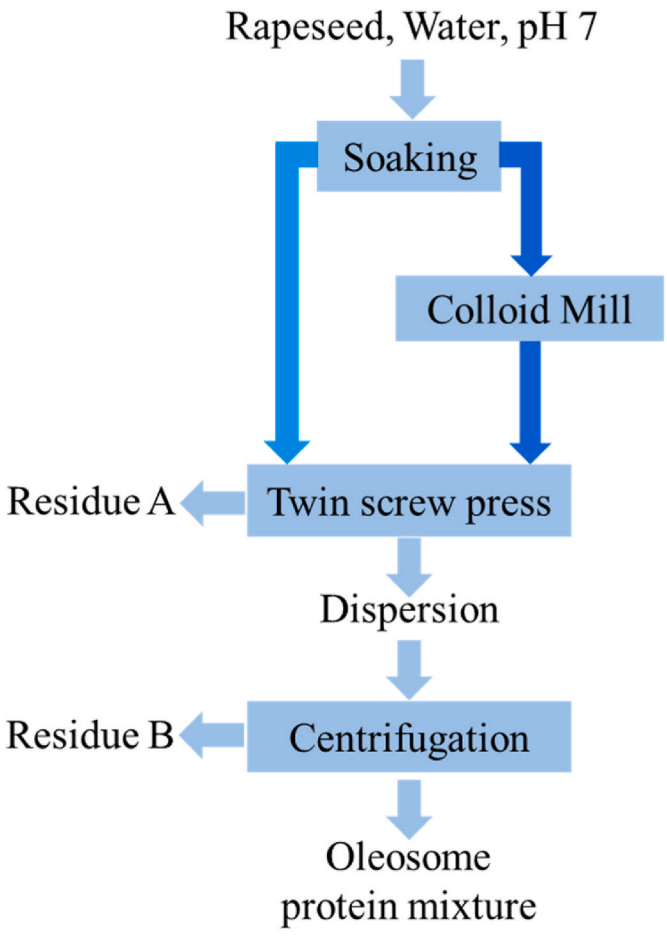


**Fig. 1.** Extraction process of rapeseed oleosomes and proteins.

### 3.2. Physical properties of oleosome and protein mixture

The ζ-potentials of the dispersions obtained from the twin screw press and the combination of colloid mill and twin screw press methods are found to be −8.7 ± 0.2 mV and −10.6 ± 0.5 mV, respectively. At the pHs of the dispersions between 5.6 and 5.8, and therefore the ζ-potential of rapeseed protein can be assumed to be around −12 mV at pH 6 (Ntone et al., 2020), and the ζ-potential of rapeseed oleosomes to be around 0 mV at pH 6 (Romero-Guzmán et al., 2020a).

Particle size distributions of the oleosome protein mixture are shown in Fig. 3 A. A main peak is present for both dispersions between 2 and 20 μm plus a small side peak at a larger size of 50–100 μm. A tiny peak (0.1–0.5 μm) appeared with the combination of the colloid mill and twin screw press. The particle size was 13.4 μm of $D_{(4,3)}$ and 9.3 μm of $D_{(3,2)}$ with only twin screw press extraction was 12.7 μm of $D_{(4,3)}$ and 3.5 μm of $D_{(3,2)}$ with the combination of the colloid mill and twin screw press extraction. This is in line with the findings by King & Keswani. (1994), that particles rotate and impinge in a shear field during colloid milling, which reduces the particle size.

SDS was used to break up the hydrogen bonds and hydrophobic interactions of aggregates (Wang et al., 2022). This changes the particle size distribution significantly (Fig. 3 A with dotted line): only one peak between the size 0.1 and 3.0 μm remained after the addition of SDS. Therefore most of the large particles (1–100 μm) were aggregates which can be dissociated by SDS. The $D_{(4,3)}$ of oleosome-protein mixture was 0.80 ± 0.01 μm with twin screw press extraction, and 0.59 ± 0.03 μm with the combination of colloid mill and twin screw press extraction after SDS was added, corresponding to the previous results of 0.7 ± 0.1 μm reported by Ntone et al. (2020).

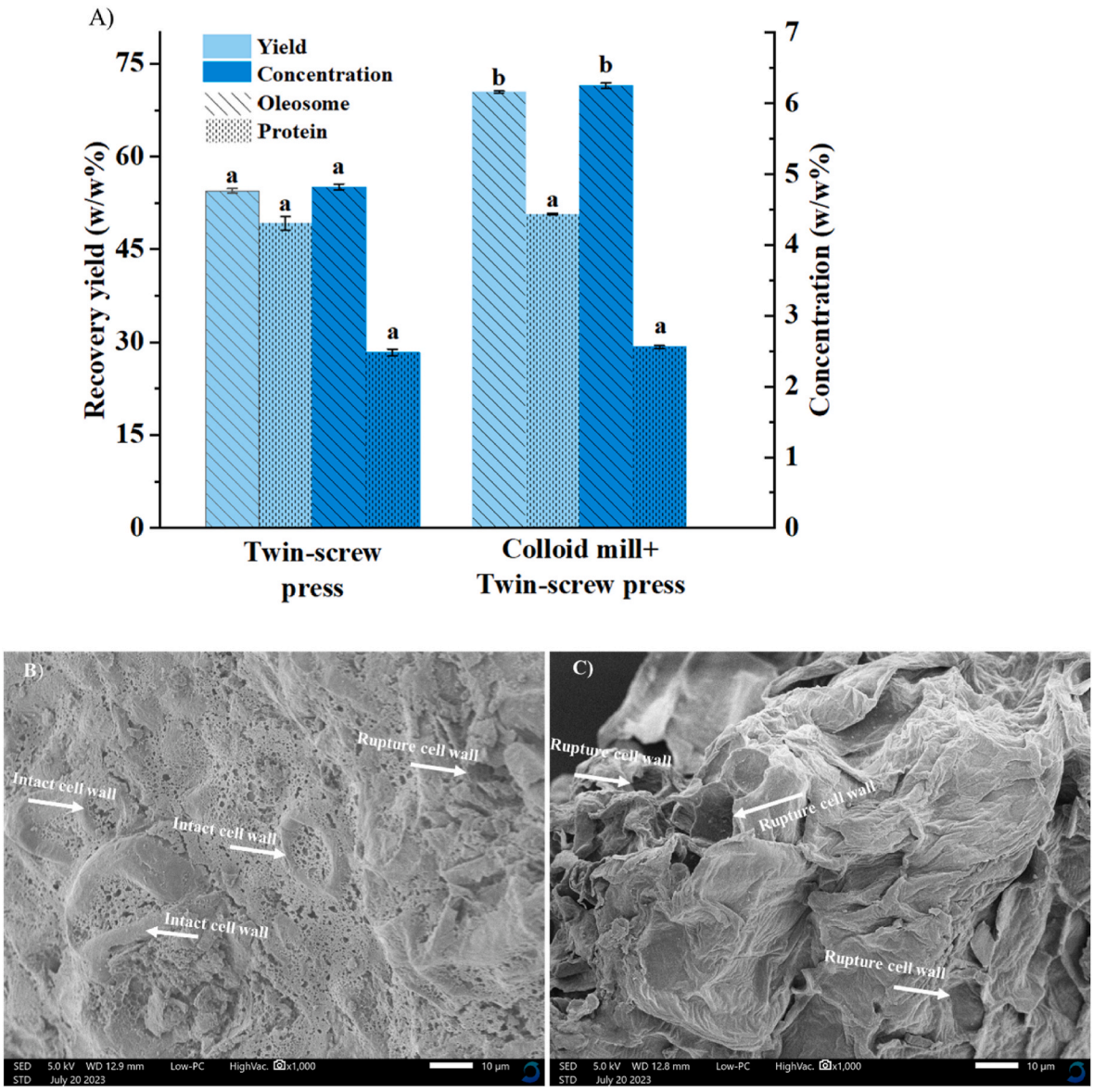


**Fig. 2.** Oleosome and protein extraction yield and concentration by twin screw press extraction and the combination of colloid mill and twin screw press extraction (A); The Scanning Electron Microscope (SEM) of the residue after twin screw press (residue A) with only twin screw press extraction(B) and combination of colloid mill and twin screw press extraction (C).
*Note: different lowercase letters indicate significant differences* with only twin screw press extraction and the combination of the colloid mill and twin screw press extraction.

CLSM was carried out to qualitatively assess the degree of aggregation and the size of the oleosomes in the oleosome-protein mixture (Fig. 3 B, C). Individual oleosome particles of around 0.8 μm can be observed, and aggregates that agree with the big peak of 50–100 μm. This aggregation could be due to lower absolute |ζ|-potential values of 8.7 mV as mentioned above in this section, which allow hydrophobic and attractive forces to dominate and lead to aggregation. By combining the colloid mill and twin screw press extractions, the level of aggregation was significantly decreased when compared to the twin screw press extraction alone (Fig. 3 C). The absolute |ζ|-potentials of dispersions obtained from the combination of the colloid mill and twin screw press was 10.6 mV, which is higher than that extracted only by twin screw press of 8.7 mV causing an increase of particle repulsion and thus reduces aggregate formation.

### 3.3. Protein profile of oleosome protein mixture

Next, SDS-PAGE was performed to investigate the protein composition of the extracted oleosome and protein mixtures (Fig. 4). Five bands were detected: the band around 11 kDa most likely belonging to albumins, also known as napin (Kohno-Murase et al., 1994) s, the bands of 18, 22, 27 and 30 kDa are most likely belong to β-polypeptides and α-polypeptides, which belong to the globulins, known as cruciferin (Simon et al., 1985). Both cruciferin and napin are storage proteins of rapeseeds (Murphy and Cummins, 1989). The bands of 18 kDa and 27 kDa are reported to be oleosin and caleosin, respectively, both of which are oleosome proteins located within the membrane of the oleosom (Tzen, 2012) e. We observed similar protein molecular weight profiles extracted under different conditions, suggesting that similar proteins were extracted irrespective of the method used (Fig. 1) corresponding to the ζ potential results seen previously (section 3.2).

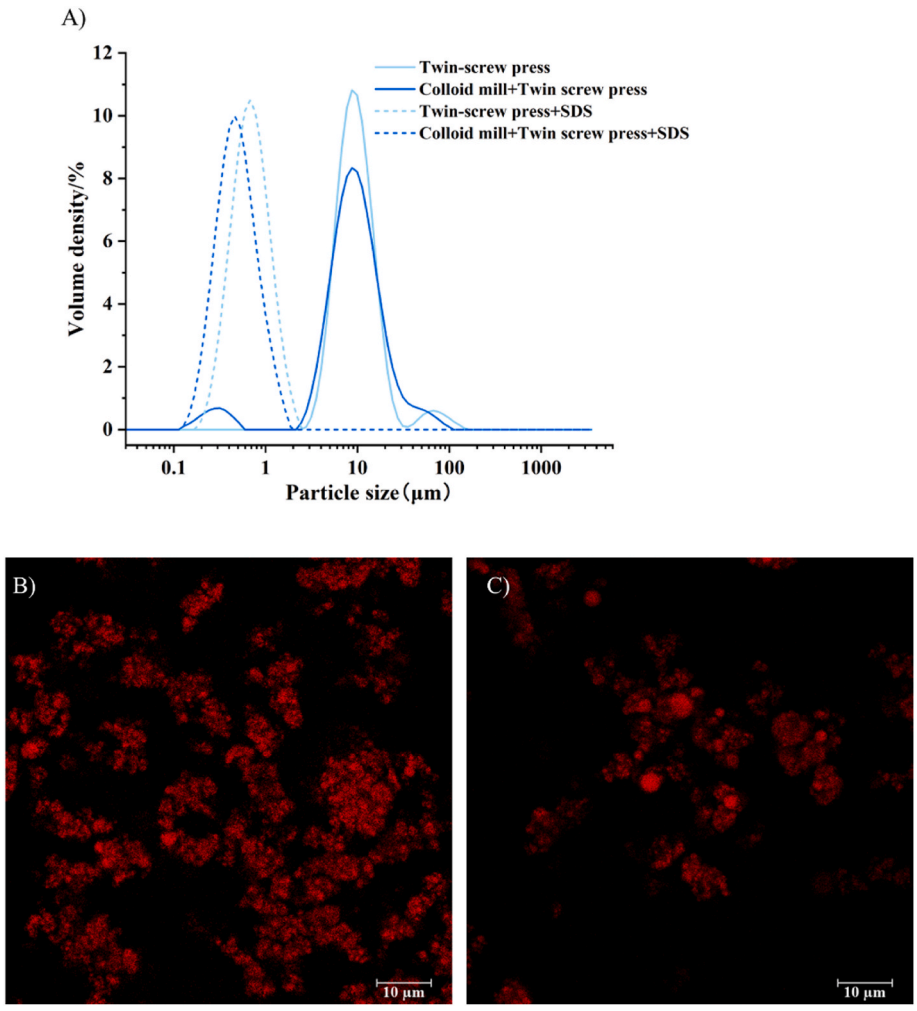


**Fig. 3.** Particle size distribution of oleosome and protein mixture extracted with only twin screw press and the combination of colloid mill and twin screw press extraction without added and with 1 wt% SDS (A); Confocal light scanning microscopy (CLSM) of oleosome and protein mixture extracted with only twin screw press (B) and the combination of colloid mill and twin screw press extraction (C).

### *3.4. A second pass of the residues from the colloid mill and the twin screw press further enhances the extraction yield*

From the results described above, the lowest oleosome extraction yield was obtained by twin screw press alone (54 % w/w), while the extraction yield was significantly enhanced by combining twin screw press and colloid mill extraction (70 % w/w) (Fig. 2). However, even with this combined approach there was still 30 % w/w oleosome unextracted from the seeds. Based on the SEM images (Fig. 2), it is probable that the remaining unextracted oleosomes are most likely still stuck inside the cell matrix and therefore not yet accessible for the aqueous extraction. Thus, based on our findings here, we speculate that additional mechanical processing to break the cell wall matrix may lead to an even higher oleosome extraction yield. We therefore tested whether further cell wall disruption could improve the efficiency of the process for continuous extraction of oleosome and protein by reloading the residues again to the colloid mill and twin screw press, after it was mixed with fresh water for steeping (Fig. 5).

Indeed, the second extraction stage achieves significantly higher oleosome and protein extraction yield compared to the first extraction (Fig. 2). Around 16 % w/w extra oleosome and 9 % w/w extra protein were extracted by the second pass, corresponding to overall extracts of 86 % w/w oleosome and 60 % w/w protein extracted from the initial rapeseeds. This shows that the incomplete extraction was indeed due to the incomplete disruption of the cell matrix. It can be expected that a third step would lead to a further, albeit slight improvement in extraction yield. In industry, this could be achieved and easily implemented by increasing the cycle number of colloid mill and increasing the length of twin screw; possibly using a counter-current configuration for the aqueous phase, to minimize the use of water and increase the overall concentration of both oleosome and proteins.

It was earlier suggested that using less refined ingredients might also be applied as functional ingredient for food application (Lie-Piang et al., 2023). The residue from rapeseed oil extraction can, for example, be used in meat analogue application, using sheared cells to form a fibrous structure, and the natural oil can be utilized within the meat analogue to provide flavour, without further phase separation due to its emulsifying nature (Jia et al., 2022).

The gain in yield presented here by adjusting the multi-step oleosome extraction process is significant, and should be further investigated as this easily implementable enhanced extraction process significantly boosts output (Fig. 5). Thus, the combination of the colloid mill and twin screw press in multi-steps provide a potential route for high yield extraction of oleosomes.

## 4. Conclusion

We explored the potential of using a continuous extraction method by combining colloid mill and twin screw press to enhance oleosome and protein extraction yield. Our results showed that 54 % w/w of oleosomes

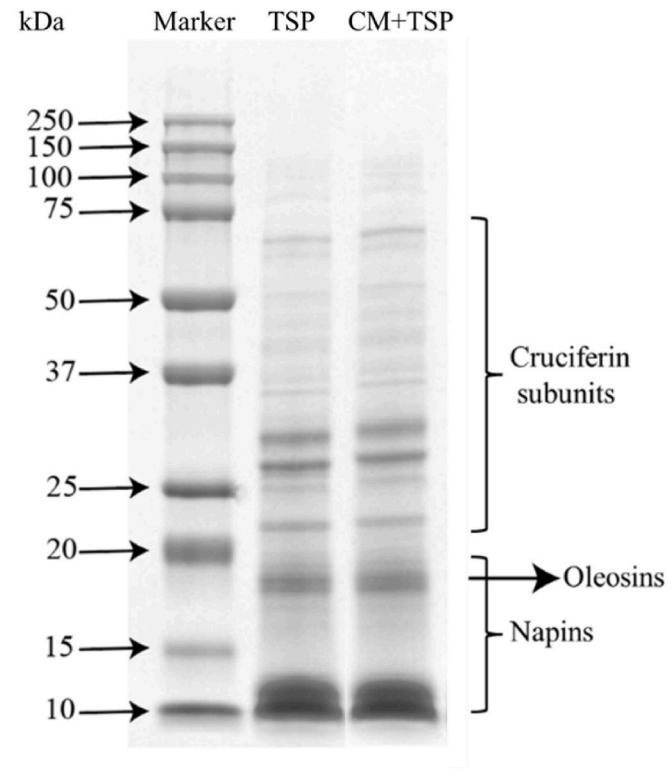


**Fig. 4.** Protein profile of oleosome and protein mixture extracted with only twin screw press (TSP) and the combination of the colloid mill and twin screw press extraction (CM + TSP).

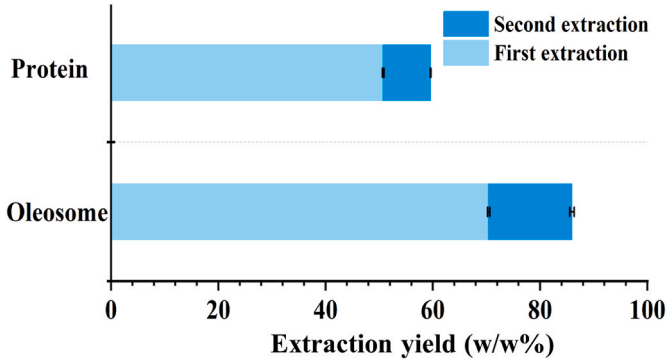


**Fig. 5.** The extraction yield of oleosome and protein by one and two passes from the combination of the colloid mill and the twin screw press.

are extracted via the twin screw press method. Significantly higher extraction yield of 70 % w/w was achieved by the combination of colloid mill and twin screw press. SEM analysis showed that this is most likely due to further disruption of the matrix, which facilitates oleosome extraction. The oleosome extraction yield was increased up to 86 % w/w by employing a second pass through the colloid mill and twin screw press. In both extraction methods, the protein extraction yield remained unchanged at 50 % w/w, probably because the pH of the extraction was around 6, close to the zero charge point of rapeseed proteins. The findings indicate that integrating and optimizing colloid mill and twin screw press extraction methods could offer more complete oleosome extraction rendering the process more feasible for larger scales.

## CRediT authorship contribution statement

**Chenqiang Qin:** Conceptualization, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing - original draft. **Rao Fu:** Formal analysis, Methodology. **Yaxin Mei:** Formal analysis, Methodology. **Xin Wen:** Methodology, Supervision, Writing - review & editing. **Yuanying Ni:** Funding acquisition, Supervision, Writing - review & editing. **Remko Marcel Boom:** Supervision, Writing - review & editing. **Constantinos V. Nikiforidis:** Conceptualization, Supervision, Validation, Writing - review & editing.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

## Acknowledgments

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jfoodeng.2023.111908.

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