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Removal of phenolic compounds from de-oiled sunflower kernels by aqueous ethanol washing

Wanqing Jia [a], Konstantina Kyriakopoulou [a], Bente Roelofs [a], Mbalo Ndiaye [b], Jean-Paul Vincken [c], Julia K. Keppler [a], Atze Jan van der Goot [a,*]

[a] Laboratory of Food Process Engineering, Wageningen University, PO Box 17, 6700 AA Wageningen, The Netherlands
[b] R&D Department, Avril Groupe, Bruz, France
[c] Laboratory of Food Chemistry, Wageningen University, PO Box 17, 6700 AA Wageningen, The Netherlands

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ABSTRACT

Selective removal of phenolic compounds (PCs) from de-oiled sunflower kernel is generally considered a key step for food applications, but this often leads to protein loss. PC removal yield and protein loss were assessed during an aqueous or aqueous ethanol washing process with different temperatures, pH-values and ethanol contents. PC yield and protein loss increased when the ethanol content was < 60% or when a higher temperature was applied. Our main finding is that preventing protein loss should be the key objective when selecting process conditions. This can be achieved using solvents with high ethanol content. Simulation of the multi-step exhaustive process showed that process optimization is possible with additional washing steps. PC yield of 95% can be achieved with only 1% protein loss using 9 steps and 80% ethanol content at 25℃. The functional properties of the resulting concentrates were hardly altered with the use of high ethanol solvents.

1. Introduction

Sunflower (*Helianthus annuus* L.) is the third most cultivated oilseed crop in the world after soy bean and rapeseed (USDA, 2020). Sunflower meal is the main by-product obtained after oil extraction. Because of its high protein content (25%–55% w/w), sunflower meal is often suggested for food applications, but it is still mainly used for animal feed (Laguna et al., 2019; Pickardt, Eisner, Kammerer, & Carle, 2015). The high content of phenolic compounds (PCs) (1%–4% on a dry basis) restricts its use in the food industry, because the presence of chlorogenic acid (CGA) in sunflower meal. This can lead to dark green and brown colouring under alkaline conditions or during aqueous processing through the formation of protein and PC complexes (Ozdal, Capanoglu, & Altay, 2013; Pedrosa et al., 2000). In addition, the formation of these complexes can lower the nutritional value of the protein by altering its digestibility and bioavailability (Karefyllakis, Salakou, Bitter, Van der Goot, & Nikiforidis, 2018), and the functionality of the protein is changed (Keppler et al., 2020; Rawel, Meidtner, & Kroll, 2005). Therefore, removal of PCs is generally considered a prerequisite for enhanced use of the sunflower proteinaceous fraction in food applications.

Previous studies have mainly focused on obtaining a purified protein isolate by complete removal of PCs (Albe Slabi et al., 2020; Pickardt et al., 2015). However, a low protein yield in these studies and the use of large amounts of water and chemicals make the process less suitable for producing refined ingredients for modern food applications (González-Pérez et al., 2002; Karefyllakis, Altunkaya, Berton-Carabin, van der Goot, & Nikiforidis, 2017). Furthermore, many food applications do not require the use of completely purified proteins. Other components, such as carbohydrates, can have a positive functionality as well, and retaining them can result in improved resource use efficiency. Washing processes using a mixture of water and ethanol are often suggested as food-grade solvents to remove PCs, yielding less refined protein concentrate (Chemat, Vian, & Cravotto, 2012; Prat et al., 2015). The ratio of water and ethanol in the solvent mixtures is used to finetune the polarity of the solvent, and thereby control the selectivity of the extraction of both PCs

Abbreviations: CGA, chlorogenic acid; CME, counter-current multi-stage extraction; DM, dry matter content; DSK, de-oiled sunflower kernel; F-C, Folin-Ciocalteu; GAE, gallic acid equivalent; HPLC, high-performance liquid chromatography; K_{pc}, equilibrium constant for phenolic compounds; K_{pro}, equilibrium constant for protein; NSI, nitrogen solubility index; PC, phenolic compound; TPC, total phenol content; SEM, scanning electron microscopy; WHC, water holding capacity.

* Corresponding author.
E-mail address: Atzejan.vandergoot@wur.nl (A.J. van der Goot).

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and proteins (Jankowiak, Trifunovic, Boom, & Van Der Goot, 2014). Protein in a polar nature can be better extracted with water compared to low-polarity solvent of ethanol (Chemat et al., 2012). In addition to solvent (mixture) selection, temperature and pH are known to influence the removal of PCs. It is reported that a higher temperature and alkaline pH can increase the amount of PCs removed in the extract by increasing their solubility (Perez, Vereijken, Koningseld, Gruppen, & Voragen, 2005; Sripad, Prakash, & Rao, 1982). However, protein solubility also increases with increased temperature and alkaline pH (Sathe, Zaffran, Gupta, & Li, 2018), which leads to protein loss. Further, protein nativity might also be affected by the use of a high temperature (Molina, Petruccelli, & Añón, 2004). Certain process conditions favour the formation of protein-PC complexes through covalent interaction, especially at alkaline pH. For this reason, process conditions at a lower pH can be beneficial to reduce protein loss and avoid covalent protein and PC interactions. The challenge therefore is to select the process conditions such that they leads to effective PC removal while minimizing protein loss.

In this study, the selective removal of PCs from de-oiled sunflower kernel (DSK) through washing is investigated. The aim was to investigate the effect of different water–ethanol content and process temperature on the effective removal of PCs from DSK while retaining as much protein as possible. The outcomes of the experiments were used to determine the equilibrium constants, which were used to simulate multi-step processes. The washed concentrates were assessed on their functional properties, such as protein nativity, microstructure, nitrogen solubility index (NSI) and water holding capacity (WHC). Both NSI and WHC are important when considering material as a protein source for modern food applications, such as meat analogues (Jia, Rodriguez-Alonso, Bianeis, Keppler, & van der Goot, 2021). It is hypothesized that the yield of PC removal and protein loss will be largely influenced by the ethanol content and temperature, and the functional properties of the resulting washed concentrates will be affected by high temperature and high ethanol content (Taha, Mohamed, Mohamed, & Mohamed, 2011; Sripad & Narasinga Rao, 1987).

2. Materials and methods

2.1. Materials

DSK, provided by Avril (France), was obtained after mechanical pressing of sunflowers. The proximate composition of DSK is 51.0% protein, 8.0% moisture, 3.9% PCs, 6.4% fat, 7.5% ash and the rest will be carbohydrates mainly. Ethanol (96%) was purchased from EMD Millipore Corporation (Darmstadt, Germany). Folin-Ciocalteu (F-C) reagent was obtained from MP Biomedicals (Illkirch, France). Analytical grade trifluoroacetic acid (for high-performance liquid chromatography [HPLC], purity 99%), gallic acid, CGA, HCl and NaOH were obtained from Sigma-Aldrich (St Louis, MO, USA). Acetonitrile ULC-MS (purity of 99.97%) was obtained from Actu-All chemicals (Oss, the Netherlands), anhydrous sodium carbonate (≥99.5%) was obtained from VWR International (Darmstadt, Germany). Ultrapure water was purified with a Milli-Q Lab Water System (Milli-Q IQ 7000 Ultrapure Lab Water System, Merck KGaA, Darmstadt, Germany) and was used in this study.

2.2. Methods

2.2.1. Sample preparation

2.2.1.1. Aqueous washing. Aqueous washing was performed under varied process conditions with different pH and temperature as follows: pH 4 at 25℃, pH 4 at 75℃; pH 7 at 25℃; pH 7 at 75℃. Milli-Q Water was preheated to 75℃ in a water bath (TW8 Water Bath, Julabo, the Netherlands) before the washing process.

Five grams of DSK was mixed with 50 mL of Milli-Q water in a 100-mL glass Schott bottle with lid, leading to a dispersion with a solid-to-liquid ratio of approximately 1:10. Then, the pH was adjusted to the desired value with 0.1 M HCl or 0.1 M NaOH. In the next step, the dispersion was mixed using a Stirring Drybath 15–250 (2mag AG, Munich, Germany) with a magnet at 400 rpm and controlled temperature for 10 min. A preliminary test showed that the washing time from 5 min to 60 min had hardly any impact on the amount of PCs being removed. The dispersion was vacuum filtrated using a vacuum pump system (SC 950; Germany) with filter paper (grade 4; Whatman, Sigma-Aldrich). The pellet remaining on the filter paper was transferred into the Schott bottle and used as the starting material for the next washing step. We tried to transfer as much material as possible into the bottle, but slight material losses were unavoidable at this stage. The extracts were collected in a Büchner flask and transferred into a 50-mL Falcon tube for further analysis. Up to 5 sequential steps were performed with the same protocol, making a total of 6 steps. After 6 washing steps, the final pellet obtained after filtration was freeze dried and stored at 4 °C. Fresh extracts of 1 mL from each step were kept in a fridge at 4℃, and the rest of the extracts were freeze dried and stored at 4 °C.

2.2.1.2. Aqueous ethanol washing. Aqueous ethanol mixtures were prepared by mixing water with ethanol. The ratio of the volume of ethanol to the total solvent volume is referred to in this study as the ethanol content: 0%, 20%, 40%, 60%, 80% and 100% (the purity of the ethanol used in this study was 96%). The mass of the mixtures was converted to the density of the aqueous ethanol mixtures: 0.998, 0.970, 0.939, 0.896, 0.851, 0.801, respectively. Water–ethanol mixtures were prepared separately with the correct volume in a cylinder, mixed in a Schott bottle and placed in the water bath at the correct temperature before adding DSK. The washing process was performed at 2 different temperatures (25℃ and 50℃) for all solvents with different ethanol content.

Five grams of DSK was mixed with 50 mL of solvent in a 100-mL glass Schott bottle with lid, creating a dispersion with a solid-to-liquid ratio of 1:10 (g/mL). Next, the dispersion was mixed using a Stirring Drybath 15–250 with a magnet at 400 rpm and controlled temperature (25℃ or 50℃). After mixing for 10 min, the dispersion was filtrated using a vacuum pump system (SC 950) with grade 4 Whatman filter paper. The extracts were collected in a Büchner flask and transferred into a 50-mL Falcon tube. The wet pellet remaining on the filter paper was transferred into the Schott bottle and used as the starting material for the next washing step. Up to 2 sequential steps were performed with the same protocol, making a total of 3 steps. After 3 washing steps, the final pellet obtained after filtration was freeze dried and stored at 4 °C. Fresh extracts of 1 mL from each step were kept in a fridge at 4℃. The rest of the extracts were pre-concentrated in a 250-mL flask and placed in rotary evaporator (RC900, KNF, Freiburg, Germany) at 40℃ and 100 rpm under an operating pressure of 150 mbar to remove most of the ethanol before freeze drying. The evaporation was carried out until the volume of the liquid remained constant, and the concentrated extracts were further freeze dried and stored at 4℃. The scheme for the aqueous washing and aqueous ethanol washing is shown in Fig. 1.

Both aqueous washing and aqueous ethanol washing process were reproduced with 2 batches. The pellet is referred to as the concentrate in the following discussion, except in Section 2.3.

2.2.2. Analysis of the extract

The TPC in the extracts was measured using the Folin-Ciocalteau (F-C) method (Drosou, Kyriakopoulou, Bimpilas, Tsimogiannis, & Krokida, 2015; Ghosh & Seijas, 2014). The CGA content was measured using HPLC (Karefyllakis et al., 2018). The protein content in the extracts was measured using the Dumas method (Pickardt et al., 2009).

2.2.2.1. Quantification of the total phenol content. Fresh extracts (100 μL) obtained from the different conditions of aqueous washing and aqueous ethanol washing process were added to 7.9 mL of water and

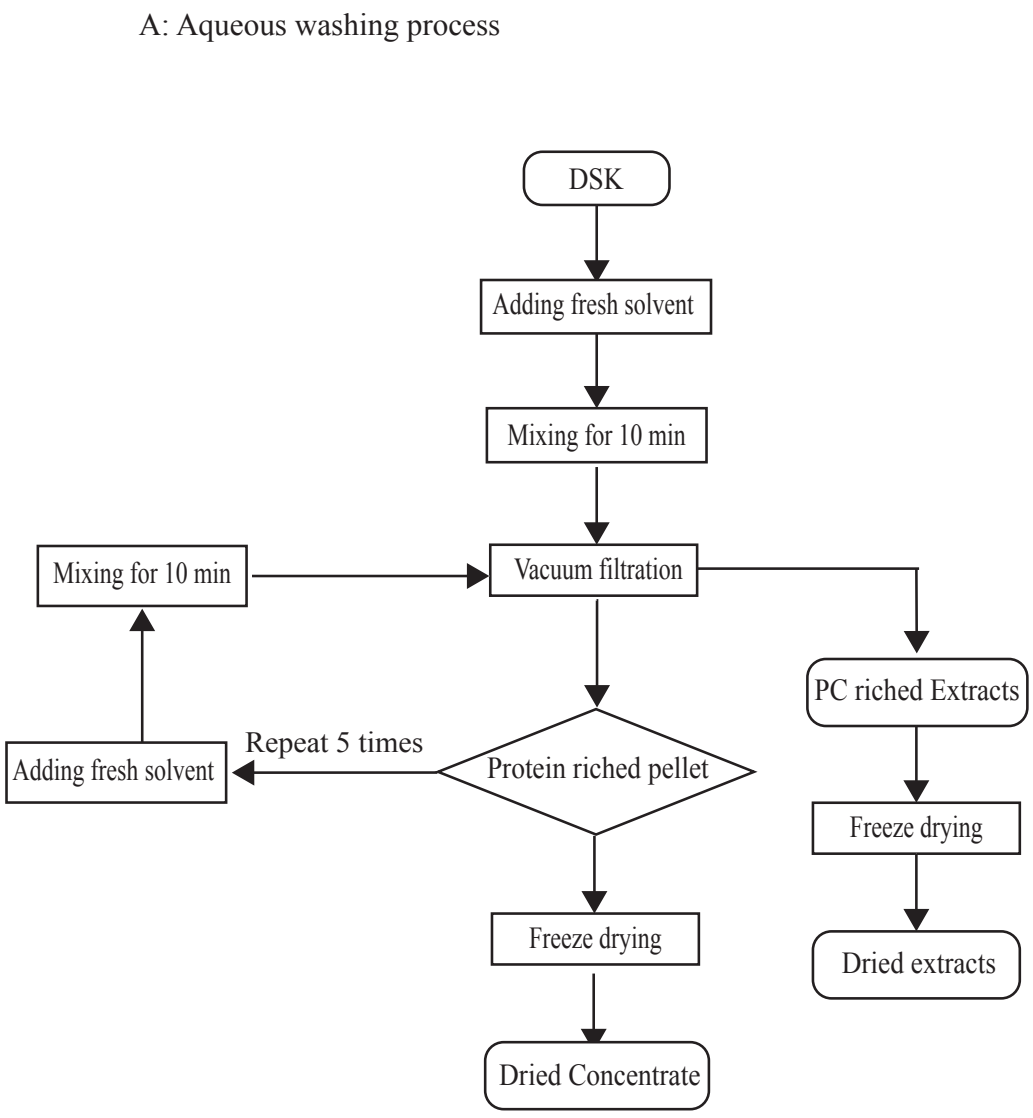

Fig. 1. Schematic overview of the aqueous washing (A) and aqueous ethanol washing (B) for de-oiled sunflower kernels (DSK).

mixed using a vortex. Subsequently, 500 μL of F-C reagent and 1.5 mL of 20% (w/v) sodium carbonate were added and mixed thoroughly with a vortex. The samples were placed in a water bath (TW8 water bath; Julabo, Boven-Leeuwen, the Netherlands) at 40℃ for 30 min. The absorbance was measured at 750 nm with a spectrophotometer (DR3900 Laboratory VIS Spectrophotometer, Hach, Loveland, CO < USA). Calibration curves with gallic acid solutions ranging from 0.025 to 4 mg/mL were made for each solvent with a different ethanol content. The total phenol content (TPC) is expressed as mg gallic acid equivalents (GAE)/g DSK on a dry basis. Each sample was measured in duplicate.

2.2.2.2. Quantification of CGA. HPLC was used to quantify the content of CGA in the extracts. A Dionex Ultimate 3000 chromatograph (Thermo Fisher, Waltham, MA, USA) was used with a Gemini 3 μm C18 phenol column at 30℃. The eluent consisted of 0.1% (v/v) trifluoroacetic acid, 23% (v/v) acetonitrile in ultrapure water. A calibration curve for CGA was drawn for concentrations of CGA ranging between 0.0125 and 0.3 mg/mL. The sample was prepared by dissolving 10 mg of freeze-dried extract in 1 mL of ultrapure water. Then dilutions (10 to 20 times) were prepared with ultrapure water based on the TPC results obtained using the F-C method. The dilution was done to achieve a final CGA concentration < 0.3 mg/mL. The samples were centrifuged at 15,000 × g for 10 min to remove the insoluble part of the extracts. The injection volume was 10 μL and the flow rate was maintained at 1.0 mL/min. The peak areas of the standard solutions and the extracts were measured at a wavelength of 295 nm using a Chromeleon Chromatography Data system (Thermo Fisher). The results are expressed as mg CGA/g DSK on a dry basis and each sample was measured in duplicate.

2.2.2.3. Protein analysis. The Dumas method was carried out by combusting a known mass of the freeze-dried extracts at 900℃ in the presence of oxygen. The nitrogen content was determined using a FlashEA 1112 NC Analyzer (Thermo Fisher). The protein content was then derived using a nitrogen conversion factor of 5.6 (Pickardt et al., 2009). Each sample was measured in triplicate.

2.2.3. Yield of PC removal and protein loss

The yield of PC removal (%) and protein loss (%) were calculated as follows:

$$\text{PC yield}(\%) = \frac{\sum_1^n (c_{\text{pc},i,\text{e}} \times m_{i,\text{e}})}{c_{\text{pc,DSK}} \times m_{\text{DSK}}} \times 100\% \quad (1)$$

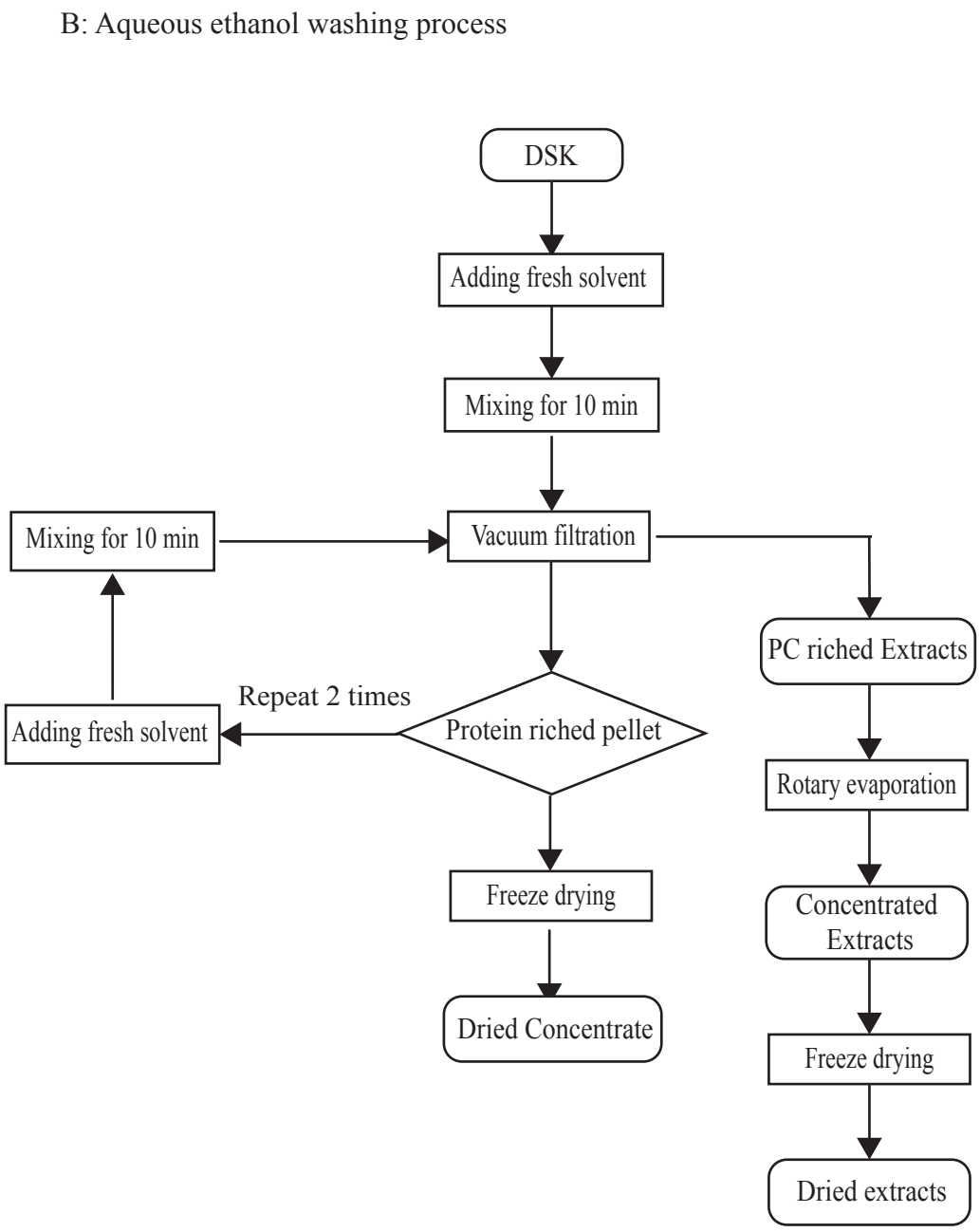

Fig. 1. (*continued*).

$$\text{Proteinloss}(\%) = \frac{\sum_1^n (c_{\text{pro},i,\text{e}} \times m_{i,\text{e}})}{c_{\text{pro,DSK}} \times m_{\text{DSK}}} \times 100\% \quad (2)$$

where $c_{\text{pc,DSK}}$ is the initial PC content in DSK. A value for $c_{\text{pc,DSK}}$ of 39.4 mg GAE/g DSK was obtained from an extensive washing process (10 steps) with 0% and 40% ethanol content. $c_{\text{pc,DSK}}$ was calculated by cumulating the average value of each washing step. The results are shown as boxplots in Fig. S1. The TPC measured was slightly lower than that reported in the literature for defatted sunflower meal (of 42 mg TPC/g dry matter) Weisz et al. (2009). The variation in the TPC determined from sunflower materials may depend on different factors, such as the variety of the sunflower crops, the de-oiling process, the process conditions for extraction of PCs, and the analytical methods. $c_{\text{pro,DSK}}$ of 510 mg/g DSK was used as the total initial amount of protein present in the DSK, which was obtained from Dumas measurement. $c_{\text{pc},i,\text{e}}$ (mg GAE/g liquid extract) and $c_{\text{pro},i,\text{e}}$ (mg/g liquid extract) are the concentration of PC and protein in the extracts from step *i*; concentrations were obtained from the F-C and Dumas methods. $m_{i,\text{e}}$ and $m_{i,\text{p}}$ are the mass of the liquid extract and pellet at step *i*. *n* is the total number of washing steps.

2.2.4. Analysis of the functionality of the concentrates

The functional properties of the concentrates were evaluated with respect to the microstructure, protein nativity, WHC and NSI. These methods were adapted from a previous study (Jia et al., 2021). Protein content was measured using the Dumas method, and the TPC recovery yield (%) in the concentrate was calculated with the amount of PC remained in the concentrate (based on equation (2)) over the amount of TPC in the DSK.

2.2.4.1. Microstructure. Scanning electron microscopy (SEM) was used to visualize the microstructure of the washed concentrates from aqueous washing and aqueous ethanol washing. The dry samples were placed onto SEM tubes (aluminium pin-type mounts 12.7 mm; JEOL, Nieuw-

Vennep, the Netherlands) using double-sided adhesive conductive carbon tabs (12 mm carbon tabs; SPI Supplies Division of Structure Probe, West Chester, PA, USA). The samples were sputter coated with gold. Compressed air was used to distribute the sample evenly on the surface of the carbon tabs. The accelerating voltage was 10 kV. In total, 8 pictures were taken for each sample analysis, from which one representative picture was selected.

2.2.4.2. Protein nativity. The protein nativity in the original DSK and concentrates from aqueous washing and aqueous ethanol washing was analysed with differential scanning calorimetry (TA instrument 250; TA Instruments, Newcastle, DE, USA). From each sample, 6 mg was placed in a high-volume pan. Ultrapure water was pipetted to create a dispersion of 15% w/w concentration. The pan was then sealed and the sample was hydrated for 1 h before measurement. The pan was heated from 25 °C to 130 °C at a heating rate of 5 °C/min. After 1 min, the pan was cooled down to 25 °C at a cooling rate of 20 °C/min. This heating and cooling process was repeated for a second time to make sure the peak indicated protein denaturation. Once the protein was denatured in the first heating step, it was not detected again in the second heating step. Duplicates were measured for each sample of concentrate. The temperature at the onset of protein denaturation (onset T), peak temperature of denaturation (T_d) and denaturation enthalpy (J/g protein) were collected by Trios data analysis software (TA Instruments).

2.2.4.3. Water holding capacity and nitrogen solubility index. DSK or concentrate from aqueous ethanol washing (1 g) was prepared in a 50-mL Falcon tube into a 2% dispersion by mixing with Milli-Q water, and rotated for 24 h (SB3 rotator; Stuart, Stone, UK) at a speed of 20 rpm for hydration. The dispersion was then centrifuged at 15,000 × *g* at 25℃ for 10 min. The supernatant was removed with a pipette and the wet pellet was transferred into an aluminium tray and dried in an oven at 105℃ for 24 h. The weight of the wet pellet ($M_{\text{wet pellet}}$) and after drying ($M_{\text{dry pellet}}$) was measured. The nitrogen content in the dry pellet ($N_{\text{dry pellet}}$) and in the original sample (N_{original}) was measured using the Dumas method (Section 2.2.2). The mass of the original sample and the pellet was measured and expressed as M_{original} and $M_{\text{dry pellet}}$. Duplicates of WHC and NSI were made for each sample.

WHC and NSI was calculated as

$$\text{WHC} = \frac{M_{\text{wet pellet}} - M_{\text{dry pellet}}}{M_{\text{dry pellet}}} [\text{gwater/gdrypellet}] \tag{3}$$

$$\text{NSI} = \frac{N_{\text{original}} \times M_{\text{original}} - N_{\text{drypellet}} \times M_{\text{drypellet}}}{N_{\text{original}} \times M_{\text{original}}} [\%] \tag{4}$$

2.3. Equilibrium kinetics and simulation

The equilibrium kinetics of the PCs and protein were calculated using the mass balance of the entire process (Fig. S2), and the PC and protein content in the extracts measured by the F-C and Dumas methods. Preliminary research has revealed that a 10-min washing time leads to almost complete removal of PCs. The equilibrium constants $K_{\text{pc},i}$ and $K_{\text{pro},i}$ are defined as the concentration of the component ($c_{\text{pc},i,\text{e}}$ or $c_{\text{pro},i,\text{e}}$) in the extracts over the concentration of the component remaining in the pellets ($c_{\text{pro},i,\text{p}}$ [mg/g dry pellet] or $c_{\text{pc},i,\text{p}}$ [mg GAE/g dry pellet]). The concentration of the component in the pellet was not measured and was therefore calculated from the ingredient mass balance (Fig. S2):

The ingredient balance:

$$m_{\text{DSK}} c_{\text{DSK}} = m_{\text{e}} c_{\text{e}} + m_{\text{p}} c_{\text{p}} \tag{5}$$

Mass of the dry pellet:

$$m_{\text{p}} = m_{\text{DSK}} - m_{\text{e}} \tag{6}$$

The equilibrium constants are given below and rewritten with known parameters using the mass balances described above:

1st step:

$$K_{\text{pc},1} \equiv \frac{c_{\text{pc},1,\text{e}}}{c_{\text{pc},1,\text{p}}} = \frac{c_{\text{pc},1,\text{e}} \times (m_{\text{DSK}} - m_{1,\text{e}})}{c_{\text{pc,DSK}} \times m_{\text{DSK}} - c_{\text{pc},1,\text{e}} \times m_{\text{sol}}} \tag{7}$$

2nd step:

$$K_{\text{pc},2} \equiv \frac{c_{\text{pc},2,\text{e}}}{c_{\text{pc},2,\text{p}}} = \frac{c_{\text{pc},2,\text{e}} \times (m_{\text{DSK}} - \sum_1^2 (m_{i,\text{e}}))}{c_{\text{pc,DSK}} \times m_{\text{DSK}} - \sum_1^2 (c_{\text{pc},i,e} \times m_{\text{sol}})} \tag{8}$$

3rd step:

$$K_{\text{pc},3} \equiv \frac{c_{\text{pc},3,\text{e}}}{c_{\text{pc},3,\text{p}}} = \frac{c_{\text{pc},3,\text{e}} \times (m_{\text{DSK}} - \sum_1^3 (m_{i,\text{e}}))}{c_{\text{pc,DSK}} \times m_{\text{DSK}} - \sum_1^3 (c_{\text{pc},i,\text{e}} \times m_{\text{sol}})} \tag{9}$$

where $m_{1,\text{e}}$, $m_{2,\text{e}}$,$m_{3,\text{e}}$ stand for the amount of dried extract obtained after freeze drying for each step. m_{sol} stands for the mass of the solvent used for each step and was calculated as the volume of 50 mL multiplied by the density of the water and ethanol mixtures. The amount of PC remaining in the pellet can be calculated by subtracting the amount of PC removed from DSK or the pellet in the previous step. Unfortunately, the loss of part of the pellet happened due to transfer of the material and incomplete removal of the solvent from the pellet, which also included soluble components, could not be avoided. Both effects, which have an opposite effect on the yields in the next steps, were therefore ignored in this calculation. The equilibrium constant K_{pro} at each step was calculated using equations similar to equations (7)–(9), where $c_{\text{pc},i,\text{e}}$ and $c_{\text{pc,DSK}}$ are replaced by $c_{\text{pro},i,\text{e}}$ and $c_{\text{pro,DSK}}$, respectively.

After the equilibrium constant was calculated, $K_{\text{pc},1}$ and $K_{\text{pro},1}$ obtained from the first step of the experiment results were used for the simulation of a multi-step exhaustive washing process to calculate the concentration of the PC and protein in the extract, expressed as $c^{\text{s}}_{\text{pc},i,\text{e}}$ (mg GAE/g liquid extract) and $c^{\text{s}}_{\text{pro},i,\text{e}}$ (mg/g liquid extract). The following assumptions were made before the simulations:

- The equilibrium constants K_{pc} and K_{pro} in each step of the multi-steps exhaustive washing process are constants.
- The amount of protein and PC that will be removed is much lower than the mass of the DSK, therefore the weight of the starting material for each step is assumed to be constant and the same as m_{DSK}.

Based on these assumptions, the concentration of PC in the extract of each step was simulated with the follow equations:

1st step:

$$c^{\text{s}}_{\text{pc},1,\text{e}} = \frac{K_{\text{pc},1} \times m_{\text{DSK}} \times c_{\text{pc,DSK}}}{m_{\text{DSK}} + K_{\text{pc},1} \times m_{\text{sol}}} \tag{10}$$

2nd step:

$$c^{\text{s}}_{\text{pc},2,\text{e}} = \frac{(c_{\text{pc,DSK}} \times m_{\text{DSK}} - c^{\text{s}}_{\text{pc},1} \times m_{\text{sol}}) \times K_{\text{pc},1}}{m_{\text{DSK}} + K_{\text{pc},1} \times m_{\text{sol}}} \tag{11}$$

n step:

$$c^{\text{s}}_{\text{pc},n,\text{e}} = \frac{(c_{\text{pc,DSK}} \times m_{\text{DSK}} - \sum_1^{n-1} c^{\text{s}}_{\text{pc},i} \times m_{\text{sol}}) \times K_{\text{pc},1}}{m_{\text{DSK}} + K_{\text{pc},1} \times m_{\text{sol}}} \tag{12}$$

where $c^{\text{s}}_{\text{pro},i,\text{e}}$ was also calculated according to equations (10)–(12) by replacing the relevant parameters for PC with the protein. The simulated PC removal yield (%) and protein loss (%) were calculated with equations (1) and (2), in which the $c_{\text{pc},i,\text{e}}$ and $c_{\text{pro},i,\text{e}}$ were replaced by $c^{\text{s}}_{\text{pc},i,\text{e}}$ and $c^{\text{s}}_{\text{pro},i,\text{e}}$.

2.4. Statistics analysis

The statistics in this paper were analysed using SPSS software, version 25.0 (IBM, Armonk, NY, USA). A univariate general linear model with the least significant difference test was carried out to investigate the significant differences with respect to Table 1 and Table 2 as well as

the total PCs being removed from the DSK in Fig S3. Differences were considered significant when $P < 0.05$ and shown as the small upper letters.

Correlation between the TPC and CGA content is shown in Fig. S1. A Pearson correlation factor was generated, and the significance differences were analysed at the $P < 0.01$ level.

3. Results and discussion

This section presents the results: (1) analysis of the extract regarding removal of PCs and protein loss under different process conditions; (2) PC removal and protein loss with multi-step washing by simulations; (3) evaluation of the functionality of the washed concentrates with respect to microstructure, protein nativity, WHC and NSI.

3.1. Analysis of the extract

The PC content is often measured using two different methods: the F-C method for the TPC and HPLC (specific CGA). In this study, the HPLC results gave a lower PC content than the F-C method. Zardo et al. (2019) also reported a lower amount of CGA after extraction from sunflower seed cake compared with the TPC results. The difference between the results for the two methods could be explained by the use of CGA as a marker component in the HPLC analysis, whereas in the F-C method, TPC was calculated on the basis of gallic acid equivalents. Nevertheless, the CGA and TPC results were highly correlated (Fig. S1); the Pearson correlation factor was 0.962, which was significant at 0.01 level. In the following results, the F-C method was selected for the discussion and simulation, because it accounted for the total PC present in the sunflower material instead of CGA only.

The results of aqueous washing are presented in Fig. 2A, with the pH and temperature varied. The TPC removed after 6 steps ranged between 23 and 36 mg GAE/g DSK; pH 7 at 25℃ exhibited the highest value and pH 4 at 25℃ showed the lowest value. Overall, the amount of PC removed at pH 7 at different temperatures was higher than at pH 4. Higher temperature at 75℃ led to higher removal at both pH values. The first step removed most of the PC with all conditions applied, and the effect of additional steps was limited, especially after 3 steps. The protein removed at pH 4 in the first step was low compared with that at pH 7, and hardly any protein was removed in additional steps at pH 4. The low solubility of sunflower protein (mainly globulin) was reported to be pH 4–6 (Albe Slabi et al., 2020; Pickardt et al., 2015; Subaşı et al., 2020), therefore protein loss at pH 4 is probably mainly due to the loss of the albumin fraction. The effect of temperature on protein extraction was more pronounced at pH 7 than at pH 4. Overall, the highest protein extraction was found at pH 7 and 75℃.

The amounts of TPC removed by aqueous ethanol washing at 25 and 50℃ are shown in Fig. 2B and 2C, respectively. The highest TPC value obtained was 34 mg GAE/g DSK from 0% and 20% ethanol content after 3 washing steps at 50℃. This accounts for 86% of the PC removal yield (Weisz et al., 2009). The amounts of TPC removed from the extract by the 1st washing step at 25℃ were similar for ethanol content between 0% and 60% and were slightly higher than the value obtained with 80% ethanol content. Hardly any PC was removed with 100% ethanol solvent. These results were in line with a previous study in which it was reported that the use of 50% v/v aqueous ethanol leads to more PC removal (30 mg GAE/g sunflower florets) than the use of 90% v/v aqueous ethanol (23 mg GAE /g sunflower florets) (Ye, Liang, Li, & Zhao, 2015). The 2nd and 3rd steps increased the total extraction of PC, and the amount removed in those steps was much lower than the

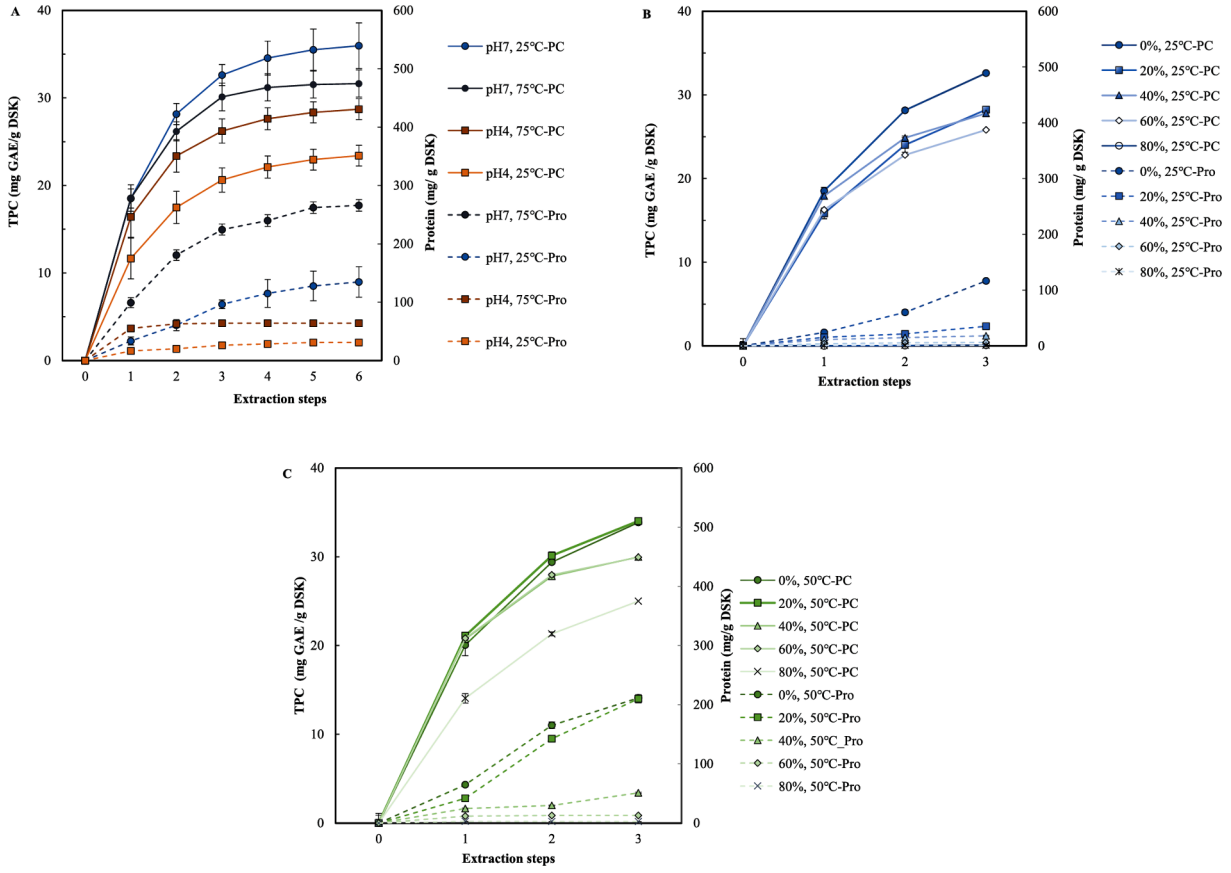

Fig. 2. The total amount of phenol content (TPC) and protein in the extracts obtained from aqueous washing (A), at the condition of pH4/25℃, pH 4/75℃, pH7/25℃, pH7/75℃; The amount of TPC and protein in the extracts obtained from aqueous ethanol washing at different ethanol contents 0%, 20%, 40%, 60% and 80% at 25℃ (B) and 50℃ (C). The TPC and protein results are expressed in mg gallic acid equivalent (GAE)/g DSK and mg protein/g DSK, respectively.

amount removed in the 1st step. The lower amount removed at high ethanol concentration might be due to the fact that the penetration of the solvent into the sample matrix was hindered at this high ethanol concentration (Zardo et al., 2019). Overall, higher TPC values were measured at temperatures above 50℃ compared with 25℃ at fixed ethanol content, but the TPC values removed were lower with increasing ethanol content. The result of 76% PC yield with 40% ethanol content at 50℃ was found to be the same as the reported CGA yield of 76% with 40% ethanol content at 90℃ after three sequential steps (Scharlack, Aracava, & Rodrigues, 2017). The effect of temperature was also in line with the results presented in previous studies (Sripad et al., 1982; Vázquez-León et al., 2019; Zardo et al., 2019).

The amount of protein in the extracts with different ethanol content and at different temperatures is also shown in Fig. 2B and 2C. The total amount of protein extracted by aqueous ethanol was lower when using solvents with higher ethanol content at both 25 °C and 50℃. The highest amount was 117 mg/g DSK achieved with 0% ethanol content at 25℃. The amount of protein extracted decreased above 20% ethanol content. This outcome might be a result of the low polarity and denaturation effect of ethanol (Chemat et al., 2012; Wagner, Andreadis, Nikolaidis, Biliaderis, & Moschakis, 2021), which decreased protein solubility and extractability in the solvent (González-Pérez, 2015). A small additional amount of protein was extracted in the 2nd and 3rd steps. This was different from the PC, which were mostly extracted in the first step. Furthermore, similar amounts of protein were found in the extracts obtained with 0% and 20% ethanol content at 50℃, which were approximately 2 times higher compared with 25℃ with 0% ethanol. At 50 °C, protein extraction was higher when using solvents containing ethanol than the extraction at 25 °C, which might be due to the change of the matrix from which it is extracted as a function of temperature and ethanol content in the solvent (Zhong et al., 2014).

3.2. Phenolic compounds removal efficiency versus protein loss

The purpose of the washing process described above is to effectively remove PCs while retaining proteins. The protein extracted is considered as loss of protein. An optimal process is thus to extract all PCs while limiting protein loss. Therefore, the 2 parameters, TPC removal yield (%) and protein loss (%), for different steps were plotted in a cumulative curve (Fig. 3A). This allows better understanding of the effect of the washing steps on both parameters and evaluation of the optimal process conditions.

Most of the data points were concentrated in the area ranging from 30% to 75% PC yield and 0% to 15% protein loss. This part of the diagram can be achieved using many different process conditions, such as 3 steps with ethanol content below 60% at 25℃ or 2 steps with water at 25℃. Increasing the ethanol content to 80% can lead to a PC yield up to 60% with hardly any loss of protein. A further increase in the PC yield of>80% can be achieved by increasing the temperature or using pure water. However, those conditions resulted in protein loss of up to 52%. From the results, one can hypothesize that the area with both high PC removal yield and low protein loss can be achieved with high ethanol contents by applying more steps. The removal of PCs was not complete with 3 washing steps, thus it would be interesting to know the effect of

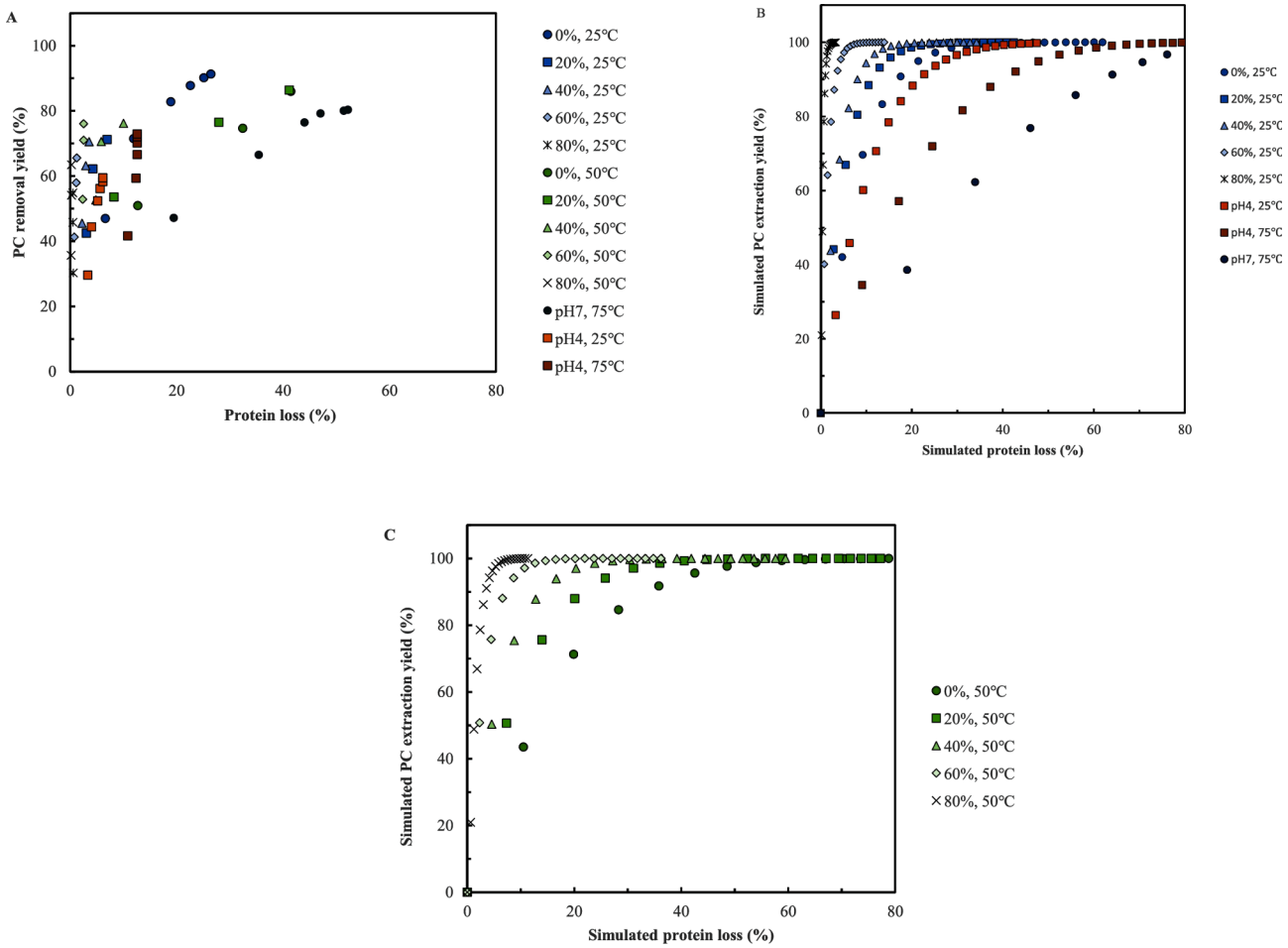

Fig. 3. Removal yield (%) of phenolic compounds (PCs) versus protein loss (%) in the extracts from the aqueous washing and aqueous ethanol washing of de-oiled sunflower kernel (DSK) (A). Simulations of multi-step exhaustive washing process for the pH4/25℃, pH 4/75℃, pH7/25℃, pH7/75℃ (B), and different ethanol content 0%, 20%, 40%, 60% and 80% at 25℃ (B) and 50℃ (C); the closed symbols for 25℃ and open symbols for the 50℃.

increasing the number of washing steps. For this reason, the equilibrium constants K_{pc} and K_{pro} were calculated and this is discussed further in the following section.

3.3. Equilibrium kinetics and simulation

The equilibrium constants K_{pc} and K_{pro} were calculated based on the experimental results for the amount of PC removed and protein extracted (Table 1). The K_{pc} corresponding to pH 4 was found to be lower than the values at pH 7 or in aqueous ethanol mixtures. For the first step of aqueous ethanol washing, K_{pc} was stable between 0.065 and 0.075 at 25℃ for different ethanol content, except for the lower value above 80% ethanol content. K_{pc} was higher at 50℃ with ethanol content between 20% and 60%, and the value was similar for both temperatures at 0% and 80% ethanol content.

The value for K_{pc} decreased with the additional washing steps, and was similar for different ethanol contents and at different temperatures. These results suggested that K_{pc} was independent of the ethanol content and temperature with regard to PC removal after the 1st washing step. The decrease in K_{pc} at the 2nd and 3rd steps suggest that the matrix changed by renewing the solvent each time (Zhong et al., 2014). Changes could be caused by extraction of other components or by altering the interaction of PCs with the matrix. The first effect most likely occurred at high water content, whereas the second effect is more likely to occur with an aqueous ethanol mixture. Nonetheless, the value for the K_{pc} was found to be similar as reported in the literature for the PCs extraction from M. oleifera leaves (Vázquez-León et al., 2019).

The K_{pro} value at pH 7 was much larger than the value at pH 4, while a higher temperature enhanced the values. K_{pro} decreased with increasing ethanol content for the first step and K_{pro} at 50℃ was larger than at 25℃. The equilibrium constants varied at pH 7 for the 2nd and 3rd steps with different ethanol content. Nevertheless, it was found that the impact of solvent, pH and temperature was more pronounced for K_{pro} than for K_{pc}. Thus, a change in the process conditions or solvent quality influenced the loss of protein more than the extraction of PCs. To better understand the consequence of this, we simulated the DSK washing process. In the simulation, K_{pc} and K_{pro} were assumed to be constant for each step and the values were taken from the 1st step. Process conditions of 0%, 20%, 40%, 60% and 80% ethanol and pH 4 of water at 25℃ were selected for the simulation, and the simulation outcomes are presented in Fig. 3.

Fig. 3B and 3C reveals that all curves can be divided into 2 regions: a region with sharp increase in the amounts of PC removal followed by levelling off. It is interesting to compare the different conditions in which similar amounts of PC are removed. Here, we analyse the protein losses at a PC yield of 95%. For example, a PC yield of 95% can be achieved by a 6 step process with pure water at pH 7, which gives a protein loss of 25%. Alternatively, the washing can be performed using pH 4 and 10 steps, but this leads to a protein loss of 27%. When aqueous ethanol mixtures were applied with the ethanol content ranging from 20% to 60%, a PC yield of 95% could also be achieved using 6 steps, but the protein loss decreased from 13% to 3% with increasing ethanol content. More steps were required to reach the 95% yield with 80% ethanol content, but then only 1% of protein was lost. Therefore, the application of a multi-steps process using high ethanol content is preferred when minimal protein loss is required. Overall, it can be concluded that when selecting process conditions, it is more important to focus on limiting protein loss rather than optimizing PC removal.

An efficient industrial translation of a multi-step batch process is a counter-current multi-stage extraction (CME). The CME process is recommended for energy and solvent efficiency compared with exhaustive extraction (Vázquez-León et al., 2019). The simulation of a multi-step exhaustive batch process presented here provides a theoretical basis and potential outlook for the CME process with respect to PC removal and protein loss.

Table 1
Equilibrium constants calculated from the experimental data for total phenol content by the F-C method and protein content by the Dumas method, indicated as K_{pc} for phenolic compounds and K_{pro} for protein with ethanol contents of 0%, 20%, 40%, 60% and 80% at 25℃ and 50℃ taking 3 washing steps into account. The values in the table are compared within the column and different upper case letters indicate a significant difference ($P < 0.05$).

Aqueous washing	K_{pc}1 (* 10^{-3})	K_{pro} 1 (* 10^{-3})	K_{pc} 2 (* 10^{-3})	K_{pro} 2 (* 10^{-3})	K_{pc} 3 (* 10^{-3})	K_{pro} 3 (* 10^{-3})
pH 4, 25℃	36.2 ± 9.0 ^{a}	3.0 ± 0.1 ^{a}	14.1 ± 1.6 ^{a}	6.1 ± 0.1 ^{a}	6.7 ± 1.7 ^{a}	1.1 ± 0.004 ^{a}
pH 4, 75℃	54.1 ± 5.5 ^{b}	9.2 ± 0.03 ^{b}	14.9 ± 1.1 ^{a}	13.5 ± 0.2 ^{b}	5.2 ± 0.4 ^{a}	0.2 ± 0.001 ^{b}
pH 7, 25℃	75.0 ± 5.7 ^{c}	4.6 ± 0.1 ^{a}	23.7 ± 0.7 ^{b}	7.2 ± 0.9 ^{a}	7.8 ± 3.9 ^{a,b}	11.9 ± 0.4 ^{c}
pH 7, 75℃	65.2 ± 11.5 ^{b,c}	19.6 ± 2.0 ^{c}	14.5 ± 1.4 ^{a}	15.9 ± 0.1^{b}	5.8 ± 1.2 ^{a}	8.3 ± 0.1 ^{d}
Aqueous ethanol washing	K_{pc}1 (* 10^{-3})		K_{pc} 2 (* 10^{-3})		K_{pc} 3 (* 10^{-3})	
Ethanol content	25℃	50℃	25℃	50℃	25℃	50℃
0%	75.0 ± 5.7 ^{a}	82.2 ± 2.5 ^{a}	23.7 ± 0.7 ^{a}	17.4 ± 3.0 ^{a}	7.8 ± 3.9 ^{a,b}	5.6 ± 0.3 ^{a}
20%	65.5 ± 2.9 ^{b}	108.7 ± 3.0 ^{b}	19.8 ± 0.6 ^{b}	19.5 ± 0.3 ^{a}	7.4 ± 0.5 ^{a}	5.9 ± 0.7 ^{a}
40%	76.4 ± 6.0 ^{a}	106.7 ± 7.0 ^{b}	18.3 ± 0.9 ^{b}	18.0 ± 1.0 ^{a}	6.5 ± 0.5 ^{a}	4.4 ± 0.1 ^{b}
60%	69.0 ± 0.7 ^{b}	105.8 ± 2.0 ^{b}	18.4 ± 0.2 ^{b}	19.6 ± 1.8 ^{a}	7.2 ± 0.2 ^{a}	4.5 ± 0.4 ^{b}
80%	46.4 ± 0.3 ^{c}	59.0 ± 1.2 ^{c}	18.8 ± 0.1^{b}	22.6 ± 2.0 ^{a}	9.7 ± 0.1 ^{b}	9.8 ± 0.6 ^{c}
Ethanol content	K_{pro} 1 (* 10^{-3})		K_{pro} 2 (* 10^{-3})		K_{pro} 3 (* 10^{-3})	
0%	4.6 ± 0.1 ^{a}	10.8 ± 0.1 ^{a}	7.2 ± 0.9 ^{a}	23.8 ± 1.7 ^{a}	11.9 ± 0.4 ^{a}	9.6 ± 0.2 ^{a}
20%	2.7 ± 0.1^{b}	7.3 ± 0.3 ^{b}	1.6 ± 0.1 ^{b}	23.5 ± 1.2 ^{a}	2.8 ± 1.1 ^{b}	15.1 ± 2.0 ^{b}
40%	2.0 ± 0.1 ^{b}	4.4 ± 0.2 ^{c}	1.0 ± 0.004 ^{c}	1.6 ± 0.2 ^{b}	0.7 ± 0.1^{c}	4.6 ± 0.3 ^{c}
60%	0.7 ± 0.04 ^{c}	2.1 ± 0.3 ^{d}	0.5 ± 0.1 ^{d}	0.5 ± 0.2 ^{c}	0.1 ± 0.03 ^{d}	N.A
80%	0.2 ± 0.03 ^{d}	0.6 ± 0.01 ^{e}	0.1 ± 0.03 ^{e}	N.A*	0.02 ± 0.01 ^{e}	N.A

* N.A indicates the results were not applicable due to not enough samples obtained.

3.4. The effect of process conditions and solvent quality on protein functionality

The morphologies of the DSK and the concentrates were visualized with SEM (Fig. 4). The original DSK particle consisted of a compact structure, which is known to be a cellular matrix, and spherical particles were present on the surface. Those spherical particles were most likely protein bodies, which have a known particle size of 0.5–10 μm (González-Pérez, 2015; Oscar Laguna et al., 2018). The cellular matrices became open laminar structures after washing with 0% ethanol at both 25° and 50℃ with all the spherical particles disappeared, leading to pores with a diameter between 10 and 50 μm. The results suggested that the protein present on the surface was fully washed away by the water, which was in line with the high protein loss (Section 2.2.3). When the ethanol content increased above 40% at 25℃ and 50℃, small spherical particles of <8 μm became visible on the surface of the structure. Here, the matrix became dense and compact under these conditions. These observation is in line with results reported for the matrix of okara, which was suggested to be in the glassy state when exposed to a solvent with high ethanol content. As a consequence, the diffusion of the PC to the solvent was hindered (Jankowiak, Kantzas, Boom, & Van der Goot, 2014), which explains why the addition of water to the matrix was needed to facilitate PC removal. At pH 4, the open cell wall matrix was

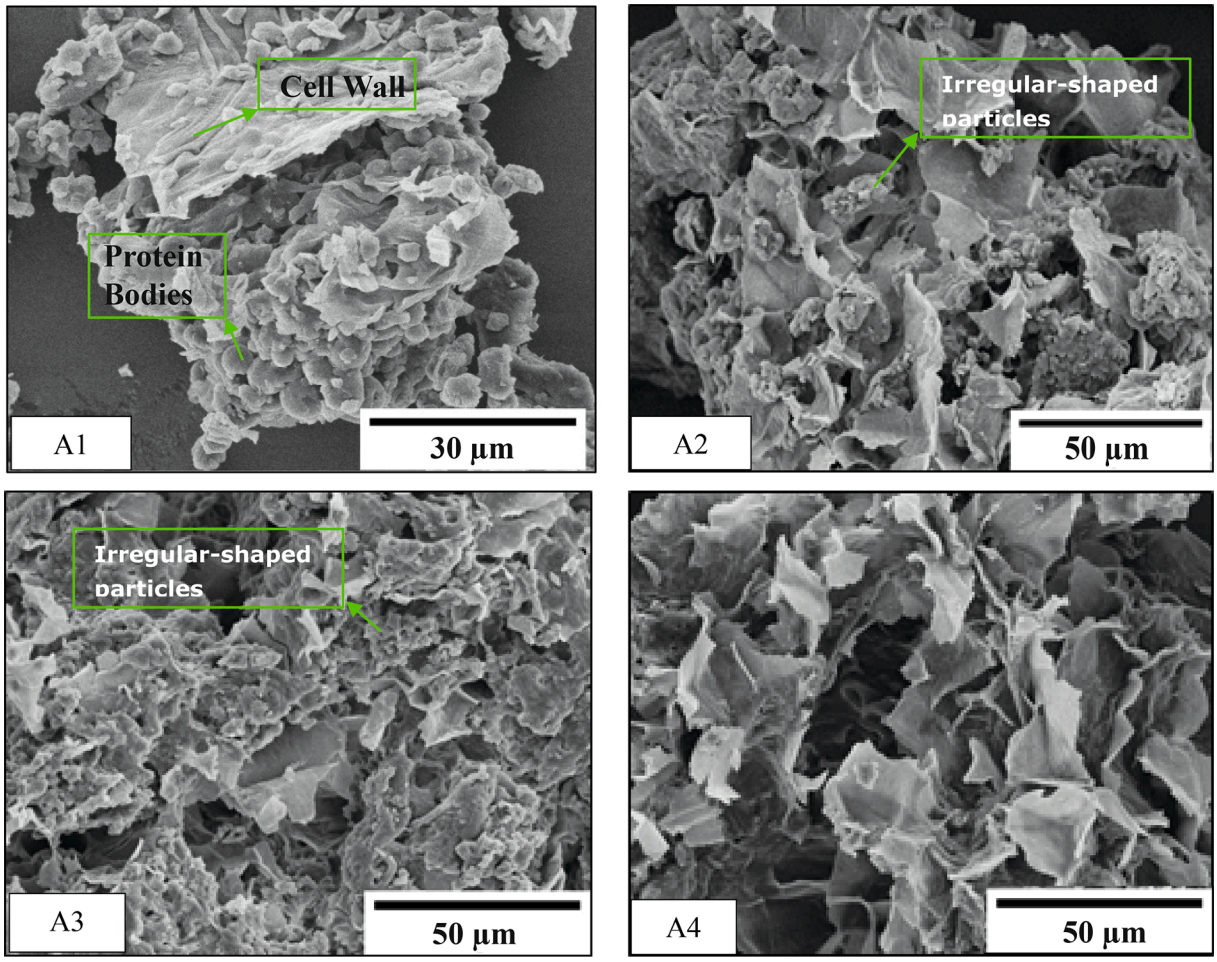

Fig. 4. Scanning electron micrographs for the DSK (A1) and aqueous washed concentrates with pH 4 and 25℃ (A2), pH4 and 75℃ (A3), pH 7 and 75℃ (A4); aqueous ethanol washed concentrates with ethanol contents of 0%, 20%, 40%, 60%, 80% and 100% at 25℃ (B1–B6) and 50℃ (C1– C6). The different scale bars of 30 μm or 50 μm were showed, because the particle size were different. Only one representative picture was selected from 8 pictures.

filled with aggregates of 10–30 μm, and the aggregates were found to be more connected when the temperature increased to 75℃. The aggregates found at pH 4 might be associated with the protein aggregation at the isoelectronic point and lowest protein solubility between pH 4 and 6 (Albe Slabi et al., 2020; Perez et al., 2005).

The functionalities analysis of the concentrate after the process are shown in Table 2. Protein content of the material was increased above 20% ethanol content at 25℃ or 40% ethanol at 50℃, due to the removal of other soluble components. The decreased protein content for concentrates obtained from low ethanol content at both temperatures relates to the high protein loss in the extracts during washing (Fig. 2). The TPC recovery yield in the concentrate became higher with increasing ethanol content, which is in line with the TPC removal in the extract (Fig. 2). No significant differences between the enthalpy of the denaturation peak of DSK and the concentrates treated with 80% ethanol content at 25℃ and 50℃ were found, which indicates that hardly any denaturation had occurred during extraction. The other conditions resulted in partial denaturation with decreased enthalpy at 25℃. It is reported that ethanol can induce protein denaturation (Liu, Li, Zhang, & Tang, 2019). Remarkably, the protein was completely denatured with 0% and 20% ethanol at 50℃ although it had not yet reached T_d. Complete denaturation was found with the concentrates obtained by aqueous washing at 0% and 20% ethanol at 50℃. Besides, complete denaturation was found with pH 7, at 75℃ and pH 4 at different temperatures (results not shown in the table). The effect of pH on protein nativity was in line with the literature (Investigacio, 2004). The results indicated that the effect of high temperature on protein nativity was more pronounced at lower ethanol content compared with a high ethanol content. The use of high temperature at low ethanol content and low pH with 0% ethanol content should thus be avoided if protein nativity is important. The DSK washed by aqueous ethanol mixture with ethanol content of 20%–60% at 25℃ and 50℃ showed similar NSI (range, 50%–56%) as the original DSK. The washed concentrates obtained using pure water and 100% ethanol content at 25℃ had slight lower NSI of 34%–41%, which aligns with the results that show partial protein denaturation. Lower NSI values of 16% and 7% were observed with the completely denatured concentrates obtained after extraction with pure water and 20% ethanol content at 50℃. The results suggested that efficient PC removal with increased ethanol content of 40%–80% can not only limit protein loss but it also preserves protein nativity and solubility. The high protein loss during washing with low ethanol content also showed reduced protein functionalities with complete protein

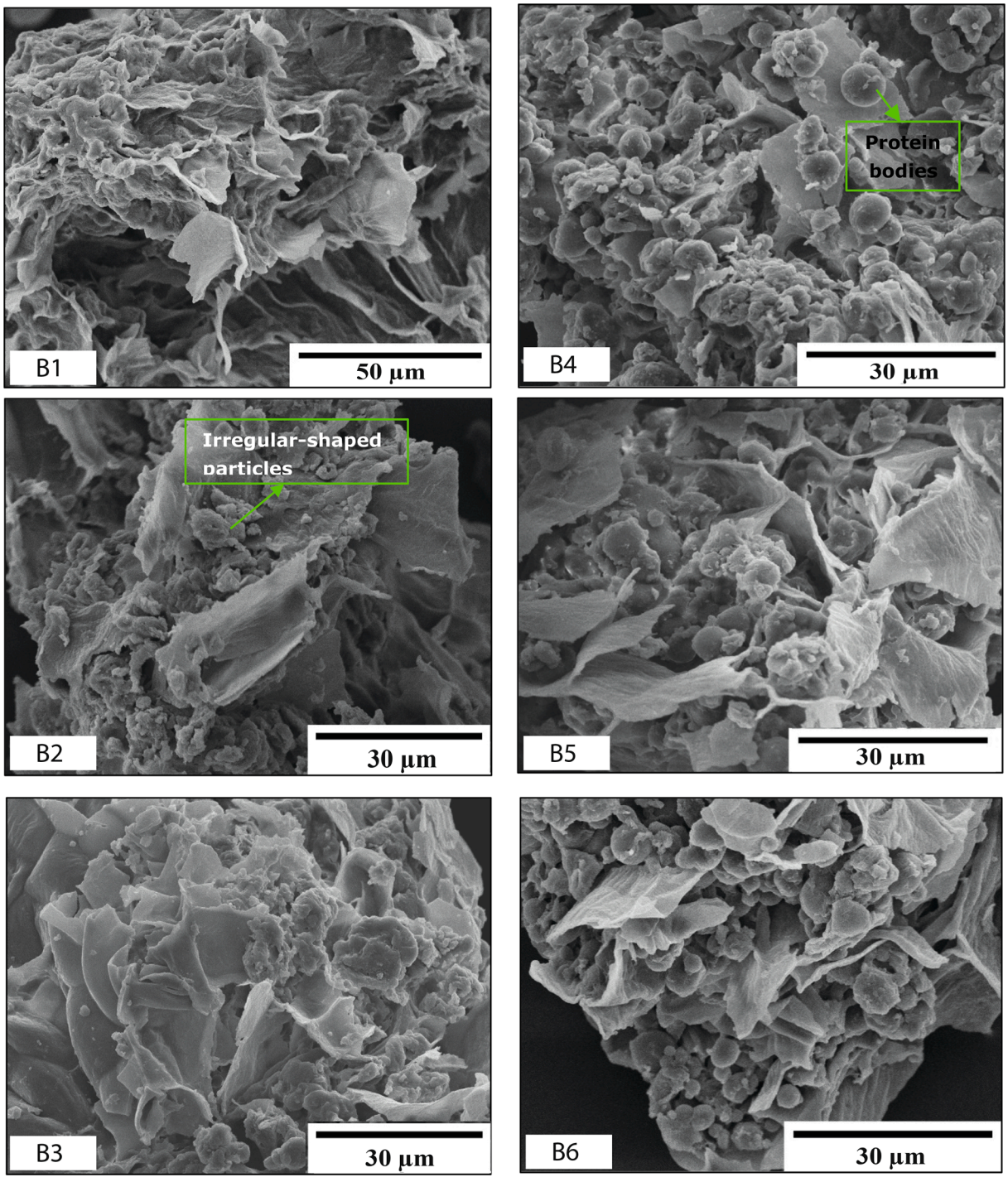

Fig. 4. (*continued*).

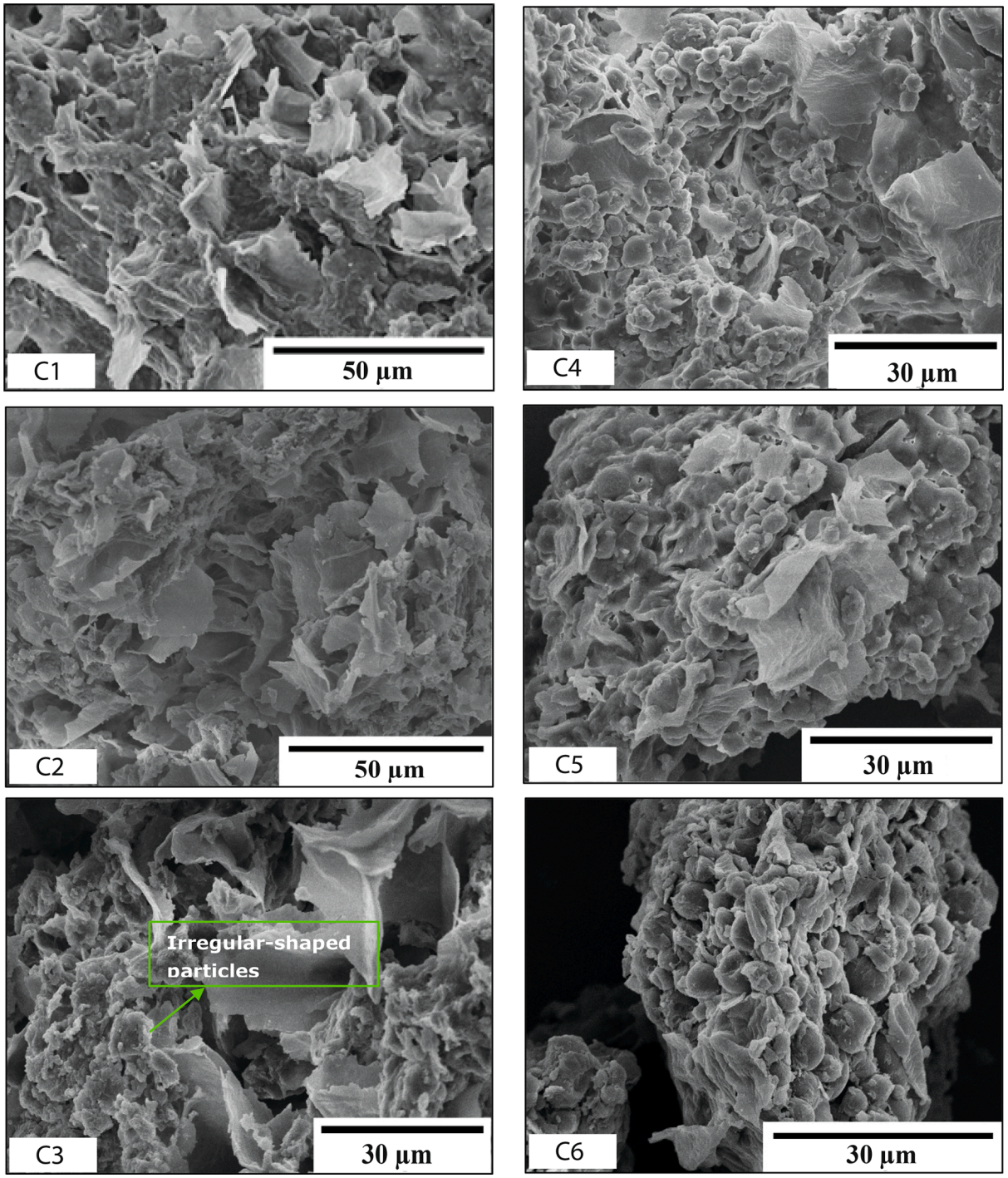

Fig. 4. (*continued*).

denaturation and low protein solubility.

The concentrates obtained from aqueous ethanol washing showed enhanced WHC under different conditions compared with the original DSK (Table 2). The highest WHC was found with 40% ethanol at 25℃ and 0% ethanol at 50℃. A slightly lower value was found with ethanol contents > 60%. The results suggest that aqueous ethanol washing in general has a positive effect on the WHC of sunflower material, whereas the effect is smaller when using higher ethanol contents. The WHC is known to be influenced by the composition, such as defatting and protein enrichment, or protein conformational change and hydrophobicity (Jia et al., 2021; Zhang, Yang, Tang, Chen, & You, 2015). The results discussed above suggest that the functionality difference of the concentrates obtained after extraction using solvents with different ethanol content or temperatures were small, except when the pure water was applied. Enhanced WHC after aqueous ethanol washing process might lead to potential structuring properties, such as meat analogue which required a high WHC (Cornet, Edwards, van der Goot, & van der Sman, 2020). Therefore, techno-functional and structuring properties of the washed concentrates by aqueous ethanol washing might be carried out in future study for this application.

Table 2
The functional properties of the original sample DSK and the washed concentrates obtained at different ethanol ratios of 0%, 20%, 40%, 60%, 80% and 100% at 25℃ and 50℃. The functional properties including protein content (%), TPC recovery yield (%), protein denaturation at onset denaturation temperature, denaturation peak temperature (T_d) and denaturation enthalpy (J/g protein), WHC (g water/ g dry pellet) and NSI (%). The values in the table are compared within the column and different upper case letters indicate a significant difference ($P < 0.05$).

T (℃)	Ethanol content (%)	Protein (g/100 g)	TPC recovery yield (%)	DSC Onset T[°C]	T_d[°C]	Enthalpy[J/g protein]	WHC(g water/ g dry pellet)	NSI (%)
25	DSK	51.0 ± 1.4 ^{a,b}	N.A	93.3 ± 0.2 ^{a}	99.8 ± 0.2 ^{a}	9.4 ± 1.5 ^{b}	5.7 ± 0.2 ^{a}	49.2 ± 0.1 ^{a}
	0%	49.8 ± 0.9 ^{a}	11.3 ± 1.2 ^{a}	93.0 ± 0.6 ^{a}	99.5 ± 0.4 ^{a}	6.1 ± 1.0 ^{a}	8.7 ± 0.2^{b}	33.7 ± 5.4^{b}
	20%	56.6 ± 0.3^{c,d}	21.3 ± 2.1^{b}	93.2 ± 0.9 ^{a}	99.1 ± 0.2 ^{a}	6.7 ± 1.1 ^{a}	8.8 ± 0.2^{b}	50.8 ± 0.3 ^{a}
	40%	57.9 ± 1.0^{c}	24.1 ± 1.9^{b}	92.9 ± 1.1 ^{a}	99.0 ± 1.0 ^{a}	6.5 ± 0.1 ^{a}	9.2 ± 0.02^{c}	55.6 ± 0.1^{c}
	60%	57.9 ± 0.5^{c}	28.9 ± 0.9^{b}	93.2 ± 0.1 ^{a}	99.6 ± 0.2 ^{a}	7.4 ± 0.4 ^{a}	8.8 ± 0.1^{b}	56.5 ± 1.5^{c}
	80%	55.7 ± 0.6 ^{d}	40.3 ± 0.4^{c}	93.4 ± 0.6 ^{a}	99.5 ± 0.1 ^{a}	9.1 ± 1.1^{b}	7.6 ± 0.04 ^{d}	51.5 ± 0.2 ^{a}
	100%	53.8 ± 1.2^{b}	89.6 ± 0.002 ^{d}	93.6 ± 0.1 ^{a}	99.5 ± 0.2 ^{a}	7.1 ± 0.6 ^{a}	7.0 ± 0.1 ^{e}	41.5 ± 1.9 ^{d}
50	0%	41.1 ± 1.0 ^{e}	10.2 ± 1.5 ^{a}	N.D*	N.D	N.D	10.0 ± 0.1 ^{d}	6.6 ± 0.9 ^{e}
	20%	44.5 ± 1.0 ^{e}	7.6 ± 1.8 ^{a}	N.D	N.D	N.D	9.4 ± 0.6^{c}	15.9 ± 0.6^{f}
	40%	55.9 ± 1.1 ^{d}	18.0 ± 1.4^{b}	93.5 ± 0.2 ^{a}	99.7 ± 0.4 ^{a}	6.6 ± 1.0 ^{a}	9.0 ± 0.3^{b,c}	53.3 ± 0.4^{c}
	60%	58.9 ± 1.1^{c}	19.4 ± 1.2^{b}	93.4 ± 0.1 ^{a}	99.6 ± 0.1 ^{a}	7.3 ± 0.1 ^{a}	8.0 ± 0.1 ^{d}	52.9 ± 1.2^{c}
	80%	55.3 ± 1.4 ^{d}	31.4 ± 1.2^{c}	93.2 ± 0.3 ^{a}	99.6 ± 0.1 ^{a}	9.8 ± 2.4^{b}	7.8 ± 0.1 ^{d}	54.0 ± 1.5^{c}
	100%	54.4 ± 0.9^{b,d}	82.2 ± 0.01 ^{d}	93.2 ± 0.1 ^{a}	99.4 ± 0.1 ^{a}	8.6 ± 1.3 ^{a,b}	7.3 ± 0.1 ^{e}	40.8 ± 2.2 ^{d}

* N.D indicates that the results were not detectable.

4. Conclusions

In this study, scientific insight was gained on the effects of the process conditions (pH and temperature) and solvent quality on removal of PCs and protein loss from DSK. The amount of PC removed from DSK was positively affected by neutral pH, a higher temperature of 50℃ and an ethanol content below 60%. However, increasing PC removal also led to higher protein loss in most of those circumstances. In addition, it was found that the effect of altering the process conditions was greater on protein loss than PC removal yield, implying that the optimal process conditions will be mostly governed by the protein loss. Experimental results showed that by fine-tuning the process conditions, the PC removal yield can reach 75% with<15% protein loss using only 3 steps. Simulations revealed that PC removal of 95% can be reached in a multi-step exhaustive washing process, with protein loss < 3% using an ethanol content > 60% at 25℃. If even more steps are allowed or a counter-current process is applied, protein losses can be reduced to < 1% using 80% ethanol. In addition to the reduced protein loss, aqueous ethanol washing with high ethanol content is also preferred, because the use of those process conditions preserves the functional properties of native protein best.

CRediT authorship contribution statement

Wanqing Jia: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - original draft, Data curation, Visualization. **Konstantina Kyriakopoulou:** Conceptualization, Supervision, Writing - review & editing. **Bente Roelofs:** Methodology, Data curation. **Mbalo Ndiaye:** Project administration, Funding acquisition, Writing - review & editing. **Jean-Paul Vincken:** Methodology, Writing - review & editing. **Julia K. Keppler:** Supervision, Conceptualization, Verification, Writing - review & editing. **Atze Jan Goot:** Conceptualization, Supervision, Methodology, Project administration, Funding acquisition, 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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2021.130204.

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