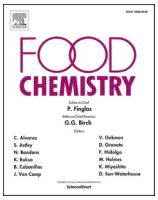
Food Chemistry 387 (2022) 132911

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem



Impact of enzymatic pre-treatment on composition of nutrients and phytochemicals of canola (*Brassica napus*) oil press residues

Ye Tian [a], Marie Kriisa [b], Maike Föste [c], Mary-Liis Kütt [b], Ying Zhou [a], Oskar Laaksonen [a], Baoru Yang [a,*]

[a] Food Chemistry and Food Development Unit, Department of Life Technologies, Faculty of Technology, University of Turku, 20014 Turku, Finland
[b] Center of Food and Fermentation Technologies (TFTAK), Akadeemia tee 15a, 12618 Tallinn, Estonia
[c] Fraunhofer Institute for Process Engineering and Packaging IVV, Giggenhauser Strasse 35, 85354 Freising, Germany

ARTICLE INFO

Keywords:
Side-stream
Canola oil press cake
Enzymatic treatment
Nutrient
Anti-nutritional agent
Valorization

ABSTRACT

The study aimed to develop a biorefining process to recover proteins and dietary fibres from a food industry side-stream, canola (*Brassica napus*) oil pressing residues. The materials were treated with commercial protease, carbohydrase, and phytase to obtain protein-rich supernatants and fibre-rich precipitates. The compositions of these fractions were analyzed using LC-MS (glucosinolates and phenolics) and GC–MS (sugars, acids, and amino acids). Compared to raw material, the supernatants were richer in proteins, sugars, acids, amino acids, phenolic acids, and flavonols; the precipitates had higher levels of minerals and dietary fibres. The enzymatic treatment decreased the contents of phytic acid, glucosinolates, and phenolic alkaloids in all fractions. The applied enzymes effectively enhanced solubility of proteins, despite the lower yield of crude proteins compared to the alkaline extraction (40–82 vs 91 g/100 g dry matters). The impact of enzymes on other chemical components was also revealed by using principal component analysis.

1. Introduction

Canola (*Brassica napus*, also called as 'double-zero rapeseed' in Europe), as main cultivated rapeseed cultivar containing considerably low contents of glucosinolates and erucic acid, has become one of the largest oilseed crops nowadays (Chmielewska et al., 2020). In year 2019–2020, approximately 69 million tons of rapeseeds were harvested worldwide, and over 39 million tons of residues were produced after mechanical oil pressing (Foreign Agricultural Service United States Department of Agriculture, 2021). Previous studies have suggested the necessity of utilizing the solid wastes of oil production due to abundance of nutrients and bioactive compounds in these materials. The residues/oil press cakes are a rich source of proteins (35–40% of dry matters), carbohydrates (30–35%), crude fibres (10–15%), and minerals (8–14%) (Lomascolo et al., 2012; Rommi, 2016). A major concern for the utilization is presence of undesirable components in these materials. Phytic acid forms complex with proteins and minerals reducing the digestion and absorption of these nutrients, whereas a high content of glucosinolates (mainly progoitrin, 4-hydroxyglucobrassicin, gluconapin) in the diet interferes with the function of the thyroid. High levels of phenolic compounds reduce bioavailability of proteins. Some of these compounds are also associated with unpleasant taste (glucosinolates, sinapine, and kaempferols) (Chmielewska et al., 2020). Although some of these compounds having potential benefits to human health, for example reducing risks of cancers and cardiovascular diseases (glucosinolates) as well as exhibiting anti-oxidative, anti-microbial, anti-mutagenic, and anti-inflammatory activities (phenolics), their contents should be reduced to appropriate levels for application in human diet (Traka, 2016; Vuorela, 2005).

At present, canola oil press cake (CPC) is used mostly as livestock feeds (Baker & Charlton, 2020), CPC has potential as a raw material for human nutrition due to the remarkably high content of essential nutrients. The European Commission recently authorizes powders of de-oiled CPC as novel food, in which the contents of glucosinolates and phytates are reduced to safe levels for human consumption (Turck et al., 2020). In food industry, valorization of CPC focuses mainly on producing protein-rich extracts (Aider & Barbana, 2011). A protein isolate (Isolexx^{TM}, BioExx Specialty Proteins Ltd., Toronto, Canada) produced from CPC has been approved by the European Commission as a novel food ingredient with an estimated intake of 2.2 g/kg body weight per day for

* Corresponding author at: Food Chemistry and Food Development Unit, Department of Life Technologies, University of Turku, FI-20014 Turku, Finland.
E-mail address: baoru.yang@utu.fi (B. Yang).

https://doi.org/10.1016/j.foodchem.2022.132911
Received 22 November 2021; Received in revised form 7 March 2022; Accepted 4 April 2022
Available online 6 April 2022

adults (Agostoni et al., 2013). Although producing protein-rich products is a fine start for CPC valorization, it may not be the best way to take full advantage of this food industry side-stream. First, refining a single nutrient from the side-stream is often not efficient, resulting in high costs and new residues. Secondly, during purification of proteins, some health-promoting ingredients in CPC (for example, dietary fibres and phenolics) are commonly removed in the process (Wanasundara et al., 2017). Yet, from the perspective of human health, these health-beneficial components are highly recommended to be retained in new products, whether used as new foods or potential food additives. Certainly, the contents of these compounds should be remained at appropriate levels, which cause no significant reduction in either nutrition values or sensorial properties of the products.

In order to exploit a low-cost application of canola oil press cake in food industry, this study was designed to pre-treat this material with bioprocessing using several commercial enzymes (a carbohydrase, two proteases, and a phytase). This was due to the fact that conventional alkaline extraction causes protein denaturation and decreases digestibility of proteins and levels of essential amino acids (Gerzhova et al., 2015). The aim of the enzymatic treatment was to enhance the extractability of functional ingredients (proteins, dietary fibres, sugars, and bioactive compounds) and to maximize the reduction of glucosinolates and phytates. In previous studies on canola press cakes/meals, spectrophotometric methods were mostly used for characterizing the composition of glucosinolates and phenolic compounds. In the present research, detailed changes in composition and contents of free sugars, acids, amino acids, phenolics, and glucosinolates were monitored using gas chromatographic (GC), liquid chromatographic (LC), and mass spectrometric (MS) approaches. The obtained products are expected to be rich in multiple nutrients and health-promoting compounds besides protein only. Different from previous research focusing only on liquid fractions after varying treatments, our study also investigated the solid residues of the treatments (rich in dietary fibres) and provided compositional information for their future utilization in food production. Our results provide new concepts for valorization of the side-streams from oil manufacturing, supporting circular economy models in food industries.

2. Materials and methods

2.1. Side-stream materials and chemicals

Canola oil press cake (CPC) was supplied by Myssyfarmi Ltd. (Haveri, Finland). Commercial enzymes, Protamex® (serine metalloendoprotease), Alcalase® (alkaline serine endoprotease), and Viscozyme® (mixture of pectinase, arabanase, cellulase, β-glucanase, hemicellulose, and xylanase) were provided by Novozymes (Bagsvaerd, Denmark). Phyzyme® XP 5000L (phytase) was provided by Danisco Animal Nutrition (Dupont, Wiltshire, United Kingdom).

All reference standards of sugars, acids, amino acids, phenolic compounds, and glucosinolates were purchased from Sigma-Aldrich (St. Louis, MO, United States). Other chemicals of LC and MS grade were purchased from VWR International Oy (Espoo, Finland).

2.2. Pre-treatments of canola oil press cake

Dried CPC was grinded into fine powders using an ultra-centrifugal mill ZM200 (Retsch GmbH, Haan, Germany) with a 0.5 mm mesh. The powder sample (75 g) was mixed with deionized water at a ratio of 1:10 (*w/v*) in 1.25 L of Biobundle bioreactor (Applikon Biotechnology B.V., Delft, Netherlands). Pre-treatments consisted of five enzymatic treatments (using Protamex®, enzymatic activity of 1.5 AU/g declared by the manufacturer; Alcalase®, 2.4 AU/g; Viscozyme®, 100 FBG/g; and Phyzyme®, 5000 FTU/g) and a conventional alkaline protein extraction. The optimal condition of applied enzymes was set based on the manufacturer's suggestions. The initial pH of incubation was adjusted by either 2 M of NaOH or 1 M of H_2SO_4, and changes of pH value were monitored during whole incubation period. The condition of each treatment is given in Supplemental Table 1. After pre-treatments, the samples were centrifuged (at 4000×*g*, for 20 min, at 4 °C), and both the supernatants and the precipitates were collected. The precipitates were lyophilized. All precipitates and supernatants were stored at –20 °C for further analyses.

2.3. Analyses of proximate composition and phytic acid

Dry matter and ash contents were determined at 105 °C and 550 °C, respectively, (Naumann et al., 2019) using a TGA 601 thermogravimetry analyzer (Leco Corp., St. Joseph, MI, United States). The protein content in the treated samples was determined with a Kjeldahl method applying a conversion factor of N × 6.25 (International Organisation of Standardization, 2009). The content of solubilized proteins (% of crude protein) was calculated using the following equation. The fat content was determined according to the Soxhlet standard procedure using *n*-hexane as solvent (Nielsen, 2017). The measurements of dietary fibers and phytic acid were carried out according to the manufacturer's instructions using enzymatic test kits (Megazyme Ltd., Bray, Ireland).

$$\text{Solubilized protein}(\%) = \frac{\text{Initial volume}\,[\text{mL}] \times \text{protein content in supernatant}\left[\frac{\text{mg}}{\text{mL}}\right]}{\text{sample mass}\,[\text{mg}] \times \text{protein content}[\%\text{db}] \times \text{dry matter}[\%]} \times 100$$

2.4. Analysis of free sugars, acids, and amino acids

Raw CPC (3.0 g) was extracted with 4 × 10 mL of aqueous methanol (methanol:water, 8:2, *v/v*), followed by 20 min of ultra-sonication and 15 min of centrifugation (at 1500×*g*). The supernatants from four times of extraction were collected and combined; the final volume was set to 50 mL using the extraction solvent. For each pre-treated CPC sample, 1 mL of supernatant (approximately 1.03 g) was taken for the analysis. Both raw CPC extract and pre-treated supernatants were filtered through 0.45 μm PTFE syringe filters for further analyses.

Sugars, acids, and amino acids were analyzed as trimethylsilyl (TMS) ethers. Identification of the studied compounds was performed by a Thermo Scientific TRACE 1310 gas chromatograph (GC) equipped with TSQ 8000 Evo mass spectrometry (MS, Thermo Fisher Scientific, Waltham, MA, United States). A SPB-1 column (30 m × 0.25 mm i.d., 0.25 μm, Supelco, Bellefonte, PA, United States) was used for separation. An aliquot of 0.4 mL of sample was diluted to 0.8 mL with Milli Q water, dried and later reacted with 600 μL of Tri-Sil reagent (Pierce, Rockford, IL, United States). A 1 μL of TMS derivatized sample was injected to GC–MS. The chromatographic condition was described in our previous study (Liu et al., 2018). The energy of ion source was set to 70 eV, and the mass range scanned was 40–400 *m/z*. The compounds were characterized by matching the obtained mass spectra with the standard NIST 08 library and by comparing GC retention times with those of the external reference standards.

A Shimadzu GC-2010 coupled with flame ionization detector (Shimadzu corp., Kyoto, Japan) was applied in the quantification of the studied compounds. Sorbitol (for sugars) and tartaric acid (for acids and amino acids) were used as internal standards. Approximately 0.4 mL of sample together with 0.2 mL of each standard were derivatized before GC analysis. The injection volume and analytical parameters were the same as applied in the GC–MS identification.

2.5. Analysis of glucosinolates and phenolic compounds

In order to investigate the effect of solvents on extraction yield, glucosinolates and phenolics were extracted from CPC with six different organic solvents, including absolute methanol, ethanol (99.5 %, *v/v*), absolute acetone, methanol:water (8:2, *v/v*), acetone:water (8:2, *v/v*), and methanol:acetone:water (7:7:6, *v/v/v*). For each solvent, CPC (2.0 g)

was extracted with 4 × 10 mL of solvent at room temperature. The extraction was assisted with 20 min of ultra-sonication and followed by 15 min of centrifugation (at 1500×*g*). The supernatant from the extraction was collected and evaporated to dryness with a vacuum rotary evaporator at 50 mbar, 30 °C. The residue was dissolved in 1.5 mL of Milli Q water. For the pre-treated samples, 1 mL of supernatants (approximately 1.03 g) were used for the analysis. The precipitates were re-extracted, following the same method as for raw CPC. All samples were filtered with 0.2 μm of RC filters for LC analysis.

Identification of the studied compounds was performed on a Bruker ultra-high performance liquid chromatography (UPLC) system, equipped with a diode-array detector (DAD), an Apollo II electrospray ion source (ESI), and a quadrupole/time-of-flight tandem mass spectrometer (Q-TOF) (Bruker Corp., Billerica, MA, United States). A Phenomenex Aeris peptide XB-C18 column (150 × 4.60 mm, 3.6 μm, Torrance, CA, United States) was used for the analysis. The mobile phase was a combination of 30 mM ammonium formate in water (pH 5.0, A) and acetonitrile (B). The injection volume was 5 (for raw CPC) or 35 μL (enzyme-treated samples). The chromatographic separation was conducted at room temperature with a total flow rate of 1 mL/min. LC gradient program was set as: 0–3 min with 0% solvent B, 3–15 min with 0–4% B, 15–20 min with 4–5% B, 20–25 min with 5% B, 25–27 min with 5–8% B, 27–32 min with 8–9% B, 32–37 min with 9% B, 37–42 min with 9–14% B, 42–47 min with 14% B, 47–52 min with 14–20% B, 52–57 min with 20–22% B, 57–62 min with 22–23% B, 62–67 min with 23–40% B, 67–68 min with 40–50% B, and 68–70 min with 50–0% B. The chromatogram was monitored at wavelength of 227 nm (for glucosinolates) and 320 nm (phenolics). The eluents of 0.3–0.4 mL/min were flown into MS system. Mass full-scan was operated under both positive and negative ionization modes, and MS^2 scan was performed using an auto MS/MS program in Q-TOF system (Supplemental Table 2).

A Shimadzu LC-30AD liquid chromatograph system was used in quantitative analysis of glucosinolates and phenolics, equipped with a SIL-30AC auto-sampler, a CTO-20AC column oven, and an SPD-M20A photodiode array detector (Shimadzu Corp., Kyoto, Japan). The chromatographic condition was same as described in LC-MS method. The identified compounds were quantified by the calibration curves of compounds as shown in Supplemental Table 3.

2.6. Statistical analyses

All results were calculated on the basis of dry matter content (DM of raw material, supernatants, or precipitates, accordingly) and expressed as mean ± standard deviation. Statistical differences among data were calculated based on one way-ANOVA and Tukey's post hoc test ($p < 0.05$) by IBM SPSS Statistics 26 for Windows (SPSS Inc., NY, United states). Principal component analysis (PCA) with full cross validation was applied by using Unscrambler 10.4 (Camo Process AS, Oslo, Norway) to investigate the correlation between chemical composition and pre-treatment.

3. Results

3.1. Chemical profiles in canola oil press cakes

As shown in Table 1, the main nutrients in CPC raw material were dietary fibres (35 g/100 g DM), mostly as insoluble fibres (83% of total fibres). Proteins and fats were abundant in CPC at high amounts of 27 and 20 g/100 g of DM, respectively. CPC contained 5510 mg/100 g DM of free sugars, mostly presenting as sucrose (Suc). The total content of the detected free acids up to 175 mg/100 g DM, consisting of both organic acid and inorganic acid. Malic acid (MaA), citric acid (CiA), and phosphoric acid (PhA) were the major acids, accounting for 67, 17, and 12% of total acids, respectively. Free amino acids in CPC were generally present at low contents (81 mg/100 g DM), mainly as glutamic acid (GlA, 80% of total content of the identified free amino acids) (Table 1, Supplemental Fig. 1).

Table 1
Concentration of nutrients and phytochemicals in raw material of CPC*.

Composition	Content
Dry matter, %	**91.1**
Proteins, g/100 g DM	**26.8 ± 0.1**
Fats, g/100 g DM	**20.1 ± 0.0**
Ashes, g/100 g DM	**6.2 ± 0.0**
Dietary fibres, g/100 g DM	**35.0 ± 0.9**
soluble	5.8 ± 0.3
insoluble	29.2 ± 0.7
Free sugars, mg/100 g DM	**5510.3 ± 72.8**
Fru	3.7 ± 0.7
Glu	54.0 ± 2.7
Suc	5432.3 ± 74.1
Ino	20.3 ± 0.7
Free acids, mg/100 g DM	**174.9 ± 5.8**
PhA	20.9 ± 0.8
free organic acids	**154.0 ± 5.4**
SuA	5.9 ± 0.2
MaA	118.0 ± 4.6
CiA	30.1 ± 0.9
Free amino acids, mg/100 g DM	**80.7 ± 9.8**
Val	3.7 ± 0.4
Pro	6.6 ± 0.8
Thr	5.9 ± 0.5
GlA	64.5 ± 8.6
Glucosinolates, mg/100 g DM	**771.3 ± 27.8**
PrN	36.4 ± 2.6
GlNFN	22.4 ± 0.3
GlNN	114.7 ± 6.0
GlAN	28.1 ± 0.5
4-OH-GlBN	458.0 ± 14.6
GlBNN	77.6 ± 3.6
GlBN	16.1 ± 0.2
GlNTN	18.1 ± 0.7
Phenolic compounds, mg/100 g DM	**1168.9 ± 3.3**
phenolic acids	**408.9 ± 2.1**
SiM	269.8 ± 2.3
SiA	19.7 ± 0.3
SiHex	8.6 ± 0.1
SiGlu	64.2 ± 0.6
SiA der 1	6.1 ± 0.1
SiA der 2	4.6 ± 0.1
diSiHexHex	6.5 ± 0.1
diSiHex 1	6.5 ± 0.1
diSiHex 2	19.3 ± 0.2
diSiHex 3	0.8 ± 0.0
SiA der 3	2.6 ± 0.1
phenolic alkaloids	**553.1±2.6**
SiN	509.2±2.6
SiN der 1	6.5±0.0
SiN der 2	7.7±0.1
SiN der 3	6.7±0.0
SiN der 4	23.0±0.1
flavonols	**206.9±0.8**
KaSopGlu	42.0±0.5
KaSiSopGlu	96.6±0.6
KaSiHexHexHex 1	17.4±0.2
KaSiHexHexHex 2	42.4±0.2
KaSiHexHexHex 3	8.5±0.1
Phytic acid, mg/100 g DM	**3907.1±105.7**

* Abbreviation is given in Appendix A. Results were shown as means (± standard deviation) of triplicate analyses.

Phytochemicals in CPC extract consisted mainly of phytic acid (3907 mg/100 g DM), phenolic compounds (1169 mg/100 g DM), and glucosinolates (771 mg/100 g DM) (Table 1, Supplemental Fig. 2**a**, Table 2). Phenolic compounds presented in the extract of CPC raw material primarily as phenolic alkaloids (47% of total content of identified phenolics), phenolic acids (35%), and flavonols (18%). Sinapine (SiN) was the main phenolic alkaloids at a content of 509 mg/100 g DM. Esterified derivatives of sinapic acid formed the major phenolic acids, such as sinapoyl malate (SiM, 270 mg/100 g DM), sinapoyl glucose (SiGlu, 64 mg/100 g DM), and disinapoyl hexose (diSiHex, three isomers

Table 2
Identification of main compounds in studied CPC samples by UPLC-DAD-ESI-Q-TOF.

No. *	Identification (abbreviation)	UV λ_{max} (*nm*)	$[M+Na]^+/[M+H]^+/[M-H]^-$ or other ions (*m/z*)	MS^{2**} (*m/z*)
Glucosinolates				
1	progoitrin (**PrN**)	226	-/-/388.0251	388.0251 → 341.0963, 332.0002, 308.0702, **290.9752**, **274.9803**, **259.0035**, **240.9936**, **195.0258**, **96.9552**, **95.9475**, **74.9869**
2	gluconapoleiferin (**GlNFN**)	212	-/-/402.0400	402.0400 → 332.0006, 322.0858, **290.9709**, **274.9805**, **259.0044**, **240.9915**, **195.0255**, **96.9552**, **95.9473**, **74.9869**
3	gluconapin (**GlNN**)	225	-/374.0595/372.0305	374.0595 → 294.0601, 132.0492 372.0305 → 292.0752, **290.9752**, **274.9797**, **259.0032**, **240.9931**, **195.0255**, **96.9553**, **95.9475**, **74.9869**
4	glucoalyssin (**GlAN**)	219	474.0559/-/450.0422	474.0559 → 394.0986, 232.0451 450.0422 → 386.0448, 370.0866, **290.9740**, **274.9807**, **259.0035**, **195.0252**, 192.0255, **96.9552**, **95.9476**, 79.9532, **74.9878**
5	4-hydroxy-glucobrassicin (**4-OH-GlBN**)	221	-/465.0649/463.0340	465.0649 → 385.1044, 223.0529 463.0340 → 383.0785, **290.9736**, 285.0078, **274.9791**, 266.9980, **259.0024**, 221.0299, **195.0259**, 169.0344, **96.9554**, **95.9492**, **74.9868**
6	glucobrassicanapin (**GlBNN**)	224	-/388.0791/386.0456	388.0791 → 146.0631 386.0456 → 341.0974, 306.0915, **290.9737**, **274.9801**, **259.0032**, 240.9932, 227.0144, **195.0256**, **96.9554**, **95.9475**, 79.9530, **74.9869**
7	glucobrassicin (**GlBN**)	225	-/-/447.0535	447.0535 → **290.9612**, **259.0115**, **195.0350**, **96.9625**, **74.9525**
8	gluconasturtiin (**GlNTN**)	219	-/-/422.0448	422.0448 → **290.9885**, **274.9918**, **259.0155**, **195.0375**, **96.9627**, **95.9471**, **74.9936**
Phenolic compounds				
9	sinapoyl malate (**SiM**)	235, 323	363.0638/341.0821/339.0701	341.0821 → **207.0629** 339.0701 → **223.0602**
10	sinapic acid (**SiA**)	228, 309	247.0569/225.0755/223.0612	225.0755 → **207.0650**, 192.0415, 175.0392, 147.0447 223.0612 → 208.0378, 193.0144
11	sinapoyl hexose (**SiHex**)	243, 328	409.1926/-/385.1144	409.1926 → 310.1629, 251.0902, **207.0645** 385.1144 → 325.0831, 265.0718, 247.0616, **223.0517**, **205.0511**
12	kaempferol 3-*O*-sophoroside-7-*O*-glucoside (**KaSopGlu**)	265, 344	-/773.2040/771.1994	773.2040 → 611.1543, 449.1034, **287.0531** 771.1994 → 609.1456, 446.0850, **284.0326**
13	sinapoyl glucose (**SiGlu**)	238, 331	409.1129/-/385.1011	409.1129 → 247.0592, 185.0434 385.1011 → 325.0808, 265.0619, 247.0519, **223.0524**, **205.0426**
14	sinapine (**SiN**)	237, 329	-/310.1595/354.1552	310.1595 → **251.0845**, **207.0620**, 175.0365, 147.0426, 119.0478, 91.0531 354.1552 → **294.1343**, **279.1107**, **264.0874**, 236.0931
15	kaempferol 3-*O*-(sinapoyl)-sophoroside-7-*O*-glucoside (**KaSiSopGlu**)	268, 334	-/979.2519/977.2536	979.2519 → 611.1486, 449.0998, 369.1112, **287.0497**, **207.0617** 977.2536 → 815.1998, 609.1417, 591.1312, 446.0845, **284.0308**
16	sinapic acid derivative 1 (**SiA der 1**)	231, 329	-/-/547.1635	547.1635 → 367.1012, **223.0603**, **205.0498**, 190.0264
17	sinapic acid derivative 2 (**SiA der 2**)	238, 327	-/-/547.1629	547.1629 → 385.1117, 367.1019, 325.0907, 295.0807, 265.0703, **223.0603**, **205.0499**, 190.0265
18	sinapine derivative 1 (**SiN der 1**)	230, 329	-/476.2199/520.2148	476.2199 → 417.1479, 221.0771, 206.0551, 177.0539, 145.0270 520.2148 → 412.1739, 403.1370, 389.1215, 355.1170, 341.1014, 264.1223, 249.0991, **207.0646**, 195.0652
19	sinapine derivative 2 (**SiN der 2**)	231, 316	-/506.2326/550.2272	506.2326 → 447.1640, **251.0896**, **207.0650** 550.2272 → 433.1492, 419.1342, **294.1339**, **279.1107**, **264.0872**, **223.0605**, 195.0660
20	kaempferol-sinapoylhexoside-hexoside-hexoside 1 (**KaSiHexHexHex 1**)	267, 322	-/-/977.2295	977.2295 → 815.1824, 653.1304, 609.1300, 447.0779, **285.0296**
21	sinapine derivative 3 (**SiN der 3**)	231, 313	-/536.2439/596.2339	536.2439 → **251.0898**, **207.0655** 596.2339 → 550.2280, 504.2224, 433.1494, 419.1346,

(*continued on next page*)

Table 2 (*continued*)

No. *	Identification (abbreviation)	UV λ_{max} (*nm*)	$[M + Na]^+/[M + H]^+/[M-H]^-$ or other ions (*m/z*)	MS^{2**} (*m/z*)
				354.1553, **294.1342**, **279.1111**, **264.0880**, **223.0607**, 195.0664
22	sinapine derivative 4 (**SiN der 4**)	229, 334	-/507.2420/559.0892	507.2420 → 447.1674, **251.0918**, **207.0657** 559.0892 → 464.0255, 357.1224, 292.0681, 232.0527, 190.0426, 172.0324, 172.0324, 146.0556, 96.9549, 79.9535
23	kaempferol-sinapoylhexoside-hexoside-hexoside 2 (**KaSiHexHexHex 2**)	265, 334	-/-/977.2580	977.2580 → 815.2037, 609.1407, 591.1352, 446.0850, **284.0332**
24	kaempferol-sinapoylhexoside-hexoside-hexoside 3 (**KaSiHexHexHex 3**)	266, 335	-/979.2575/977.2597	979.2575 → 817.2073, 655.1584, 369.1149, **287.0532**, **207.0645** 977.2597 → 652.1439, 609.1462, **285.0405**
25	disinapoyl hexose-hexose (**diSiHexHex**)	239, 330	-/-/753.2020	753.2020 → 529.1408, 487.1297, 265.0615, 247.0527, **223.0535**, **205.0420**, 190.0200
26	disinapoyl hexose 1 (**diSiHex 1**)	239, 330	615.1602/-/591.1541	615.1602 → 391.0960, **251.0902** 591.1541 → 367.1038, **223.0618**, **205.0519**
27	disinapoyl hexose 2 (**diSiHex 2**)	239, 330	615.1703/-/591.1541	615.1703 → 391.1008, 369.1188, 351.1076, 229.0471, **207.0653** 591.1541 → 367.0905, **223.0523**, **205.0427**, 190.0194, 164.0412
28	disinapoyl hexose 3 (**diSiHex 3**)	239, 327	-/-/591.1542	591.1542 → 385.0997, 367.0899, **223.0535**, **205.0426**, 190.0197
29	sinapic acid derivative 3 (**SiA der 3**)	238, 326	261.0691/239.0879/237.0753	239.0879 → **207.0623**, 192.0385, 175.0373, 147.0423, 119.0473 237.0753 → 222.0518, 207.0284, 179.0340
B1	unknown phenolic alkaloid (**unknown**)	227, 313	-/532.2076/576.2079	532.2076 → 473.1371, 319.0762, 275.0508 576.2079 → 427.1033, 304.1189, 203.0718
B2	kaempferol-sinapoylhexoside-hexoside-hexoside 4 (**KaSiHexHexHex 4**)	267, 327	-/979.2563/977.2552	979.2563 → 611.1528, 449.1020, 369.1132, **287.0515**, **207.0632** 977.2552 → 815.2012, 610.1451, 446.0836, 285.0355
B3	kaempferol-hexoside-hexoside (**KaHexHex**)	265, 344	-/611.1536/609.1439	611.1536 → 449.1030, **287.0517** 609.1439 → **284.0316**
B4	kaempferol-sinapoylhexoside-hexoside 1 (**KaSiHexHex 1**)	267, 327	839.1890/-/815.2040	839.1890 → 553.1465, 391.0963 815.2040 → 609.1441, **284.0318**
B5	kaempferol-hexoside (**KaHex**)	267, 344	-/449.1034/447.0917	449.1034 → **287.0531** 447.0917 → **284.0324**, 255.0292, 227.0342
B6	kaempferol-sinapoylhexoside-hexoside 2 (**KaSiHexHex 2**)	266, 326	-/817.2081/815.2055	817.2081 → 449.1042, 369.1151, **287.0533**, **207.0645** 815.2055 → 609.1471, **284.0333**
B7	kaempferol-sinapoylhexoside-hexoside 3 (**KaSiHexHex 3**)	267, 334	-/817.2035/815.2054	817.2035 → 655.1546, 369.1120, 351.1020, **287.0510**, **207.0626** 815.2054 → 653.1515, 447.0942, 285.0407
Other compounds				
B1′	cytidine	219, 271	266.0732/244.0916/242.0763	244.0916 → 112.0506 242.0763 → 198.1498, 174.9541, 146.9609, 110.0344, 109.0393, 81.0462, 67.0352, 61.0558, 41.8516
B2′	uridine	214, 261	267.0575/245.0760/243.0596	245.0760 → 113.0352, 70.0300, 57.0342, 55.0186 243.0596 → 200.0545, 179.9373, 152.0342, 140.0342, 124.0395, 110.0248, 82.0304, 66.0352
B3′	guanine derivative	214, 252, 275	-/346.0520/344.0380	346.0520 → 152.0569 344.0380 → 150.0413
B4′	guanosine	214, 252, 275	-/284.0962/282.0837	284.0962 → 152.0572 282.0837 → 150.0440
B5′	tryptophan	219, 270	-/205.0953/203.0814	205.0953 → 188.0687, 146.0592 203.0814 → 186.0551, 159.0915, 142.0656, 116.0501, 74.0247
B6′	adenosine	213, 258	290.0807/268.0992/266.0898	268.0992 → 136.0603 266.0898 → 134.0495

* The number of peaks is referred to Supplemental Fig. 2.
** The MS^2 spectra of identified glucosinolates and phenolics are given in Supplemental Fig. 4.

altogether 27 mg/100 g DM). Compared to these derivatives, the content of sinapic acid in free form (SiA) remained at a low level (20 mg/100 g). Flavonols in raw CPC were present mostly as glycosylated kaempferols, kaempferol 3-*O*-(sinapoyl)-sophoroside-7-*O*-glucoside (KaSiSopGlu, 97 mg/100 g DM) and kaempferol 3-*O*-sophoroside-7-*O*-glucoside (KaSopGlu, 42 mg/100 g DM) being the dominant. Other kaempferol sinapoyl-acylated glycosides were also detected at high contents. Among all identified glucosinolates, 4-hydroxy-glucobrassicin (4-OH-GlBN) showed the highest content, accounting for 59% of total content of glucosinolates, followed by gluconapin (GlNN, 15%) and glucobrassicanapin (GlBNN, 10%).

3.2. Chemical profiles in supernatants of pre-treated canola oil press cakes

Determined by total nitrogen analysis (Table 3), conventional

alkaline extraction (pH 9.5) resulted in the highest content of proteins (91 g/100 g of DM of the supernatants). Addition of proteases resulted in a medium protein contents in supernatants, ranging from 74 to 82 g/100 g DM. Compared to alkaline extraction, only two enzymatic treatments significantly enhanced the solubility of proteins. After 20 h of co-incubation using carbohydrase (Viscozyme) and phytase (Phyzyme), almost 90% of crude protein was solubilized in the supernatant, although protein content remained at a lower level. Adjusting pH value during Alcalase treatment solubilized 86% of crude protein.

The highest content of free sugars was quantified from the samples of alkaline extraction (30830 mg/100 g DW, Table 3). Sucrose (Supplemental Fig. 1) represented for 87% of total content of analyzed sugars. Monosaccharides (and derivatives) were detected at higher contents, including glucose (Glu, 2412 mg/100 g DM), galactose (Gal, 631 mg/100 g DM), fructose (Fru, 526 mg/100 g DM), and *myo*-inositol (Ino, 384 mg/100 g DM). The total content of identified sugars in enzyme-treated supernatants ranged from 21370 to 25145 mg/100 g. Protease hydrolysis produced lower amount of free sugars than alkaline extraction; however, the content of *myo*-inositol was increased by 4–5 times after incubation with Protamex. Surprisingly, addition of Viscozyme did not increase the total content of analyzed sugars, but contributed to degradation of sucrose into monosaccharides, such as Glu, Fru, arabinose (Ara), Gal, xylose (Xyl), and galacturonic acid (GaA). Additionally, increasing contents of *myo*-inositol and mannitol were detected after Viscozyme-Phyzyme co-incubation, suggesting the role of Phyzyme in degrading phytic acid. Although the contents of dietary fibres were not determined in the supernatants, the changes in dry matter content provide some indication on the content of dietary fibres. After Viscozyme and Viscozyme-Phyzyme treatments, the dry matter content in the supernatants was 1.8-fold higher compared to the samples obtained with alkaline extraction and protease treatments. This may have been due to increasing content of solubilized fibers caused by the carbohydrase activity.

The supernatants after pre-treatments contained approximately 4100 mg/100 g DM of free acids on average (Table 3). Among individual acids (Supplemental Fig. 1), phosphoric acid was concentrated in Viscozyme-Phyzyme-treated supernatant, the content of which was 2–3 times higher than that in other samples due to the action of phytase. The supernatant of alkaline extraction showed the highest content of organic acids (3059 mg/100 g DM), whereas the samples after carbohydrase hydrolysis had the lowest (1178–1368 mg/100 g DM). The composition of organic acids was similar among all supernatants where malic acid (54–57% of total content of analyzed organic acids) and citric acid (31–35%) being dominant.

The total content of the identified free amino acids in supernatants varied from 1296 to 6922 mg/100 g DM (Table 3). Low levels of free amino acids were found in the samples after conventional alkaline extraction and carbohydrase treatment (with or without addition of phytase), whereas protease hydrolysis using either Alcalase or Protamex led to a significant accumulation of these nutrients (Supplemental Fig. 1). Pre-treatment also influenced the composition of amino acids in supernatants. The supernatant of alkaline extraction mainly contained proline (Pro) and aspartic acid (AsA). Leucine (Leu) was dominant in protease-hydrolyzed samples, followed by alanine (Ala), valine (Val), aspartic acid, and proline. Moreover, glutamic acid, methionine (Met), phenylalanine (Phe), and tyrosine (Tyr) missing from the alkaline extraction sample had higher concentration after protease treatments.

In the CPC supernatants, phenolic contents varied significantly among pre-treatments (Table 3). Addition of enzymes effectively enhanced the release of phenolic compounds from plant tissue, compared to alkaline extraction. The increasing effects were ranked in the order of Viscozyme-Phyzyme (750 mg/100 g DM) < Viscozyme (795 mg/100 g DM) < Alcalase (1370–1905 mg/100 g DM) < Protamex (1935 mg/100 g DM). Although varying in total contents, the identified phenolic compounds in supernatants were phenolic acids, phenolic alkaloids, and flavonol glycosides (Table 2, Supplemental Fig. 2**b**). The major phenolics in enzyme-treated supernatants were sinapoyl malate, representing for 37–48% of total content of the analyzed phenolics (Table 3).

Glucosinolates identified from CPC raw material were not detected in all supernatants from the pre-treatments. The molecule ions of these compounds, together with typical glucosinolate fragment ions (at 291, 275, 259, 241, 195, 97, 96, and 75 *m/z*) were absent in the MS spectra of supernatants. The phytic acid content in supernatants varied from 466 to 903 mg/100 g DM (Table 3). The contents after protease-treatment were almost 2-fold lower than that obtained from the alkaline extraction, whereas a medium level of phytic acid was detected from the sample of Viscozyme-Phyzyme co-incubation.

3.3. Chemical profiles in precipitates of pre-treated canola oil press cakes

Table 4 presents profiles of nutrients and phytochemicals in the precipitates of pre-treated CPC. Dietary fibre content was determined only in the solid residues of alkaline extraction and protease hydrolysis. As the major nutrients in precipitates, the total content of dietary fibres was in a range of 55–58 g/100 g DM. No significant deviation was observed among the different pre-treatments applied. Insoluble fibres accounted for 95–98% of total content of dietary fibres. The pre-treated CPC contained a low content of proteins, and the highest value was in the carbohydrase-hydrolyzed samples. Moreover, an increase in the mineral content as determined by total ashes was also found in studied precipitates from 12 to 13 (after carbohydrase/phytase incubation), 8–9 (protease) to 7 g/100 g DM (alkaline extraction).

Low levels of phenolic compounds remained in the precipitates, ranging from 273 to 483 mg/100 g of dry matters (Table 4). The protease-hydrolyzed precipitates had higher contents of total phenolics than that obtained from carbohydrase treatments and alkaline extraction. The most abundant compounds were sinapine (25–46% of total phenolics) and sinapoyl malate (19–28%).

Regarding the undesired components, glucosinolates were not detected in the pre-treated CPC precipitates (Table 4). Nevertheless, a high amount of phytic acid was present in the precipitates after alkaline extraction and most of enzymatic treatments. The content of phytic acid was decreased by applying Phyzyme simultaneously with carbohydrase. In contrast, Viscozyme, Alcalase, and Protamex had no effect on phytic acid.

3.4. Compositional variation among raw material and pre-treated canola oil press cakes

PCA models were created to visualize the variation of individual components in the studied samples. Chemical deviation between the CPC raw material and the pre-treated samples is presented in Fig. 1. Different from all the supernatants of the pre-treatments, the raw material was rich in glucosinolates and phytic acid, exhibiting positive correlations with the compounds in Fig. 1**a**, where 79% of chemical components were included in PC-1 & PC-2. The raw material had lower content of proteins and most of the identified free sugars, acids, and amino acids. Among the phenolic compounds, total content of phenolic alkaloids was positively correlated with the CPC raw material owing to the high level of SiN. Some phenolics, such as SiGlu, kaempferol-sinapoylhexoside-hexoside-hexoside 1 (KaSiHexHexHex 1), disinapoyl hexose-hexose (diSiHexHex), sinapine derivative 4 (SiN der 4), and diSiHex 2 & 3, were found associated positively with the raw material but negatively with the supernatants. This was likely due to the fact that these compounds presented originally in the raw material were degraded during pre-treatments. In contrast, kaempferol-sinapoylhexoside-hexoside 2 (KaSiHexHex 2) and an unknown phenolic alkaloid (unknown), which were both produced in pre-treatments, showed strong correlations only to pre-treated samples.

Compared to precipitates of pre-treatments, the CPC raw material correlated strongly with the contents of proteins, glucosinolates, and

Table 3
Concentration of nutrients and phytochemicals in the supernatants of pre-treated CPC samples*.

Composition	alkaline extraction	1% Alc. No pH control	9% Visc. No pH control	1% Alc. pH control	1% Prot. pH control	9% Visc. + 0.5% Phyz. pH control
Dry matter, %	**1.9 ± 0.1^{c}**	**2.5 ± 0.1^{b}**	**4.0 ± 0.1^{a}**	**2.3 ± 0.1^{bc}**	**2.4 ± 0.1^{bc}**	**4.1 ± 0.2^{a}**
Proteins, g/100 g DM	**91.2 ± 1.6^{a}**	**73.8 ± 0.4^{c}**	**40.2 ± 0.5^{d}**	**81.7 ± 0.9^{b}**	**77.2 ± 1.9^{bc}**	**45.4 ± 1.4^{d}**
solubilized protein, % of proteins	75.6 ± 1.3^{b}	83.1 ± 0.4^{ab}	76.3 ± 0.9^{b}	85.6 ± 0.9^{a}	81.7 ± 2.0^{ab}	88.5 ± 2.6^{a}
Free sugars, mg/100 g DM	**30830.0 ± 128.5^{a}**	**23032.1 ± 220.4^{bc}**	**21370.1 ± 84.1^{c}**	**24560.9 ± 542.1^{b}**	**25059.2 ± 322.8^{b}**	**25144.5 ± 818.9^{b}**
Ara	–	–	3139.7 ± 37.6^{a}	–	–	3145.5 ± 57.9^{a}
Fuc	–	–	176.7 ± 2.8^{a}	–	–	183.7 ± 17.6^{a}
Xyl	–	–	662.6 ± 10.1^{b}	–	–	727.9 ± 8.2^{a}
Fru	526.4 ± 6.9^{b}	347.7 ± 3.8^{b}	3214.7 ± 177.9^{a}	474.4 ± 12.6^{b}	785.8 ± 43.1^{b}	3830.8 ± 235.4^{a}
Gal	631.1 ± 2.4^{b}	418.5 ± 3.6^{c}	2446.5 ± 25.6^{a}	462.7 ± 6.8^{c}	479.7 ± 16.1^{bc}	2573.8 ± 61.6^{a}
Glu	2412.2 ± 19.9^{c}	1742.2 ± 25.5^{c}	10705.7 ± 16.5^{b}	1956.8 ± 40.7^{c}	2267.2 ± 63.7^{c}	11970.7 ± 381.0^{a}
Suc	26876.3 ± 96.9^{a}	20190.3 ± 196.0^{bc}	–	21276.4 ± 472.0^{b}	19918.1 ± 186.9^{c}	–
GaA	–	–	544.1 ± 0.5^{a}	–	–	543.2 ± 11.1^{a}
Man	–	–	–	–	–	552.8 ± 2.7^{a}
Ino	384.0 ± 2.4^{bc}	333.3 ± 1.0^{c}	480.2 ± 1.7^{b}	390.6 ± 10.0^{bc}	1608.3 ± 13.1^{a}	1616.1 ± 43.4^{a}
Free acids, mg/100 g DM	**4568.7 ± 35.4^{ab}**	**3784.9 ± 29.7^{d}**	**2848.4 ± 6.9^{e}**	**4147.7 ± 78.6^{cd}**	**4389.0 ± 77.0^{bc}**	**4807.5 ± 129.9^{a}**
PhA	1509.8 ± 8.9^{cd}	1255.9 ± 14.4^{e}	1670.0 ± 2.0^{bc}	1375.5 ± 24.8^{de}	1893.2 ± 45.1^{b}	3439.3 ± 89.1^{a}
organic acids	**3058.9 ± 26.5^{a}**	**2529.0 ± 15.3^{c}**	**1178.4 ± 5.0^{e}**	**2772.1 ± 53.8^{b}**	**2495.8 ± 31.9^{c}**	**1368.2 ± 40.9^{d}**
SuA	46.5 ± 0.8^{a}	36.4 ± 0.0^{b}	15.7 ± 0.1^{c}	38.4 ± 1.1^{b}	36.8 ± 1.2^{b}	18.2 ± 0.6^{c}
MaA	1755.2 ± 8.7^{a}	1356.9 ± 14.0^{c}	660.9 ± 0.6^{e}	1533.7 ± 24.5^{b}	1370.3 ± 10.8^{c}	757.5 ± 22.2^{d}
CiA	988.2 ± 6.6^{a}	776.9 ± 5.1^{c}	402.4 ± 3.1^{e}	858.7 ± 12.2^{b}	795.5 ± 9.8^{c}	475.3 ± 13.4^{d}
FuA	268.9 ± 10.5^{c}	358.8 ± 3.8^{a}	99.4 ± 1.1^{d}	341.3 ± 16.0^{ab}	293.2 ± 12.6^{bc}	117.3 ± 4.6^{d}
Free amino acids, mg/100 g DM	**1365.6 ± 29.1^{c}**	**4219.8 ± 33.5^{b}**	**1295.5 ± 23.2^{c}**	**6921.8 ± 210.8^{a}**	**6765.9 ± 221.9^{a}**	**1426.8 ± 46.6^{c}**
Ala	90.1 ± 2.3^{c}	250.1 ± 1.3^{b}	85.7 ± 1.4^{c}	649.3 ± 20.3^{a}	646.8 ± 22.8^{a}	98.3 ± 3.1^{c}
Gly	94.6 ± 1.7^{c}	85.0 ± 0.2^{c}	28.2 ± 0.5^{d}	139.1 ± 4.4^{b}	244.1 ± 5.1^{a}	36.3 ± 1.0^{d}
Val	88.2 ± 1.4^{c}	250.5 ± 0.6^{b}	67.0 ± 1.1^{c}	602.0 ± 16.0^{a}	622.9 ± 17.4^{a}	75.7 ± 2.0^{c}
Leu	131.2 ± 1.6^{e}	733.5 ± 0.1^{c}	225.9 ± 4.5^{de}	1140.8 ± 30.7^{a}	994.2 ± 33.0^{b}	250.4 ± 6.4^{d}
Pro	450.1 ± 16.4^{a}	467.7 ± 12.4^{a}	148.7 ± 3.5^{b}	534.7 ± 21.0^{a}	531.4 ± 30.8^{a}	178.3 ± 6.8^{b}
Iso	71.4 ± 0.1^{d}	191.1 ± 1.3^{c}	51.9 ± 0.7^{d}	396.1 ± 9.7^{b}	481.1 ± 15.3^{a}	58.1 ± 1.3^{d}
Ser	90.9 ± 1.1^{d}	185.5 ± 0.2^{c}	62.1 ± 0.8^{d}	391.6 ± 11.6^{b}	482.4 ± 15.5^{a}	69.6 ± 2.3^{d}
Thr	72.5 ± 0.8^{d}	156.2 ± 0.1^{c}	54.4 ± 1.2^{d}	280.1 ± 7.4^{b}	413.1 ± 11.3^{a}	55.4 ± 3.4^{d}
GlA	–	507.8 ± 0.0^{b}	80.0 ± 0.8^{c}	658.7 ± 21.3^{a}	552.9 ± 17.6^{b}	36.9 ± 1.3^{c}
AsA	276.4 ± 3.8^{c}	561.1 ± 2.8^{b}	145.1 ± 1.6^{d}	730.8 ± 18.3^{a}	536.2 ± 14.8^{b}	149.7 ± 5.6^{d}
Met	–	153.3 ± 4.1^{b}	48.4 ± 1.6^{c}	287.3 ± 8.1^{a}	270.3 ± 9.1^{a}	49.7 ± 1.8^{c}
Phe	–	317.9 ± 9.1^{b}	160.6 ± 3.1^{c}	521.8 ± 24.5^{a}	491.5 ± 10.1^{a}	172.1 ± 4.3^{c}
Tyr	–	360.1 ± 5.6^{c}	137.5 ± 2.4^{d}	589.6 ± 17.6^{a}	499.0 ± 19.1^{b}	196.4 ± 7.2^{d}
Glucosinolates, mg/100 g DM	**–**	**–**	**–**	**–**	**–**	**–**
Phenolic compounds, mg/100 g DM	**446.1 ± 3.7^{d}**	**1905.0 ± 4.1^{a}**	**795.3 ± 25.3^{c}**	**1370.3 ± 16.5^{b}**	**1935.4 ± 10.8^{a}**	**750.4 ± 11.5^{c}**
phenolic acids	**168.9 ± 2.9^{f}**	**893.1 ± 2.0^{b}**	**432.6 ± 10.0^{e}**	**584.8 ± 6.5^{c}**	**1029.4 ± 0.2^{a}**	**477.1 ± 3.6^{d}**
SiM	168.9 ± 2.9^{e}	719.2 ± 2.2^{b}	349.7 ± 4.9^{d}	507.8 ± 5.6^{c}	761.6 ± 1.6^{a}	359.6 ± 0.0^{d}
SiA	–	98.8 ± 0.4^{b}	32.1 ± 3.7^{d}	32.1 ± 0.7^{d}	208.2 ± 0.3^{a}	67.5 ± 3.7^{c}
SiHex	–	16.2 ± 0.1^{a}	3.8 ± 0.1^{c}	–	10.9 ± 0.4^{b}	3.4 ± 0.1^{c}
SiA der 1	–	17.1 ± 0.1^{a}	16.1 ± 0.2^{ab}	15.2 ± 0.3^{ab}	14.2 ± 0.7^{b}	17.4 ± 0.9^{a}
SiA der 2	–	24.3 ± 0.7^{a}	25.4 ± 0.7^{a}	19.1 ± 0.1^{b}	19.2 ± 0.5^{b}	23.3 ± 0.6^{a}
diSiHex 1	–	17.6 ± 0.1^{a}	–	10.5 ± 0.4^{c}	15.3 ± 0.6^{b}	–
SiA der 3	–	–	5.5 ± 0.4^{a}	–	–	5.9 ± 0.4^{a}
phenolic alkaloids	**–**	**482.5 ± 1.4^{a}**	**248.8 ± 11.3^{d}**	**301.0 ± 7.0^{c}**	**395.0 ± 4.8^{b}**	**167.2 ± 7.2^{e}**
unknown	–	115.8 ± 0.7^{b}	20.0 ± 2.9^{e}	74.4 ± 1.8^{c}	159.2 ± 0.5^{a}	33.0 ± 2.6^{d}
SiN	–	318.0 ± 0.7^{a}	228.7 ± 8.5^{b}	164.1 ± 5.3^{c}	183.5 ± 3.4^{c}	134.2 ± 4.6^{d}
SiN der 1	–	14.4 ± 0.1^{b}	–	16.9 ± 0.1^{a}	15.0 ± 0.7^{b}	–
SiN der 2	–	17.1 ± 0.3^{c}	–	23.4 ± 0.1^{a}	22.6 ± 0.0^{b}	–
SiN der 3	–	17.2 ± 0.3^{b}	–	22.1 ± 0.1^{a}	14.7 ± 0.3^{c}	–
flavonols	**277.2 ± 6.6^{c}**	**529.4 ± 0.7^{a}**	**113.9 ± 4.0^{d}**	**484.5 ± 2.9^{b}**	**511.0 ± 5.8^{a}**	**106.1 ± 0.7^{d}**
KaSopGlu	96.2 ± 4.5^{a}	8.0 ± 0.0^{c}	2.4 ± 0.0^{c}	43.3 ± 0.6^{b}	3.6 ± 0.1^{c}	5.2 ± 0.2^{c}
KaSiSopGlu	–	138.6 ± 0.2^{b}	19.8 ± 0.7^{d}	111.8 ± 1.2^{c}	149.9 ± 0.8^{a}	–
KaSiHexHexHex 4	–	32.5 ± 0.1^{a}	14.6 ± 0.8^{d}	24.8 ± 0.5^{b}	–	17.6 ± 0.3^{c}
KaHexHex	181.0 ± 2.2^{a}	109.2 ± 0.1^{b}	28.2 ± 0.0^{c}	106.8 ± 0.2^{b}	108.5 ± 1.0^{b}	28.5 ± 0.8^{c}
KaSiHexHex 1	–	50.4 ± 1.0^{b}	–	47.3 ± 0.7^{b}	59.3 ± 0.9^{a}	–
KaSiHexHexHex 2	–	45.6 ± 0.9^{b}	–	50.0 ± 1.2^{b}	90.2 ± 1.3^{a}	–
KaHex	–	–	15.2 ± 0.2^{b}	–	–	22.7 ± 0.5^{a}
KaSiHexHexHex 3	–	121.4 ± 0.6^{a}	–	80.4 ± 0.9^{b}	80.9 ± 1.0^{b}	–
KaSiHexHex 2	–	23.8 ± 0.0^{a}	21.0 ± 1.5^{ab}	20.0 ± 0.2^{ab}	18.6 ± 0.6^{b}	21.4 ± 0.8^{ab}
KaSiHexHex 3	–	–	12.9 ± 0.7^{a}	–	–	10.6 ± 0.7^{b}
Phytic acid, mg/100 g DM	**903.3 ± 15.1^{a}**	**465.9 ± 2.2^{c}**	**675.1 ± 41.3^{b}**	**518.0 ± 23.8^{c}**	**484.7 ± 49.6^{c}**	**766.6 ± 27.7^{b}**

* Abbreviation is given in Appendix A. Results were shown as means (± standard deviation) of duplicate analyses. Statistical differences were conducted based on one way-ANOVA and Tukey's post hoc test ($p < 0.05$), and shown with superscript letters a–e.

soluble dietary fibres as shown in Fig. 1**b** (PC-1 & PC-2 containing 90% of variables). Nevertheless, the contents of minerals and total fibres (mainly insoluble dietary fibres) closely correlated to the precipitates, and lower amounts of these components were detected in the CPC raw material. Other distinguishing components were phenolic compounds, primarily SiM, SiN, KaSiSopGlu, KaSopGlu, and KaSiHexHexHex 2.

PCA models in Fig. 2 shows the comparison of samples from different pre-treatments. In Fig. 2**a** (PC-1 & PC-2 containing 84% of chemical components), the supernatant of alkaline extraction strongly correlated to total sugars. For phenolic compounds, KaSopGlu was rich in the alkaline-treated supernatant; but SiN, sinapic acid derivatives (SiA der 1 & 2), and KaSiHexHex 2, were absent from this sample. Among the enzyme-treated samples, the supernatants obtained after protease hydrolysis (Alcalase & Protamex) had positive correlation with free amino acids and most of the phenolic compounds. The highly-correlated phenolics included SiM, SiA, diSiHex 1, KaSiHexHex 1, KaSiSopGlu, KaSiHexHexHex (2 & 3), SiN der (1, 2, & 3), and the unknown phenolic alkaloid. On the contrary, Viscozyme-treated supernatants were rich in monosaccharides, but low in proteins and organic acids. Certain phenolics, such as SiA der 3, kaempferol-hexoside (KaHex), and KaSiHexHex 3, were found only in these samples.

Among pre-treated precipitates (Fig. 2**b** containing PC-1 & PC-2 where 74% of variables included), the samples obtained after protease treatments were associated strongly with total content of phenolics, primarily as KaSiSopGlu, KaSiHexHexHex 2, KaSiHexHex 1, and the unknown alkaloid. Both treated with Alcalase, the sample from non-pH-controlled incubation had higher contents of diSiHex 1, SiN, and KaSiHexHexHex 3, whereas KaSopGlu was accumulated mainly in pH-adjusted process. The viscozyme-hydrolyzed precipitates highly correlated to contents of proteins, minerals, and phenolic acids (mainly as SiA and SiA der 3). Positive correlation of Viscozyme hydrolysis was also observed with some kaempferol glycosides (KaHex and KaSiHexHex 2 & 3).

Comparison between enzyme-treated supernatants and precipitates was given in Fig. 2**c**, where 82% of variables were represented within PC-1 and PC-2. All the supernatants from the enzymatic treatments correlated negatively to the content of phytic acid, compared to the corresponding precipitates. The total content of proteins and the content of most of the identified phenolics had strong positive correlations with the protease-hydrolyzed supernatants, due to their high contents in these samples.

3.5. Effect of pH adjustment in Alcalase hydrolysis

The Alcalase-treated samples were compared by PCA models in Fig. 2**d & 2e**. For supernatants (99% of variables shown in PC-1 and PC-2 of Fig. 2**d**), remaining initial pH value during Alcalase incubation contributed to the increase of protein content. All identified amino acids and sugars correlated strongly to pH adjusted hydrolysis. The total content of identified acids was also associated with pH control, mainly as PhA, succinic acid (SuA), MaA and CiA. In contrast, fumaric acid (FuA) correlated positively to non-pH adjusted treatment. Controlling pH value resulted in a remarkable reduction of phenolic contents, including all studied groups. Yet, certain phenolics, such as KaSopGlu, KaSiHexHexHex 2, and SiN der (1, 2, & 3), had higher concentration in the supernatants of pH-controlled hydrolysis, which explained why these compounds were close to pH adjustment in the loading plots.

Unlike supernatants, the plots of corresponding precipitates (Fig. 2**e**, two PCs presenting 96% of variables) showed that protein content was associated strongly with the treatment without pH control. This might have been due to that the pH adjustment enhanced the solubility of protein, and the insoluble ones were concentrated mainly in the solid residues. Phenolic compounds generally had positive correlations with non-pH control. Adjusting pH value correlated positively to the contents of dietary fibres (mostly as insoluble fibres), minerals, some phenolics (KaSopGlu, SiN der 1, 2, & 3, and SiA der 3), and phytic acid.

3.6. Effect of extraction on yields of glucosinolates and phenolics

Yields of glucosinolates and phenolics were associated with varying extraction methods. Supplemental Fig. 3 shows the variation in yields of the studied compounds after extraction using different solvents. The concentration of each identified compound in various extracts is listed in Supplemental Table 4. Among all the extraction solvent studied, the highest yields of phenolics and glucosinolates were observed using a mixed solvent of methanol/acetone/water (7:7:6, *v/v/v*), where 15 mg of glucosinolates and 23 mg of phenolics were obtained from 2 g of raw material (Supplemental Fig. 3). Aqueous methanol (8:2, *v/v*) was also effective in extracting these two groups of compounds. The corresponding yields were 13 and 24 mg for glucosinolates and phenolic compounds, respectively. Additionally, approximately 90% of the compounds (including glucosinolates and phenolics) were extracted in the first three extractions with methanol/acetone/water or aqueous methanol. In contrast, extraction with aqueous acetone (8:2, *v/v*) resulted in a higher yield of phenolic compounds but a lower one of glucosinolates. Absolute ethanol and acetone were inferior in extracting both glucosinolates and phenolics, giving low yields of the compounds.

4. Discussion

4.1. Chemical profile of canola oil press cakes

Composition of nutrients and phytochemicals in rapeseed oil press residues has been studied extensively. Data from different research have shown a large variation, but the mean values of major components have been given by Lomascolo et al. (2012) after summarizing numerous previous reports. In general, rapeseed press cakes contain 35 g/100 g DW of crude proteins, 12 g/100 g DW of crude fibres, and 3 g/100 g DW of crude lipids (Lomascolo et al., 2012). Compared to the data in literature, our results in Table 1 suggested a lower protein content but higher amounts of fibres and lipids in the studied CPC raw material. This difference may have been caused by different rapeseed cultivars and varying methods used for oil-pressing and chemical analyses. Ashayerizadeh et al. (2018) and Wang et al. (2019) investigated amino acid composition in rapeseed meals by using an automated analyzer after hydrolyzing the samples with 6 M HCl at 110 °C for 24 h. Seventeen amino acids were determined and the total content was in a range of 25–34 g/100 g DW. As the most abundant compound, glutamine accounted for 24–26% of total content of amino acids (Ashayerizadeh et al., 2018; Wang et al., 2019). In our study, no acid hydrolysis was applied, and free amino acids were extracted together with sugars and simple acids by using aqueous methanol. This may explain the considerably lower level of amino acids (80 mg/100 g DW) determined from our CPC raw material sample (Table 1).

For phytochemicals, the studied CPC raw material contained 1170 mg/100 g DW of phenolic compounds, mainly as sinapine (509 mg/100 g DW, Table 1). This was in agreement with report of Laguna et al. (2018), where 19 phenolics were identified from rapeseed meals with a total content ranging from 1560 to 1660 mg/100 g of DW. Sinapine was also found as the major compound, representing for 75% of total phenolics (Laguna et al., 2018). Phytic acid content in rapeseed press cakes was in a range of 2635–4670 mg/100 g DW as reported previously by Ashayerizadeh et al. (2018) and Mohammadi et al. (2020). Likewise, approximately 3900 mg/100 g DW of phytic acid was presented in our CPC samples (Table 1). Total content of glucosinolates in the studied material was up to 771 mg/100 g DW (Table 1, the value equals to 19 μmol/g DW, sinigrin hydrates as external reference standard, molecule weight 397.46 g/mol), whereas previous research suggested the total content of glucosinolates varied from 1 to 76 μmol/g DW (Ashayerizadeh et al., 2018; Lücke et al., 2019; Mohammadi et al., 2020; Wang et al., 2019).

It is noticed that, in the present study, no inactivation of myrosinase (*e.g.* by heating at 70 °C or boiling water bath) was applied before

Table 4
Concentration of nutrients and phytochemicals in the precipitates of pre-treated CPC samples*.

Composition	alkaline extraction	1% Alc. No pH control	9% Visc. No pH control	1% Alc. pH control	1% Prot. pH control	9% Visc. + 0.5% Phyz. pH control
Dry matter, %	**91.4 ± 0.1^{d}**	**97.1 ± 0.1^{b}**	**95.2 ± 0.4^{c}**	**98.2 ± 0.0^{a}**	**97.3 ± 0.1^{ab}**	**94.7 ± 0.2^{c}**
Proteins, g/100 g DM	**18.1 ± 0.2^{d}**	**17.2 ± 0.2^{e}**	**23.0 ± 0.6^{a}**	**16.3 ± 0.1^{f}**	**19.7 ± 0.3^{c}**	**21.7 ± 0.2^{b}**
Ashes, g/100 g DM	**6.7 ± 2.2^{b}**	**8.4 ± 0.1^{ab}**	**12.6 ± 0.0^{a}**	**8.5 ± 0.0^{ab}**	**7.8 ± 0.0^{ab}**	**11.9 ± 0.1^{a}**
Dietary fibres, g/100 g DM	**57.8 ± 2.4^{a}**	**54.9 ± 1.5^{b}**		**57.7 ± 2.9^{a}**	**57.2 ± 5.9^{a}**	
soluble	1.3 ± 0.8^{bc}	1.9 ± 0.1^{b}		2.9 ± 0.3^{a}	1.2 ± 0.6^{c}	
insoluble	56.5 ± 1.7^{a}	53.0 ± 1.6^{c}		54.8 ± 2.8^{b}	56.0 ± 6.4^{a}	
Glucosinolates, mg/100 g DM	–	–	–	–	–	–
Phenolic compounds, mg/100 g DM	**311.1 ± 1.8^{d}**	**482.7 ± 2.7^{a}**	**333.7 ± 4.8^{cd}**	**361.8 ± 2.4^{c}**	**419.6 ± 1.7^{b}**	**273.4 ± 13.5^{e}**
phenolic acids	**119.3 ± 0.8^{bc}**	**135.8 ± 2.4^{ab}**	**153.3 ± 1.5^{a}**	**86.7 ± 0.0^{d}**	**114.8 ± 0.7^{c}**	**153.9 ± 8.4^{a}**
SiM	88.3 ± 1.7^{bc}	102.2 ± 2.0^{a}	79.6 ± 1.3^{cd}	67.8 ± 0.2^{e}	90.5 ± 0.1^{b}	71.8 ± 3.1^{de}
SiA	11.5 ± 0.7^{cd}	18.9 ± 0.1^{b}	36.1 ± 0.6^{a}	6.7 ± 0.2^{d}	12.8 ± 0.8^{c}	41.2 ± 2.3^{a}
SiHex	0.1 ± 0.0^{d}	0.5 ± 0.0^{bc}	0.6 ± 0.0^{b}	0.1 ± 0.0^{d}	1.4 ± 0.0^{a}	0.4 ± 0.0^{c}
SiA der 1	2.7 ± 0.1^{a}	2.8 ± 0.2^{a}	2.9 ± 0.1^{a}	2.6 ± 0.0^{a}	1.9 ± 0.1^{b}	1.7 ± 0.1^{b}
SiA der 2	4.8 ± 0.1^{b}	5.5 ± 0.2^{a}	5.7 ± 0.0^{a}	4.7 ± 0.1^{b}	3.9 ± 0.1^{c}	4.6 ± 0.2^{b}
diSiHex 1	1.6 ± 0.2^{c}	3.4 ± 0.0^{a}	0.8 ± 0.0^{d}	2.2 ± 0.0^{b}	2.9 ± 0.0^{a}	0.3 ± 0.0^{d}
SiA der 3	10.3 ± 0.1^{c}	2.4 ± 0.0^{d}	27.5 ± 0.3^{b}	2.6 ± 0.0^{d}	1.5 ± 0.0^{d}	33.8 ± 2.7^{a}
phenolic alkaloids	**129.6 ± 1.7^{d}**	**264.5 ± 2.3^{a}**	**140.5 ± 2.7^{d}**	**204.1 ± 1.7^{c}**	**228.0 ± 1.4^{b}**	**87.0 ± 3.3^{e}**
unknown	8.4 ± 0.1^{d}	25.4 ± 0.5^{b}	14.3 ± 0.2^{c}	22.6 ± 0.9^{b}	31.8 ± 1.2^{a}	16.8 ± 0.7^{c}
SiN	114.5 ± 1.3^{d}	224.2 ± 2.7^{a}	120.6 ± 2.5^{d}	165.4 ± 0.6^{c}	181.1 ± 0.1^{b}	68.3 ± 2.5^{e}
SiN der 1	2.2 ± 0.1^{c}	4.6 ± 0.0^{b}	2.2 ± 0.0^{c}	5.0 ± 0.0^{a}	4.9 ± 0.1^{a}	1.2 ± 0.1^{d}
SiN der 2	2.2 ± 0.0^{c}	5.9 ± 0.0^{b}	1.5 ± 0.0^{d}	6.3 ± 0.0^{a}	6.1 ± 0.1^{ab}	0.7 ± 0.0^{e}
SiN der 3	2.3 ± 0.3^{b}	4.3 ± 0.1^{a}	2.0 ± 0.0^{b}	4.8 ± 0.1^{a}	4.1 ± 0.1^{a}	–
flavonols	**62.2 ± 0.9^{c}**	**82.4 ± 2.6^{a}**	**40.0 ± 0.6^{d}**	**71.0 ± 0.7^{b}**	**76.7 ± 1.1^{ab}**	**32.5 ± 1.8^{d}**
KaSopGlu	4.4 ± 0.2^{ab}	3.9 ± 0.2^{b}	0.3 ± 0.0^{c}	5.1 ± 0.1^{a}	0.5 ± 0.0^{c}	0.3 ± 0.0^{c}
KaSiSopGlu	12.9 ± 0.2^{c}	22.7 ± 1.3^{a}	4.7 ± 0.1^{d}	18.9 ± 0.2^{b}	24.7 ± 0.3^{a}	5.6 ± 0.1^{d}
KaSiHexHexHex 4	6.3 ± 0.2^{a}	6.3 ± 0.5^{a}	4.6 ± 0.1^{b}	5.8 ± 0.2^{ab}	–	4.5 ± 0.3^{b}
KaHexHex	14.7 ± 0.1^{a}	11.9 ± 0.0^{b}	4.8 ± 0.0^{c}	11.6 ± 0.1^{b}	14.1 ± 0.5^{a}	5.0 ± 0.3^{c}
KaSiHexHex 1	3.9 ± 0.2^{d}	6.9 ± 0.1^{b}	3.2 ± 0.0^{e}	6.2 ± 0.1^{c}	8.2 ± 0.1^{a}	–
KaSiHexHexHex 2	3.0 ± 0.2^{c}	6.9 ± 0.2^{b}	–	6.4 ± 0.1^{b}	14.2 ± 0.1^{a}	–
KaHex	–	–	5.6±0.0^{a}	–	–	4.1±0.2^{b}
KaSiHexHexHex 3	13.5 ± 0.3^{b}	19.7 ± 0.2^{a}	–	13.6 ± 0.1^{b}	12.2 ± 0.2^{c}	–
KaSiHexHex 2	3.4 ± 0.2^{c}	4.1 ± 0.0^{c}	11.7 ± 0.3^{a}	3.4 ± 0.0^{c}	2.9 ± 0.0^{c}	9.6 ± 0.8^{b}
KaSiHexHex 3	–	–	5.1 ± 0.1^{a}	–	–	3.5 ± 0.0^{b}
Phytic acid, mg/100 g DM	**3697.1 ± 382.2^{ab}**	**3545.5 ± 719.0^{ab}**	**4453.2 ± 48.8^{a}**	**3903.4 ± 244.1^{ab}**	**2932.7 ± 76.5^{b}**	**418.0 ± 71.6^{c}**

* Abbreviation is given in Appendix A. Results were shown as means (± standard deviation) of duplicate analyses. Statistical differences were conducted based on one way-ANOVA and Tukey's post hoc test ($p < 0.05$), and shown with superscript letters a–e.

determination of glucosinolates from the CPC raw material. Thus, the determined glucosinolate content may be lower than the actual value. Yet, it is questionable whether active myrosinase is retained in the studied material. Some previous studies have revealed that high pressure can deactivate the enzyme, although the inactivation effect depends mostly on the source of myrosinase. In cabbage, brussels sprouts, and broccoli sprouts, inactivation of myrosinase started when pressure was increased up to 300 MPa (at 10 °C for 10 min), 350 MPa (30 °C, 6.5 min), and 400 MPa (30 °C, 3 min), respectively (Ghawi et al., 2012; Wang et al., 2018; Westphal et al., 2017). For mustard seeds, the enzyme deactivation required higher pressure. The enzyme was significantly deactivated when pressure was higher than 500 MPa (15 °C, 10 min) (Okunade et al., 2015). In this study, high levels of glucosinolates were found in the studied CPC samples. MS did not detect the presence of isothiocyanates, the breakdown products of glucosinolates by myrosinase, suggesting the absence of significant level of myrosinase activity in the CPC.

4.2. Enzymatic treatments of canola oil press cakes

Utilizing enzymes is a preferred method for recovering nutrients and bioactive compounds from agro-industrial side-streams. For valorization of CPC, the original idea of enzymatic treatment is to assist protein extraction (Baker & Charlton, 2020), but it is also able to enhance the extractability of other nutrients and the degradation of undesired compounds.

Pre-treatment of protein-rich plant matrices with various carbohydrases can efficiently increase protein extractability as reported by Rommi et al. (2014). Using pectinolytic enzymes can increase protein extractability up to 1.7-fold compared to treatments without enzymes by disintegrating the plant cell walls (Rommi et al., 2014). In the study, a carbohydrase (Viscozyme) with wide enzymatic activities was applied at relatively high dose (9% based on dry matter content of CPC raw material) and long incubation time (20 h). Yet, no improvement in protein extractability was detected in the carbohydrase-treated samples. This might be due to the nature of the studied CPC and its remained protein solubility during oil pressing, allowing to solubilize relatively high protein content already at alkaline conditions without any additional pre-treatments (Östbring et al., 2020). Minor improvement in protein solubilization was observed if phytase treatment was applied simultaneously with carbohydrase treatment. CPC is relatively high in phytic acid content, which can have strong interactions with proteins due to the high density of negatively charged phosphate groups, and thus affect protein solubility and bioavailability (Kies et al., 2006). Decreasing phytic acid content can lead to a shift in the protein solubility curve and thus increase the solubility at different pH values, especially at low pH (Kortekangas et al., 2020; Rosa-Sibakov et al., 2018). Aside from proteins, carbohydrase treatment influences sugar composition of rapeseed press residues. Harith et al. (2019) reported that the contents of glucose, galactose, arabinose, and xylose were significantly increased by treatment of rapeseed meals with Viscozyme (enzyme concentration was 1–15%, *v/v*) for 24 h (Harith et al., 2019). Similar increases were also observed in our study (Table 3). Applying Phyzyme in carbohydrase treatment enhanced the release of *myo*-inositol into the liquid fraction (Table 3), which was due to complete hydrolysis of phytic acid producing one molecule of inositol and six molecules of inorganic

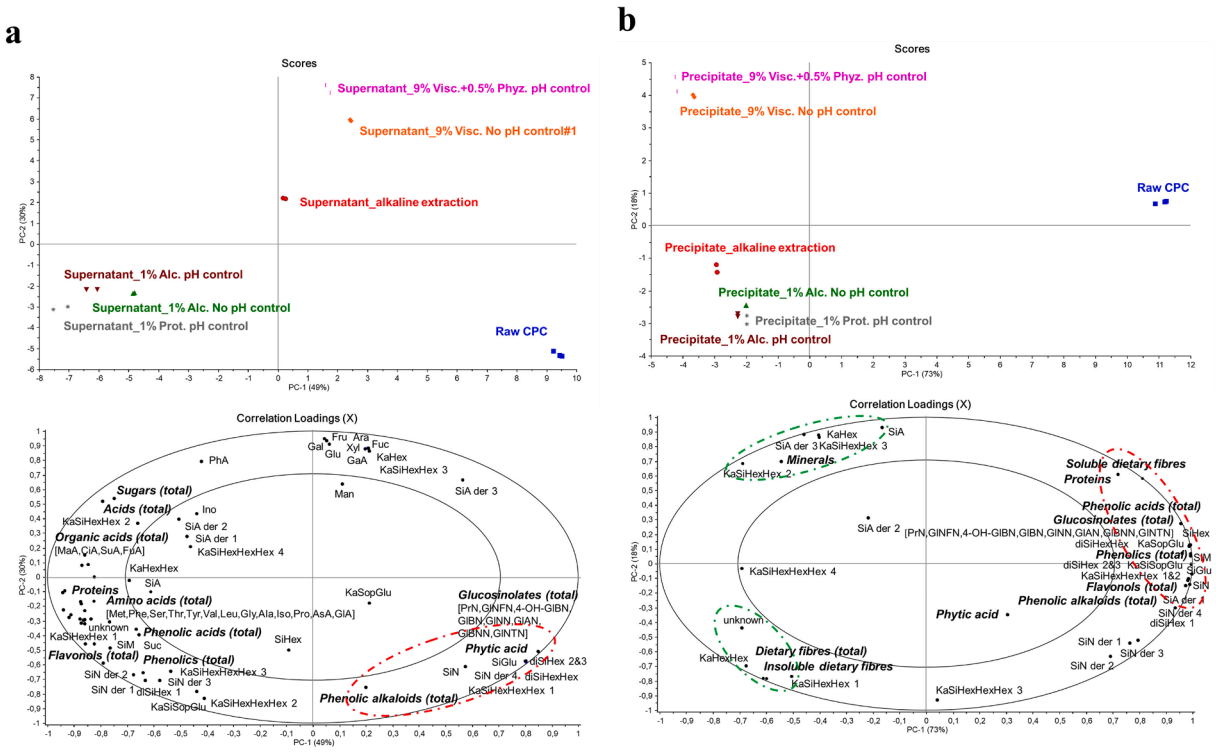


Fig. 1. PCA models for comparison between raw material and pre-treated CPC samples: a. raw CPC vs. supernatants of pre-treated CPC; b. raw CPC vs. precipitates of pretreated CPC. The abbreviation is given Appendix A.

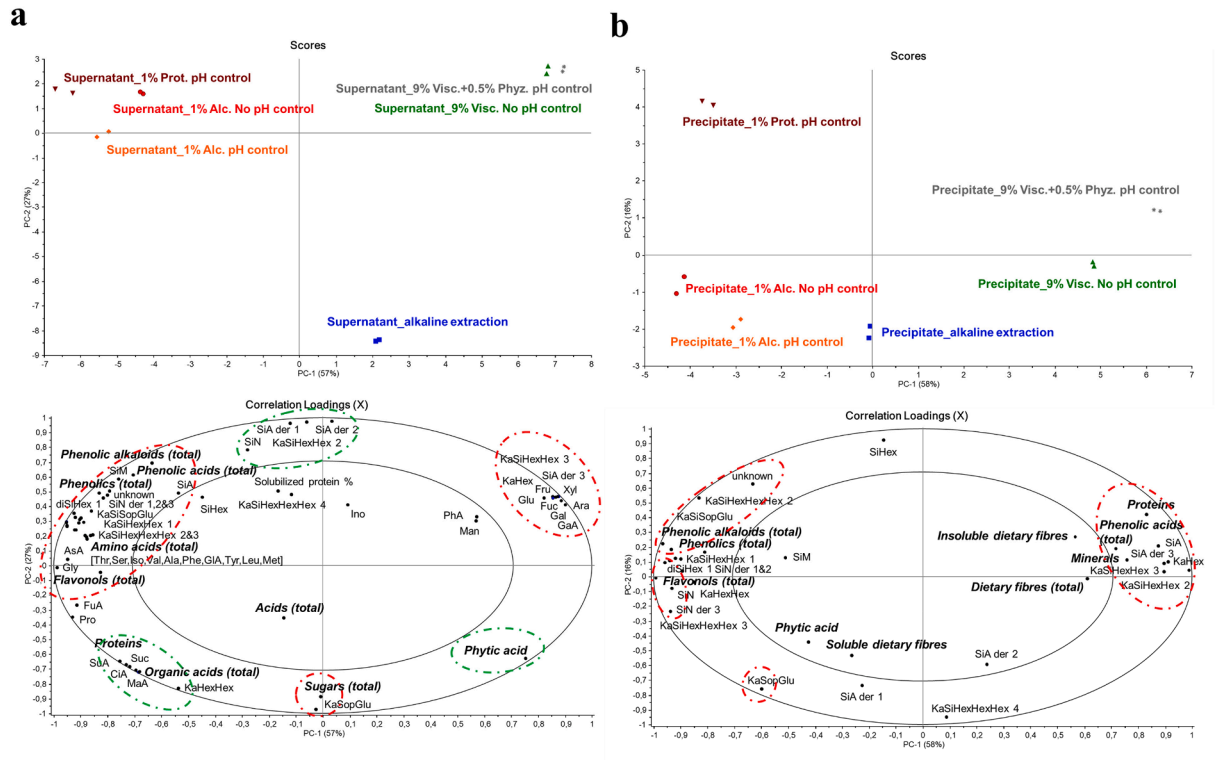


Fig. 2. PCA models for comparison among pre-treated CPC samples: a. supernatants of pre-treatments; b. precipitates of pre-treatments; c. supernatants and precipitates of enzymatic treatments; d. supernatants of Alcalase hydrolysis; e. precipitates of Alcalase hydrolysis. The abbreviation is given in Appendix A.

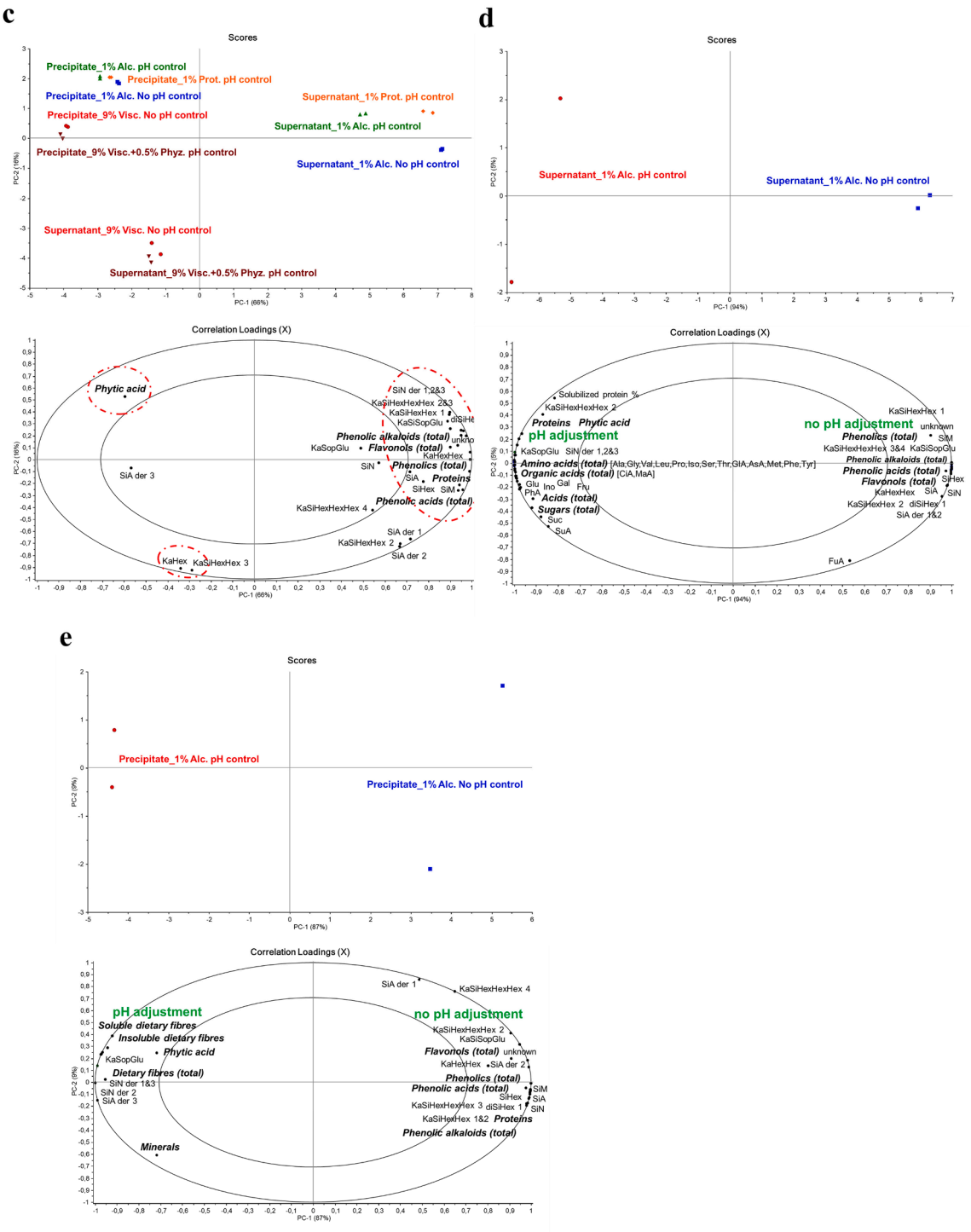


Fig. 2. (*continued*).

phosphate (Rao et al., 2009). In addition, Viscozyme (simultaneously with or without Phyzyme) treatments led to an increase in dry matter content of the supernatant fractions, which may indicate effective carbohydrate solubilization. Nevertheless, similar findings have not been reported by previous research.

Proteases are commonly applied for protein isolates to improve their techno-functional properties or increase their bioactivity (Alashi et al., 2013; Chabanon et al., 2007). Extensive studies have been performed for direct protein hydrolysate preparation from rapeseed meals (Niu et al., 2012; Rivera et al., 2015; Sari et al., 2013). In this study, two commercial enzymes, Alcalase and Protamex, with wide endopeptidase activities were used (Table 3, Table 4). The total nitrogen determination gave an overview of the total concentration of proteinaceous fraction (water-soluble peptides and free amino acids) in the supernatants (Table 3). Generally, the studied protease treatments had similar effect and more than 80% of crude protein was solubilized. This was in accordance with other similar studies where addition of proteases efficiently increased protein extraction yield (Niu et al., 2012; Sari et al., 2013). The free amino acid composition after protease treatments was also comparable to the most dominant amino acids (*e.g.*, GlA, AsA, or Leu) described in different canola isolates and hydrolyzates (Chabanon et al., 2007). It was also demonstrated that hydrolysis at constant pH

value increased the protein content and was important for the accumulation of free amino acids. This is most likely due to more favored reaction conditions for the enzymes where higher pH promotes hydrolysis and solubilization of proteins. Nevertheless, a higher degree of protein hydrolysis can negatively affect further emulsifying or foaming properties of the proteins (Chabanon et al., 2007), as well as decrease the anti-oxidative, anti-wrinkle, or anti-inflammatory properties of the peptides (Rivera et al., 2015). Thus, the most suitable reaction conditions should be chosen depending on the final aim.

For the undesired components, the common method of reducing these compounds is solid-state fermentation using a wide range of microorganisms, such as *Aspergillus niger*, *Bacillus* species (*subtilis*, *licheniformis*), *Candida utilis*, *Enterococcus faecalis*, *Lactobacillus* species (*acidophilus*, *delbrueckii*, *salivarius*), *Rhizopus oligosporus*, and *Saccharomyces cerevisiae*. Degradation of the anti-nutritional compounds was not ascribed to the various strains applied, but the enzymes synthesized by which, such as phytase, cellulase, xylanase, and glucanase (Lücke et al., 2019; Olukomaiya et al., 2019; Wang et al., 2019). Also, the growth of microorganisms inevitably causes nutrient loss in treated CPC samples. Therefore, directly employing the enzymes seems a better option than fermentation. Few studies have reported the effect of enzymatic treatment on reducing CPC anti-nutritional components. Chabanon et al. (2007) used Alcalase to remove major anti-nutritional compounds from rapeseed protein isolates (Chabanon et al., 2007). After Alcalase incubation, the content of total polyphenols was decreased to 0.2–0.3% of dry matters in isolates compared to raw material (1.3%), and phytic acid was not detected from final products.

As shown in both Table 3 and Table 4 of our study, phytase (Phyzyme) was confirmed as an effective way for reduction of phytic acid in different CPC fractions, as reported previously (Mohammadi et al., 2020). The treatment using carbohydrase (Viscozyme) or protease (Protamex and Alcalase) caused a significant reduction of glucosinolates compared to the CPC raw material. The glucosinolate content in both enzyme-hydrolyzed supernatants and precipitates was below the detection limit (Table 3 & 4). Yet, the contribution of the studied enzymes to glucosinolate degradation is questionable. This was due to the fact that no glucosinolates were found in supernatants or in precipitates of the alkaline extraction, where CPC was incubated at pH 9.5 and 50 °C for 30 min. Temperature and pH might be the major factors causing the degradation of glucosinolates in pre-treated samples. Again, myrosinase deactivation was not conducted before varying pre-treatments; however, no isothiocyanates were detected in either the supernatants or the precipitates of enzyme treatments. Therefore, it is not likely that this enzyme causes the absence of glucosinolates in the pre-treated samples. The omission of inactivating myrosinase was based on the concern that heating might have negative influence on certain nutrients and bioactive compounds of CPC. For examples, long-time heating could cause protein denaturation and deterioration, amino acid destruction, and reactions between proteins and non-protein components. For phenolic compounds, heating process might result in transformation of sinapine into lignane-type compounds (Dijkstra et al., 2003) as well as thermal degradation of flavonol glycosides and phenolic acid esters, which would influence the nutritional value and sensory properties of the final products of valorization.

Our results suggested that phenolic acids, phenolic alkaloids, and flavonol glycosides were present mainly in the supernatants. Certain supernatants of protease hydrolysis showed higher phenolic contents than the level in the raw material; however, phenolic compounds in most of the pre-treated samples remained at low levels. This is not comparable to previous research, since previous studies fail to provide phenolic composition of enzymatically treated CPC samples (Chabanon et al., 2007). It is also noticed that the phenolic contents in the most of previous studies were determined only by colorimetric method using Folin-Ciocalteu reagent (Ashayerizadeh et al., 2018; Chabanon et al., 2007; Lücke et al., 2019; Mohammadi et al., 2020), which were likely interfered by other compounds containing free hydroxyl groups (*e.g.*, reducing sugars).

It is still debatable to classify all phenolic compounds into undesired factors in CPC valorization, considering a large structural variation of phenolics. There is no doubt that tannins are major concern due to its ability of interfering protein digestion. Nevertheless, as primary phenolics present in CPC, sinapic acid (including its choline and glycoside derivatives) has also been reported as potent anti-allergic, anti-inflammatory, anti-atherogenic, anti-thrombotic, vasodilatory, anti-microbial, and anticarcinogenic effects (Chmielewska et al., 2020), which can be retained in CPC valorized products as functional ingredients. Hald *et al.* reported that kaempferol 3-*O*-(2-*O*-sinapoyl-*β*-sophoroside) was responsible for unpleasant taste of canola protein isolates (Hald et al., 2019). Nevertheless, for most of phenolic in CPC, the sensorial properties have not been determined yet. Moreover, this negative effect of phenolics is associated with their threshold concentration in products, and the presence of other components (such as free sugars) may reduce the negative impact of these compounds to some extent.

5. Conclusion

The study provided a new concept for valorization of canola oil press cake (CPC) instead of producing protein isolates only. Two types of crude products, protein-rich supernatants and fibre-rich precipitates were obtained after low-cost and efficient enzymatic pre-treatments. Aside from the dominant nutrients, the enzyme-treated fractions contained considerably high amounts of phenolic compounds but low levels of phytic acid and glucosinolates. These fractions showed potential to be used in certain food products to fortify nutrient level or to supply health-promoting properties. Further studies on the CPC crude extracts are needed to select proper food products and to develop new product concepts, as well as to study the consumer acceptance.

To our best knowledge, this is the first study systematically investigating the effects of carbohydrase, protease, and phytase on a wide range of nutrients, bioactive compounds, and anti-nutritional components in the CPC. Sugars, acids, amino acids, phenolic compounds, and glucosinolates in CPC samples were thoroughly investigated on molecular level, showing variation of these compounds to be highly dependent on the different enzyme treatments. This novel approach of bioprocessing for nutrient enhancement and simultaneous reduction of anti-nutritional factors also provides an important reference as an innovative process for valorizing other side-streams of food industry.

CRediT authorship contribution statement

Ye Tian: Methodology, Investigation, Writing – original draft, Visualization, Writing – review & editing. **Marie Kriisa:** Methodology, Investigation, Writing – original draft. **Maike Föste:** Conceptualization, Methodology, Investigation, Writing – original draft. **Mary-Liis Kütt:** Conceptualization, Writing – original draft. **Ying Zhou:** Formal analysis, Visualization. **Oskar Laaksonen:** Project administration, Writing – review & editing. **Baoru Yang:** Conceptualization, Supervision, 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.

Acknowledgements

We are grateful to MSc. Juuso Korpela, MSc. Mikael Fabritius, and PhD Annelie Damerau (University of Turku) for assistance in analyses of sugars, acids, amino acids, glucosinolates, and phenolic compounds. We appreciate Anastassia Taivolsalo (TFTAK) for the help with the CPC pretreatment experiments and analyses.

Funding

This research belongs to the project "Prowaste: Protein-fibre biorefinery for scattered material streams". The project was initiated by the ERA-NET Cofund FACCE SURPLUS (in the frame of the Joint Programming Initiative on Agriculture, Food Security and Climate Change (FACCE-JPI). FACCE SURPLUS has received funding from the European Union's Horizon 2020 research and innovation programme (under grant agreement No. 652615). The study of anti-nutritional compounds has been also funded by Turku University Foundation (identification No. 080888).

Appendix A. Abbreviation used

The abbreviation used in the article includes: **Alc.**, Alcalase; **Phyz.**, Phyzyme; **Prot.**, Protamex; **Visc.**,Viscozyme; **Ara**, arabinose; **Fuc**, fucose; **Xyl**, xylose; **Fru**, fructose; **Gal**, galactose; **Glu**, glucose; **Man**, mannitol; **GaA**, galacturonic acid; **Ino**, *myo*-inositol; **Suc**, sucrose; **PhA**, phosphoric acid; **SuA**, succinic acid; **MaA**, malic acid; **CiA**, citric acid; **FuA**, fumaric acid; **Ala**, alanine; **Gly**, glycine; **Val**, valine; **Leu**, leucine; **Pro**, proline; **Iso**, isoleucine; **Ser**, serine; **Thr**, threonine; **GlA**, glutamic acid; **AsA**, aspartic acid; **Met**, methionine; **Phe**, phenylalanine; **Tyr**, tyrosine; **PrN**, progoitrin; **GlNFN**, gluconapoleiferin; **GlNN**, gluconapin; **GlAN**, glucoalyssin; **4-OH-GlBN**, 4-hydroxy-glucobrassicin; **GlBNN**, glucobrassicanapin; **GlBN**, glucobrassicin; **GlNTN**, gluconasturtiin; **SiM**, sinapoyl malate; **SiA**, sinapic acid; **SiHex**, sinapoyl hexose; **SiGlu**, sinapoyl glucose; **SiA der**, sinapic acid derivative; **diSiHexHex**, disinapoyl hexose-hexose; **diSiHex**, disinapoyl hexose; **SiN**, sinapine; **SiN der**, sinapine derivative; **unknown**, unknown phenolic alkaloid; **KaSopGlu**, kaempferol 3-*O*-sophoroside-7-*O*-glucoside; **KaSiSopGlu**, kaempferol 3-*O*-(sinapoyl)-sophoroside-7-*O*-glucoside; **KaSiHexHexHex**, kaempferol-sinapoylhexoside-hexoside-hexoside; **KaHexHex**, kaempferol-hexoside-hexoside; **KaSiHexHex**, kaempferol-sinapoylhexoside-hexoside; **KaHex**, kaempferol-hexoside.

Appendix B. Supplementary data

The supplemental material for the article includes: Pre-treatment condition of CPC raw material (**Supplemental Table 1**); ESI-QTOF analytical condition of glucosinolate and phenolic analyses (**Supplemental Table 2**); External standards applied in quantification of glucosinolates and phenolics (**Supplemental Table 3**); Concentration of glucosinolates and phenolics in raw CPC extracted with different solvents (**Supplemental Table 4**); GC chromatograms of free sugars, acids, and amino acids in studied CPC samples (**Supplemental Fig. 1**); LC chromatogram of main compounds in raw material CPC and supernatants & precipitates of pre-treated CPC (**Supplemental Fig. 2**); Yields of glucosinolates and phenolics in raw CPC extracted by different solvents (**Supplemental Fig. 3**); and MS^2 spectra of the identified glucosinolates and phenolic compounds (**Supplemental Fig. 4.**). Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2022.132911.

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