

Cite this: *Food Funct.*, 2020, **11**, 6273

Mechanical cell disruption of mustard bran suspensions for improved dispersion properties and protein release†

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Mustard bran, a by-product of mustard production, is still rich in valuable compounds. The high-pressure homogenization treatment was tested as a mechanical cell disruption (MCD) technique to unlock valuable intracellular compounds. An aqueous suspension of mustard bran was treated by shear mixing, followed by high-pressure homogenization at different pressure levels (50–150 MPa) and number of passes (1–10), and using different homogenizing systems. The moderate-intensity treatment (up to 100 MPa and 3 passes) can deliver significant changes in the mustard bran suspension, inducing (a) a more homogeneous and smooth appearance due to the disruption of individual cells, (b) a better structuring ability in the suspension, through the increase in viscosity and storage and loss moduli G' and G'' , as well as (c) a remarkable enhancement of protein release, up to 72% of total proteins. The controlling factor in the extent of MCD was found to be the specific energy transferred to the mustard bran suspensions, whereas no significant differences were recorded when varying the homogenization system. The MCD process of mustard bran, based on its physical treatments using only water as a suspension medium, can be regarded as a safe, clean and environmentally friendly technology platform, which contributes to reaching the zero-waste concept by transforming agro-food by-products into value-added ingredients, with enhanced functionality and bioactive content.

Received 1st April 2020,
Accepted 24th June 2020
DOI: 10.1039/d0fo00852d

rsc.li/food-function

Introduction

Mustard is extensively used in the preparation of mustards and salad dressings, because of its emulsification and water-binding capacity, deriving from the high mucilage content.^{1,2} The mucilage, primarily contained in the seed hull and bran (up to 20–25%), consists of a complex of cellulose and acid polysaccharides, characterized by high viscosity, and thickening and stabilization properties.³ Mustard bran represents a valuable by-product of mustard production also because it is rich in proteins and hydrophilic polysaccharides with proteinaceous moiety with high surface activity,¹ as well as in high-

molecular-weight polysaccharides and uronic acid, with remarkable antioxidant power.²

However, the valorization of mustard bran is a challenging task, because most of its high value-added components are tightly locked inside the bran cells. Conventional processes of recovery of intracellular compounds are usually based on the use of organic solvents in combination with agitation and, eventually, high-temperature processing, causing the degradation of target compounds,⁴ or use of enzymes, with an increase of the operating costs.^{5,6} However, novel methods, based on the selective permeabilization of the cell membranes, such as Pulsed Electric Fields (PEF),⁷ might not be sufficient to extract the compounds located in the inner bodies of the cells, *i.e.* vacuoles and lipid vesicles, such as proteins and phenols,⁸ whereas more intensive or selective processes are needed.^{9,10}

Mechanical cell disruption (MCD) technologies represent a natural and sustainable approach, which, using minimal heating and no chemical treatments, enables the exploitation of the insoluble parts of the bran. An MCD treatment, requiring a high mechanical energy dose to be transferred to the individual plant cells, needs to be based on high-energy-density wet-milling methods, such as high-pressure homogenization of aqueous suspensions of plant material, to achieve cell deagglomeration, disruption of cell walls and membranes,¹¹ and cellulose defibrillation,^{12,13} with the final goal of

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†Electronic supplementary information (ESI) available. See DOI: 10.1039/d0fo00852d

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unlocking intracellular compounds, including proteins and mucilage, for improved digestion and absorption,^{14,15} or for exploiting their techno-functional properties.^{16,17}

Remarkably, MCD technologies may rely on the use of water as an extraction solvent, rather than organic solvents, which are often toxic and require to be completely removed from the exhaust material before its use or disposal.¹⁸ However, the use of co-solvents, co-solutes or even oil might be beneficial to enhance either extraction yield or selectivity.¹⁹

Different green technologies have been reported in the micronization of plant tissue in suspension to recover intracellular bioactive compounds with high extraction yields. These include high-pressure homogenization (HPH), either with piston valves, orifice valves or microfluidizers,^{7,20,21} but also ultra-high pressure extraction (UPE),²² negative pressure cavitation (NPC),²³ high voltage electrical discharges (HVED),²⁴ pulsed electric fields (PEF),⁹ and ultrasounds (US).²⁵ All these techniques were reported to enhance the mass transfer rate of intracellular compounds, through the mechanical damage of cell walls and membranes in an environmentally friendly way. They mainly differ in the degree of cell damage, which ranges from simple cell membrane permeabilization (PEF, UPE and NPC and lower intensity US) to complete cell disruption (HPH, HVED and higher intensity US), and therefore can be selected based on the cell structure of the plant material and the location within the cells of the targeted compounds. Overall, HPH offers significant advantages in terms of ease of operation, scalability, robustness, and consistent performance.^{26–28}

MCD treatments by HPH were previously applied to different plant cell aqueous suspensions, including corn bran,²⁹ wheat bran,^{30,31} rice bran,³² corn gluten meal,³³ broccoli,³⁴ broccoli seeds,³⁵ soy slurry, and soybean okara,^{36,37} tomato peels and spent coffee grounds,^{20,38} intending to develop, through the release of intracellular compounds, a secondary product with improved properties, in terms of appearance, structuring capability, nutritional value, or health-beneficial effects, including antioxidant activity or capability to bind heavy metal ions.

This work aims at assessing, for the first time, the role of a purely physical MCD treatment based on HPH in sustainably improving different technological and nutritional properties of yellow mustard bran, without any waste stream being generated, and using only water as process medium, hence avoiding the use of any environmentally harmful reagent. In particular, the main operating parameters of HPH processing, including valve geometry, pressure, and number of passes are investigated in terms of the effect on size distribution, rheological behavior, and protein release, also during simulated gastric digestion, of yellow mustard bran suspensions.

Experimental

Materials

Yellow mustard bran (*Sinapis alba*) was a kind gift from G.S. Dunn Ltd (product code 402) and was used as received.

Mechanical cell disruption

The MCD treatment consisted of sequential treatments of shear mixing (SM) and high-pressure homogenization in a piston valve system (HPH, followed by S1 for 1-stage valve and S2 for 2-stage valve) or by microfluidization (MF).

Yellow mustard bran was suspended in Milli-Q water (2 wt%), without any pH adjustment, similarly to the conditions used in previously reported microfluidization treatments on wheat or corn bran.^{29,30}

SM treatment was carried out on 500 g of suspension, using a Silverson L4RT-W shear mixer, mounting the emulsor screen with fine perforation, operated at 7000 rpm for 5 min at room temperature (20 °C).

HPH treatment was carried out on the SM-treated suspension (500 g), using a Panda Plus Niro Soavi homogenizer, equipped with flat-head piston, adjustable valves, at a process pressure between 50 and 150 MPa, for a number of passes $N = 1–10$, with intermediate cooling in a water bath. The homogenizer was operated either with a single-stage homogenization valve or with a two-stage valve (two valves in series), with the pressure drop in the second valve set to 30 MPa.

MF treatment, using a Microfluidics Corporation Pneumatic Microfluidizer Model 110S, equipped with a Z-disruption chamber (87 μm), was conducted at 120 MPa, at room temperature, with intermediate cooling in a water bath. However, to reduce the possibility of blocking the fixed-geometry chamber of the Microfluidizer homogenizer, a first HPH pass at 50 MPa was carried out in the Panda Plus homogenizer, followed by 1–4 MF passes, for a total N of up to 5 passes.

Particle size analysis

Mean particle size and size distribution were measured by laser diffraction using a Mastersizer 2000 (Malvern Instruments, Worcestershire, UK). Particle size analysis of each sample was determined based on the Mie theory, setting the refractive index of sample to 1.50 and of dispersant aqueous phase to 1.33 (refractive index of water), which gave the best fit based on product specifications. The refractive index of the sample is in agreement with that reported for oat insoluble dietary fibers.³⁹ From the particle size distribution, the D_{10} , D_{50} , and D_{90} diameters, corresponding to the 10, 50 and 90 percentile of the cumulative distribution, the volume mean diameter ($D_{4,3}$), and the surface mean diameter ($D_{3,2}$) were calculated, as previously described.²⁰ Measurements were repeated three times on two independently prepared samples.

Mean diameter and polydispersity index (PdI) of the supernatant of processed and unprocessed mustard bran suspensions (2 wt%), obtained by centrifugation (5 min at 11 400g in an Eppendorf Micro Centrifuge 5415 C) were measured by dynamic light scattering using a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK). Measurements were performed at 25 °C with an equilibrium time of 60 s, without any dilution. Measurements were made in triplicate on two independently prepared samples.

Rheology

Rheological tests were carried out using a controlled stress rheometer (AR, 2000; TA Instruments, Newcastle, DE) at 20 °C. Parallel plate geometry with 40 mm plate diameter was used, with the gap size set to 1 mm, in consideration of the solid particles suspended in the aqueous phase. The measurement procedure consisted of a time sweep test under continuous oscillation at 1 Hz frequency and 1.0% strain stress as a conditioning step for the sample, followed by a continuous ramp step up from a shear rate of 0.1 s⁻¹ to 500.0 s⁻¹ for 2 min, and a continuous ramp step down back to a shear rate of 0.1 s⁻¹ for 2 min, and a final strain sweep test, conducted under continuous oscillation at 1 Hz frequency from a strain of 0.1% to 500.0%, under log conditions. All the measurements were repeated three times.

Light microscopy

Mustard bran suspensions, processed and unprocessed, were deposited as a single drop on a standard microscope slide and covered with a coverslip. All slides were examined in the bright field using a Nikon Eclipse 55i light microscope (Nikon Corp., Tokyo, Japan) equipped with a digital camera (Nikon Digital Sight DS-Fi1-L2), through a 100× lens (Nikon).

Determination of total proteins release

The total proteins released during MCD processing were quantified using the BCA protein assay⁴⁰ for the supernatant of processed and unprocessed mustard bran suspensions (2 wt%), obtained by centrifugation (5 min at 11 400g in an Eppendorf Micro Centrifuge 5415 C).

Depending on processing conditions, two or three layers were formed upon centrifugation: at the bottom, a layer made of dark sediment was always present, while above it either only one supernatant aqueous layer was formed, or two separate aqueous layers (one clear and one turbid). The dark sediment was left in the Eppendorf tubes, while the entire aqueous fraction was withdrawn for analysis. The supernatant fluids were thoroughly mixed by vortexing for 1 min, and then were diluted 10 times with Milli-Q water and mixed with the reagent of the Pierce BCA Protein Assay Kit (0.1 mL of diluted sample in 1 mL BCA reagent) in UV-Vis semi micro-cuvettes.

The cuvettes were incubated for 30 min at 37 °C, then cooled to 20 °C in a water bath, and absorbance was read at 562 nm using a UVIKON XL UV-Vis spectrophotometer (SECOMAM, France).

Aqueous solutions of bovine serum albumin at different concentrations (20–2000 µg ml⁻¹) were used as calibration standards.

Total proteins, both in the mustard bran suspension and in the supernatant, were also determined by Kjeldahl method, through the quantitative determination of nitrogen, according to the standard procedure, and multiplying the total Kjeldahl nitrogen content with a factor 6.25, as already reported elsewhere.⁴¹

Protein release in digestive fluids

The mustard bran suspensions (2 wt%) were sequentially exposed to gastric and intestinal fluids, under conditions simulating digestion, according to a previously tested procedure.⁴² In the case of gastric fluids, a carefully weighted sample of mustard bran suspension was mixed with 10 mL pepsin–HCl solution (2.5 mg mL⁻¹ pepsin in 0.05 M HCl) in a 50 mL centrifuge tube, containing five 1 cm³-glass beads. The samples were incubated in a shaking (100 rpm) water bath at 37 °C for 120 min. Aliquots of the digestion mixture were collected for analysis and gastric digestion was stopped by snap freezing with dry ice.

After 120 min, the solution was neutralized with 1 mL 0.5 M NaOH, and 5 mL of bile solution (30 mg mL⁻¹ bile extract in 0.1 M PBS buffer at pH = 6.5) and 5 mL of pancreatin solution (2.5 mg mL⁻¹ pancreatin in 0.1 M buffer) were added to simulate the intestinal fluids. The samples were incubated in a shaking (100 rpm) water bath at 37 °C for 120 min. Aliquots of the digestion mixture were collected for analysis and intestinal digestion was stopped by snap freezing with dry ice.

Since protein release was measured using the BCA protein assay on whole mustard bran suspensions instead of isolated supernatant (as in the previous Section), an additional centrifugation step (5 min at 11 400× g) was added before absorbance reading at 562 nm, to remove suspended particles.

The modification of the experimental procedure is based on the hypothesis that the released proteins include not only the soluble proteins but also those released from the bran matrix but still bound to solid particles (*i.e.* cell wall debris), which are able to react with BCA reactant.

Data analysis

All experiments were performed in triplicate, unless differently specified, and the results are given as mean values ± standard deviation. EzANOVA software was used to perform one-way Analysis of Variance (ANOVA) tests followed by the Tukey method, with the overall significance level set at 0.05.

Results and discussion

Mechanical cell disruption treatment of yellow mustard bran suspensions

The application of the MCD concept to yellow mustard bran suspensions was demonstrated by carrying out HPH and MF treatments of different intensity, in comparison with unprocessed samples or SM-treated samples.

Preliminary tests were performed to tune the SM treatment, in terms of processing time, with the results reported in Fig. 1. The unprocessed mustard bran suspension (as received and suspended in water) exhibited a size distribution centered around 310 µm (*D*₅₀), ranging from about 690 µm (*D*₉₀) to about 90 µm (*D*₁₀), with *D*_{4,3} and *D*_{3,2} corresponding to 360 µm and 140 µm, respectively (time 0 min in Fig. 1).

The results of Fig. 1 show that a measurable effect on the particle size distribution of the mustard bran suspension was

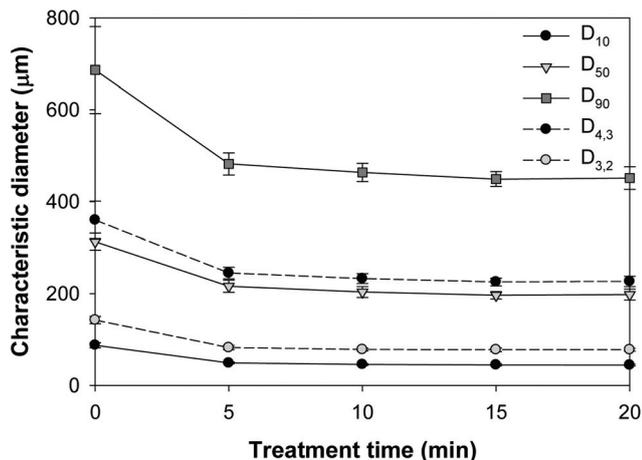


Fig. 1 Mean particle size, expressed as characteristic diameters of the size distribution, of mustard bran suspensions (2 wt%) as a function of SM processing time.

observed only for processing times ≤ 5 min, while for longer times all the characteristic diameters under examination tended towards an asymptotic value. Therefore, an SM processing of 5 min was selected for all the following experiments.

Moreover, Fig. 1 also shows that the size distribution was centered around 200 μm (D_{50}) and that both $D_{4,3}$ (230 μm) and $D_{3,2}$ (80 μm) were of the order of magnitude of individual bran cells (typically in the 10–100 μm range, as in the case of wheat bran⁴³), hence suggesting that, in the case of mustard bran, SM processing is able to disaggregate cell clusters but it is not sufficient to disrupt the individual cells.

This is confirmed in Fig. 2a and b. Fig. 2a compares the visual appearance of unprocessed mustard bran suspension with SM- and HPH-treated suspensions. The grainy texture of unprocessed mustard bran, with darker particles visible to the naked eye, corresponding to large cell aggregates with size distribution above the detectability limit by the human eye (100 μm), was only blandly modified by SM processing, which is not able to break the individual cells but can only improve the dispersion of the particles and disrupt larger cell aggregates, which are still visible in the suspension. This is confirmed by the microscopic images of Fig. 2b, showing that cell aggregates were still present in the suspension after SM processing.

The HPH treatments, instead, caused a visible improvement of the homogeneity of the bran suspensions, as shown in Fig. 2a for 3 passes.

Previous studies, carried out to characterize the particle size distribution of tomato peel suspensions treated at different HPH passes,¹⁸ showed that above 10 passes no significant changes can be observed in particle size distributions. Therefore, a maximum number of 10 passes was also set in this work, which is, anyway, largely exceeding what is usually considered suitable for industrial applications, where, ideally, a single pass should be applied.

The images of Fig. 2a show that the HPH-treated mustard bran suspension is significantly smoother than SM-treated one. HPH processing was able to efficiently disrupt the individual cells and release the intracellular content, differently from unprocessed bran and SM processing, where still several cells are intact, and often present in the agglomerated form. In particular, Fig. 2b supports that the mean size of the mustard

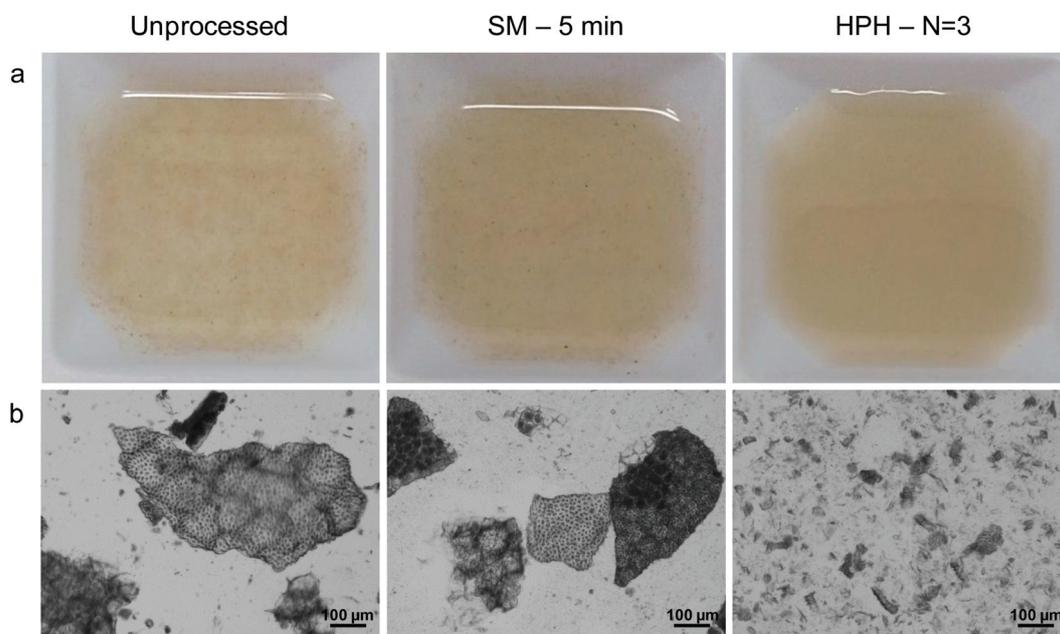


Fig. 2 Images of mustard bran aqueous suspensions immediately after preparation (reference), after SM (5 min) and HPH (S1, 150 MPa, 3 passes) treatments (a) and micrographs of the same suspensions (b), showing the cell disruption induced by the HPH treatment.

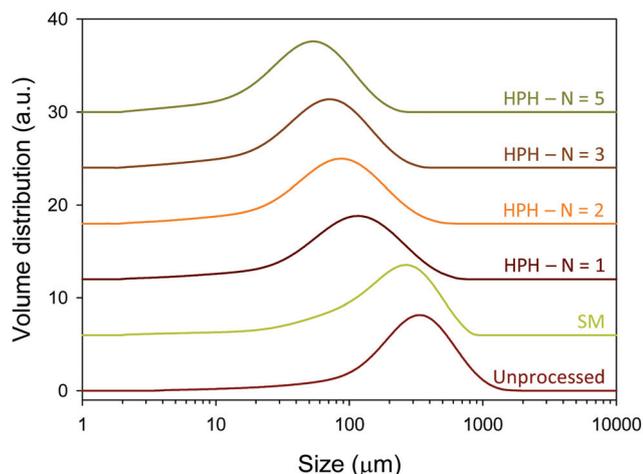


Fig. 3 Volumetric size distribution of mustard bran suspensions (2 wt%), as a function of different HPH passes (S1, 150 MPa) in comparison with unprocessed and SM-treated suspensions.

bran cells is around 100 µm, and therefore, a size distribution below this value is an indirect confirmation of the occurrence of cell disruption.

The observations of Fig. 2a and b can be correlated to the size distributions reported in Fig. 3, which compares the unprocessed sample with SM and HPH up to 5 passes, for a pressure of 150 MPa. Remarkably, a significant reduction in particle size and the distribution span was observed as the process intensity increased, with a shift from a range comprised between 100 and 1000 µm for unprocessed and SM sus-

pensions, to a range comprised between 10 and 300 µm for HPH-treated suspensions.

Further studies were also carried out to identify the most suitable processing conditions of micronization of the mustard bran suspension, varying not only the number of passes but also the homogenization pressure and valve geometry (1- or 2-stages HPH treatment in the Panda Plus or MF treatment in the Microfluidizer).

The phenomenology of the MCD treatment on bran suspension at different processing conditions can be examined through Fig. 4, which reports the micrographs of the suspensions processed by HPH from 50 to 150 MPa, for a number of passes from 1 to 10, and including the use of the 2-stage valve, in comparison with unprocessed and SM-treated sample.

As already discussed based on Fig. 1–3, SM treatment caused only the fragmentation of the initial large cell aggregates into smaller aggregates, with negligible effects on cell integrity. The application of the HPH treatment, instead, caused the fragmentation of the individual cells, broken into fine debris, which became finer as the process intensity (intended as pressure or number of passes) increased. Above a critical operating limit, corresponding to a number of passes $N = 10$ at 50 MPa, $N \geq 7$ at 100 MPa and $N \geq 5$ at 150 MPa, the formation of flocs was observed, likely as a consequence of the increasing release of fine cellulose particles or microfibrils, which tended to aggregate. Previous results have shown that the application of high shear forces, for example, based on HPH, to cellulose dispersions caused the formation of highly entangled fibrils, which spontaneously connected into aggregates to form mechanically strong networks.⁴⁴

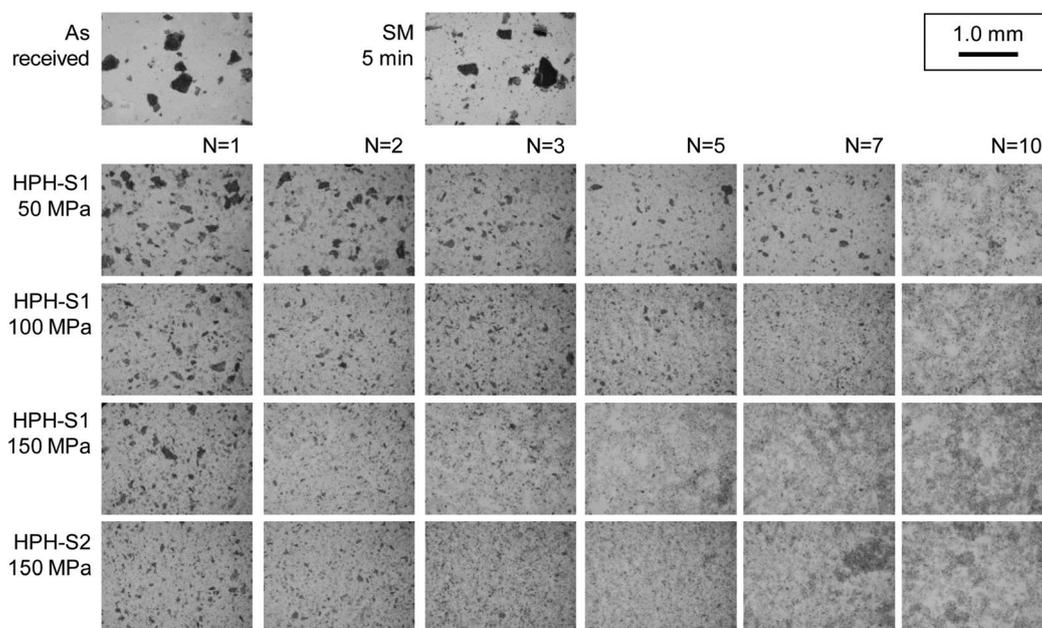


Fig. 4 Micrographs of mustard bran suspensions, as a function of different HPH treatment conditions (50–150 MPa, 1–10 passes, 1 or 2 stage valves) in comparison with unprocessed and SM-treated suspensions.

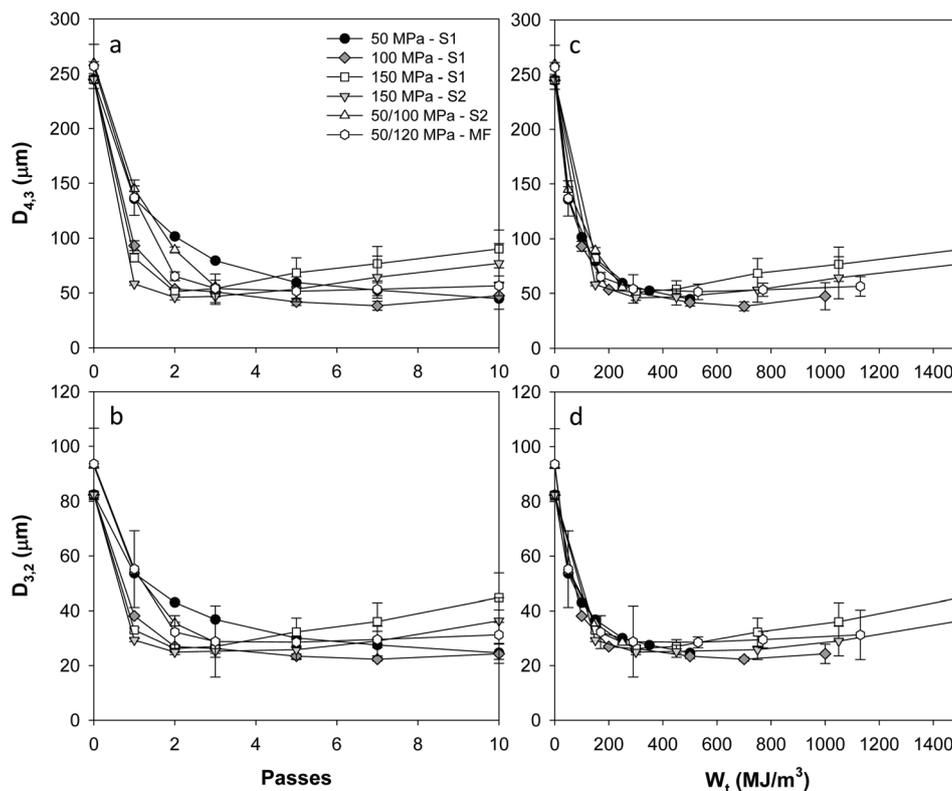


Fig. 5 Mean particle size, expressed as characteristic diameters of the size distribution $D_{4,3}$ (a, c) and $D_{3,2}$ (b, d), of mustard bran suspensions (2 wt%) as a function of MCD treatment conditions and number of passes (a, b) or of total specific energy delivered per kg of bran (W_t) for different treatment conditions (c and d). Legend: 50/100 or 50/120 indicates that the first pass was carried out at 50 MPa with 1-stage valve, followed by subsequent passes at the indicated pressure (100 or 120 MPa). Reference (0 passes, or 0 MJ m^{-3}) corresponds to SM.

The extent of size reduction as a function of MCD processing is quantified in Fig. 5, which reports the mean particle size in terms of $D_{4,3}$ and $D_{3,2}$ of mustard bran suspensions as a function of operating pressure, number of passes and type of homogenization valve. The main size reduction is observed to take place within the first 3 passes and is more significant at higher operating pressure. Moreover, especially when comparing the 1-stage and 2-stage valve configuration at 150 MPa, it is also evident that the use of a 2-stage valve (a common feature in Panda Plus Niro Soavi homogenizers³⁶) improved the mechanical disruption process, reducing the final size, in comparison with the 1-stage valve, already after the first pass.

Less severe MCD conditions were also tested, by processing the suspension initially at 50 MPa for a single pass with the 1-stage valve, and then by 2 additional passes at 150 MPa with the 2-stage valve (pressure drop split with 120 MPa in the first valve and 30 MPa in the second valve). Interestingly, after 3 passes the minimum values of both $D_{4,3}$ and $D_{3,2}$ were reached, but most of the size reduction occurred after 2 passes (the first at lower and the second at higher pressure). These process conditions are less efficient than the process carried out entirely at 150 MPa (where only 2 passes were needed to

reach the minimum size values) but the initial passage at 50 MPa helps in preventing the risk of clogging of the valve, hence ensuring smoother processing. In contrast, the treatment of the SM suspension directly at 150 MPa caused the need for frequent interruptions of the operations and release of the pressure (valve opening), because larger particles clogged the piston valve, which is set at a significantly smaller gap than at 50 MPa.²⁸ Less intense conditions are also preferable in terms of industrial scalability of the MCD process.

Similarly, MF processing also required a preliminary passage in the Panda Plus system at 50 MPa. Without this preliminary step, it was not possible to carry out the treatment, because the proprietary, fixed-geometry Z-disruption chamber (with a diameter of the microchannel of 87 μm) quickly clogged and required to be dismantled and unclogged to continue the operations. With this preliminary step, MF processing was as efficient as the HPH treatment, achieving, after 3 overall passes, similar values of $D_{4,3}$ and $D_{3,2}$.

Therefore, the results of Fig. 5 clearly show that the differences between MCD processing in the Microfluidizer and Panda Plus are small and both machines can be used with comparable efficiency.

In Fig. 5c and d, the comparison of the characteristic diameters obtained in the suspension for different operating conditions is also reported as a function of the specific pressure energy delivered. Such specific energy can be expressed as the sum of the pressures applied in each pass (eqn (1)):

$$W_t = \sum_1^N \Delta P_j \quad (1)$$

where W_t is the total energy delivered per m^3 of suspension, ΔP_j is the applied pressure in each pass j , and N is the total number of passes.^{10,45,46}

Remarkably, it can be observed that, when $D_{4,3}$ and $D_{3,2}$ are plotted as a function of W_t , the curves obtained for different operating pressure and type of valve collapse in a single curve. The maximum size reduction, both in terms of $D_{4,3}$ and $D_{3,2}$, is reached for a specific energy value of 200 MJ m^{-3} . At specific energies $W_t > 400 \text{ MJ m}^{-3}$, instead, not only did no significant contribution to size reduction is observed, but the occurrence of aggregation phenomena is triggered.

In the perspective of industrial application, it must be noted that being the specific energy primarily dependent on the volume of processed fluid, when increasing concentration, the required energy will increase only moderately, as only a very small fraction of the pressure energy is used to disrupt the solid particles, and the rest is dissipated in the fluid as frictional heating. Considering a temperature increase between 0.15 and 0.20 °C per MPa,^{10,28,47} in the case of water as a continuous phase ($c_p = 4.186 \text{ kJ kg}^{-1} \text{ K}^{-1}$), the frictional heating corresponds to 63–84% of the specific pressure energy. The effect of bran concentration on the efficiency of the MCD process is currently part of ongoing work and will be discussed in a subsequent paper.

Rheological properties

The MCD treatment of yellow mustard bran suspensions is able to significantly affect its rheological behavior, as a consequence of the disruption of individual plant cells, with consequent size reduction of the suspended particles and release of macromolecules (mucilage and proteins), as well as of the effect of high shearing on plant materials (especially fibers).

As shown in Fig. 6, the effect of MCD processing on the mustard bran suspension can be observed in terms of an increase in viscosity η (evaluated at a shear rate of 1.0 s^{-1} , but reported in the entire range investigated in the ESI, Fig. S1†) as well as in the values of storage and loss moduli G' and G'' for a strain of 10% (but reported in the entire range investigated in the ESI, Fig. S1†) in comparison to unprocessed and SM-treated samples.

The results of Fig. 6 clearly show that MCD-treated samples exhibited a significantly higher viscosity than unprocessed or SM-treated samples, of the order of 10 and 100 times higher, respectively. However, no significant difference in viscosity was observed for the different MCD conditions tested, including (a) different pressure levels and (b) number of passes, (c) use of 1 or 2-stage HPH valve, or (d) MF processing.

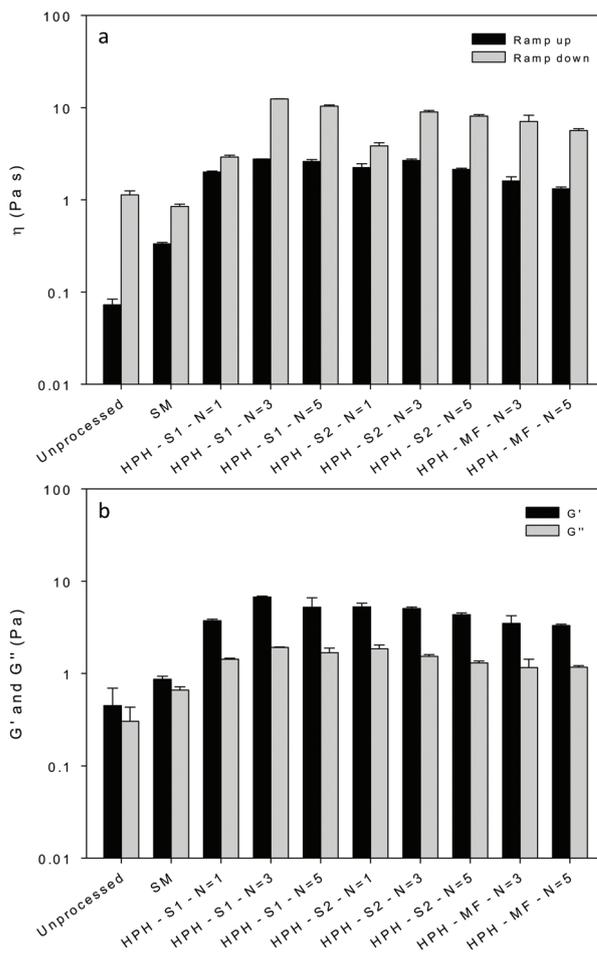


Fig. 6 Rheological behavior of the mustard bran suspensions (2 wt%) for different treatment conditions in terms of viscosity at 1.0 s^{-1} shear rate (a), obtained from experiments of shear ramping up, and down (Fig. S1a and b of ESI†), of G' and G'' (b) at a strain of 10%, extracted from experiments in strain sweep (Fig. S1c and d of ESI†).

Similar behavior was observed for G' and G'' : both the storage and loss moduli were significantly increased already after the milder HPH treatments, with only minor variations when increasing HPH process intensity or changing type of valve.

These data show that the mustard bran suspension processed by MCD exhibited an improved structuring ability, which makes it a more natural and sustainable alternative to industrial thickening agents. Previous studies have shown that, when processing soybean okara by HPH, a 10-fold increase in viscosity (measured at 100 s^{-1}) was observed when operating pressure was increased from 50 to 100 MPa.³⁷ It must be highlighted that mustard bran suspensions, despite the lower suspension concentration (2 vs. 10 wt%) exhibits a significantly higher viscosity, related to its composition rich in fibers and mucilage. When HPH is applied to systems without any fiber content, such as in the case of microalgal biomass, a decrease in viscosity is observed, as a consequence of the HPH effect on disrupting intercellular polymer interactions.⁴⁸ In the

case of mustard bran, instead, HPH treatment likely caused the progressive disruption and defibrillation of fibers, reducing the particle size and increasing their interfacial area, and hence promoting their interconnection in a structured network, as previously observed for HPH processing of soybean hull insoluble polysaccharides.¹² Previous studies have also shown that cellulose treatment by HPH already at 60 MPa caused the decrease in size of micro-ribbon of cellulose fibrils, with significant effects on rheological and textural properties of cellulose aqueous suspensions, because of the stronger interactions among small cellulose-based particles, which naturally tend to aggregate.⁴⁹

Protein release

The centrifugation of the MCD-processed mustard bran suspensions enabled to recover a supernatant fraction, which was rich in soluble proteins, as well as in mucilage, which is shown in Fig. 7 as the whitish, cloudy intermediate layer, appearing after HPH treatments.

As the MCD process intensity increased, the volume of the pellet fraction decreased, as the cells were increasingly disrupted and the intracellular material was released and recovered in the supernatant. Despite the proteins being more concentrated in the top clearer layer, preliminary experiments showed that some were also contained in the intermediate layer, and therefore, to determine the total proteins released due to MCD processing, the pellet was separated from the clear and whitish layers, and these two layers were thoroughly mixed and used for further analysis.

Preliminarily, the size distribution of the supernatant was determined by Dynamic Light Scattering, with the results being reported in Fig. 8 in terms of mean diameter and polydispersity index (PDI). Remarkably, both the mean diameter of the particles suspended in the supernatant and their polydispersity significantly decreased as the number of HPH passes increased. As expected, HPH treatment affected also the colloidal aggregates released from the disrupted cells into the aqueous phase, whose mean size decreased to below 1 μm .



Fig. 7 Images of the centrifuged mustard bran suspension (2 wt%): comparison of the unprocessed sample, SM-treated, and HPH-treated (1–5 passes at 150 MPa).

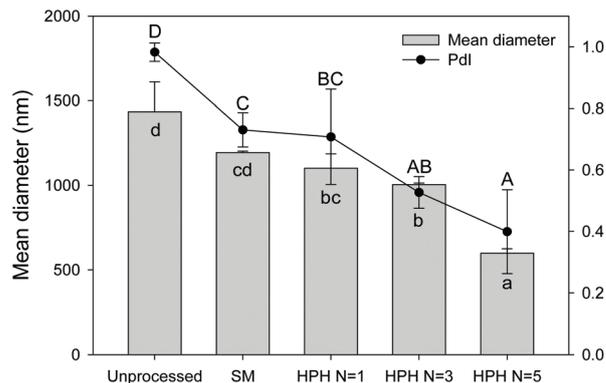


Fig. 8 Mean diameter (as measured by DLS) and polydispersity index (PDI) of the supernatant recovered from mustard bran suspensions (2 wt%) unprocessed, treated by SM and after processing by HPH for 1–5 passes at 150 MPa (S1).

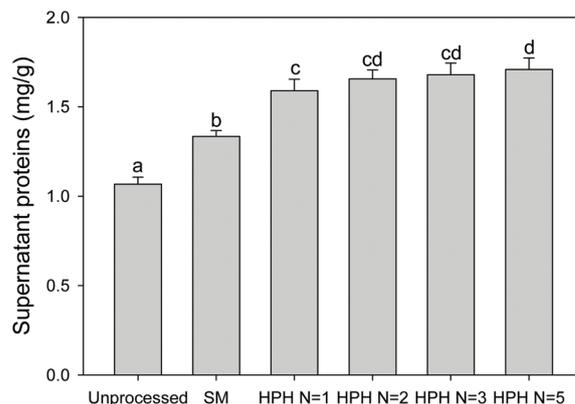


Fig. 9 Total proteins released in the supernatant recovered from mustard bran suspensions (2 wt%) unprocessed, treated by SM and after processing by HPH for 1–5 passes at 150 MPa (S1).

At the same time, the concentration of total proteins released in the supernatant significantly increased in comparison with the unprocessed and the SM-treated suspensions (Fig. 9).

The results of Fig. 9 show that a significant increase in the protein release was determined by SM treatment in comparison with unprocessed suspension, and by HPH treatment in comparison with SM-treated suspension. Moreover, the HPH treatment caused a +60% increase in the release of proteins in comparison to the unprocessed suspension and a +20–30% increase (depending on HPH conditions) in comparison to SM. The results of Fig. 9 were also confirmed through the Kjeldahl method for total N determination. The Kjeldahl analysis classified the samples in two significantly different clusters, corresponding to 1 mg g^{-1} for unprocessed and SM-treated suspension, and to 2 mg g^{-1} for the HPH-treated samples.

Notably, when considering that the total protein content of the suspension was 3.2 mg g^{-1} , as the yellow mustard bran

contained 16 wt% of proteins, as determined through the Kjeldahl method, a maximum of 53% of the total proteins was released by HPH processing. These proteins were released from the mustard bran cells in the supernatant, either as soluble proteins or as colloidal complexes with cellulose particles or fibrils, as suggested by the size distribution data of Fig. 8. In comparison to protein extraction from soybean okara, for which recovery of proteins between 82%³⁶ and 90%³⁷ was reported, the lower protein recovery in mustard bran could likely be explained in terms of the lower content of soluble proteins than in okara.

The increased protein release caused by HPH treatment on mustard bran suspensions might have important repercussions on the technological properties of the mustard bran suspension, such as structuring and emulsification ability, because of the increased surface activity associated with higher protein content. Besides, a higher amount of released proteins might also imply potential health benefits, because of the consequently improved protein digestibility.

Finally, it must be remarked that similarly to what observed for size distribution, also in the case of protein release, no significant difference was observed between HPH and MF processing (Fig. S2 of the ESI†), hence further supporting the concept that, for MCD of mustard bran, there is no need for specific equipment, as far as the required minimum amount of specific pressure energy is delivered.

Protein release in digestive fluids

The mustard bran suspensions, unprocessed and treated by SM and HPH (1 and 3 passes, conditions of 50/100 MPa – S2 of Fig. 5), were sequentially exposed to gastric and intestinal digestive fluids, to investigate the potential advantages of the MCD technology upon consumption and digestion. Fig. 10 reports the characteristic diameters ($D_{4,3}$ and $D_{3,2}$) of the control suspensions, and after exposition for 1 h to gastric fluids and for 1 h and 2 h to intestinal fluids.

As expected, on the basis of the well-known limited digestibility of bran, the digestive fluids did not significantly affect the size distribution of the mustard bran (see also D_{10} , D_{50} , and D_{90} in Fig. S3 of ESI†), as shown by the relatively small changes in the characteristic diameters. However, some aggregation phenomena were observed, due to the action of enzymes and bile salts, which were reflected in some small changes, especially in terms of $D_{3,2}$, which is more sensitive to the presence of small particles than $D_{4,3}$.

The protein release during digestion is reported in Fig. 11, in comparison with protein release from the control samples. The total protein release in Fig. 11 is higher than the total proteins released in the supernatant (Fig. 9) because the BCA assay was conducted on the whole, un-centrifuged suspension rather than on supernatant. This means that, in this analysis, the BCA assay was sensitive not only to the proteins released in the aqueous phase but also to those, which are bound to the external surface of the suspended particles (cells and cell debris) that are removed together with the pellet during centrifugation.

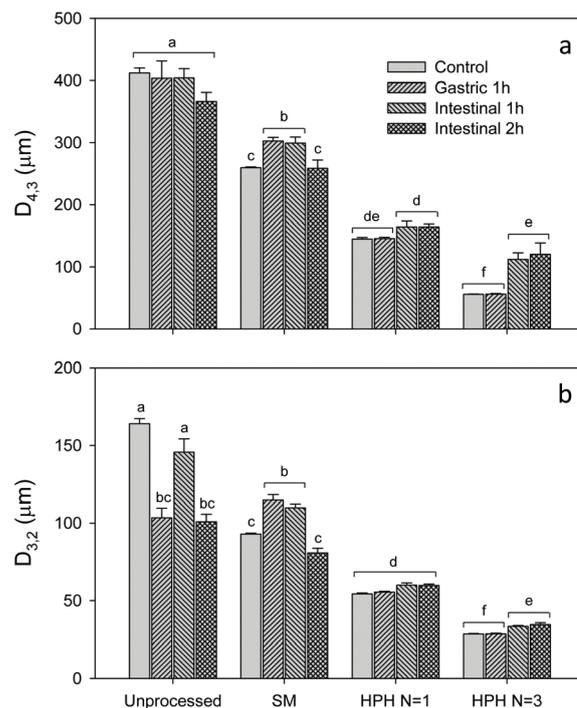


Fig. 10 Characteristic diameters $D_{4,3}$ (a) and $D_{3,2}$ (b) of the mustard bran suspensions (2 wt%) unprocessed, treated by SM and after processing by HPH for 1 or 3 passes (conditions of 50/100 MPa – S2 in Fig. 6), before and after exposition to gastric and intestinal fluids.

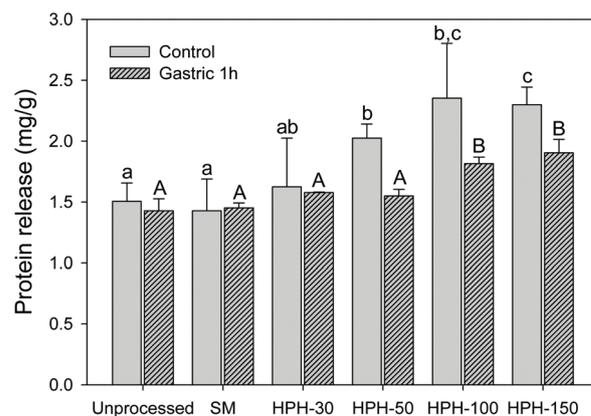


Fig. 11 Protein release from the mustard bran suspensions (2 wt%) unprocessed, treated by SM and after processing by 1 HPH pass at different pressures (S1, 30–150 MPa) before and after simulated gastric digestion for 1 h.

Remarkably, Fig. 11 shows that up to 72% of total proteins was released, as calculated for total protein content of the mustard bran suspension of 3.2 mg g^{-1} , which is closer to the data reported for soybean okara.^{36,37} Proteins are located inside the plant cells, in regions, which might be not easily accessible. Some proteins are attached to the cell wall or membrane, which are more readily accessible, while other proteins are stored in bodies inside the vacuoles of the cells, and their

release is more difficult because it requires the disruption of the cell wall and vacuole membrane.

In the human digestive system, the nutrients are degraded by specific digestive enzymes,⁵⁰ which are able to come in contact with their substrates only if they are bioaccessible, and therefore if the enzymes can penetrate the cell wall or the nutrients are released from the food matrix.¹⁴ The degree of intactness with which the plant-based foods reach the gastric environment, and therefore the extent to which these are accessible to digestion, greatly depends on processing conditions (*e.g.* cooking and milling) and mastication. Cooking is considered to increase cell wall porosity and activate endogenous enzymes,^{51,52} whereas high-intensity MCD processes, such as those based on HPH or US, might be required for the physical rupture of the cell wall, depending on the fracture properties of the plant tissue.⁵³ Therefore, food processing can be exploited to manipulate the digestive barriers and modulate the release of various nutrients and metabolites.⁵⁴

Based on the results of Fig. 9 and 11, it can be concluded that MCD processing is able to significantly enhance protein solubilization and release from the bran matrix, by mechanical disruption of the mustard bran cells, making them more accessible to digestive enzymes.¹⁴

In unprocessed and in SM-processed samples, the released proteins were about 1.5 mg g⁻¹ of suspension, which is less than 50% than total protein content. MCD processing by single-pass homogenization at 30 MPa was not able to significantly increase the protein release. However, more intense MCD conditions caused an evident increase in their release. For example, a single HPH pass at 50 MPa caused an increase in released proteins to about 2.0 mg g⁻¹ of suspension, whereas a single HPH pass at 100 and 150 MPa caused a further increase to about 2.3 mg g⁻¹ of suspension (more than 70% of total proteins of the suspension).

The gastric fluids were responsible for a slight reduction in the measured protein release, in comparison to control (undigested sample), which is especially evident in the case of HPH-treated suspensions in the 50–150 MPa range. This reduction can be explained in terms of the action of the proteolytic enzymes, accessing the proteins and hydrolyzing them in peptides and aminoacids.¹⁴ However, HPH treatment at 100 and 150 MPa still ensured a higher protein release after gastric digestion than control and mildly processed suspensions. Analysis of the released proteins of bran exposed to intestinal fluids was not carried out because of the presence of pancreatin, which hindered the BCA protein analysis.

These results suggest that MCD processing might be a technology that enables also the improvement of protein digestibility, because the proteins in MCD-treated suspensions, when reaching the stomach, have been released from the bran matrix and are accessible to the proteolytic enzymes to be digested, and do not require an additional stage of release from the often indigestible fibrous cell structures, with potential consequences also on the nutritive value of the mustard bran.

Conclusions

The mechanical cell disruption (MCD) technology is an intrinsically safe, clean, and environmentally friendly method, as it avoids using toxic reagents, strong acids or bases, making it safe to be used as a dietary product. In this work, the MCD concept was applied through high-pressure homogenization (HPH) processing to yellow mustard bran suspensions to unlock their functionality by releasing value-added intracellular compounds.

The MCD technology was based on the preliminary milling to the coarse size of the bran, as often resulting from mustard debranning stages, its suspension in water and the subsequent disruption of the individual cells by applying mechanical energy sufficient to cause the rupture of cell walls and cell and vacuole membranes, to enhance the release of valuable intracellular compounds, as well as to promote novel functionalities.

In the case of mustard bran suspensions, significant changes were observed in suspension appearance, protein release, and structuring ability, with the additional advantage that MCD did not require any additional chemical or heat treatment. A relatively simple MCD processing (*i.e.* 3 HPH passes at 50/100 MPa) resulted in a smooth and homogeneous bran suspension, with rheological properties significantly different from unprocessed or SM-treated suspensions, and that, therefore, can be exploited as a natural thickening or structuring agents. Moreover, MCD processing might also significantly contribute to improving protein digestibility, by enhancing protein release from the indigestible cell structures.

Therefore, this work contributes to advancing the knowledge about more natural and sustainable methods to exploit agri-food by-products and residues for the production of fully natural food ingredients, whose functionality and added value is obtained only by physical methods and using only water as a solvent.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

This work was financially funded by the Marie Curie Intra-European Fellowship (PIEFGA-2013-626421) within the 7th European Community Framework Programme.

Henk Husken and Liesbeth Bouwens are gratefully acknowledged for the support with the high-pressure homogenization processing, and the protein bioaccessibility experiments, respectively.

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