

## **A comparison in protein extraction from four major crop residues in Europe using chemical and enzymatic processes-a review**

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1 **A comparison in protein extraction from four major crop residues in Europe using**  
2 **chemical and enzymatic processes**

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10

11 **Highlights**

12 Separation of crop waste into different constituents containing higher protein contents

13 High protein yields using protein protocols relying on organic solvents, alkalis and acids

14 Proteases appear to be effective in recovering high protein yields

15 Lower yields with carbohydrases but functionality likely to be retained

16 Mechanical pre-treatments can increase protein yields during an enzymatic approach

17

18 **Abstract**

19

20 The agricultural production of olives, rapeseed, tomatoes and citrus fruits within Europe is  
21 significant, resulting in a considerable amount of residual material. Rapeseed contains a high  
22 proportion of protein but the presence of anti-nutritional components, including glucosinolates,  
23 limits its use in food and feed applications. In contrast, the protein quantities associated with the  
24 other crop residues are much lower, although each of the residues could be separated into  
25 different constitutive parts where some have shown higher protein contents. . A variety of  
26 different enzymatic based approaches to deconstruct crop residues have shown to be effective in  
27 increasing the yields of protein recovered. These studies show that valorisation of selected crop  
28 components could form the basis of a crop biorefinery process to capture proteins and other  
29 potentially useful compounds.

30

### 31 **Keywords**

32 Protein; enzyme-assisted; olive; rapeseed; glucosinolate; Celluclast

33

### 34 **1. General Introduction**

35

36 Food waste in the EU is estimated to be 38% of the whole crop yields with the majority  
37 occurring during the processing stage and the EU directive has pledged more effort in developing  
38 strategies to recover higher value components, including proteins, fibres and bioactive molecules  
39 from agricultural waste (Anon, 2019). These bioactives may have potential applications in the  
40 food and pharmaceutical sectors as antimicrobials, anti-oxidants and natural colorants (Baiano,  
41 2014). Among the most commonly grown crops in Europe, excluding cereal crops, are olives,

42 rapeseeds, tomatoes, and citrus fruits, where each comprise the bulk of crop waste within each of  
43 their specific categories (Fig. 1). Within the olive industry two phase or three phase extraction  
44 of the oil is generally deployed, resulting in the formation of olive mill cake and olive mill waste  
45 water (Souilem et al., 2017). Smaller companies may also perform destoning in order to produce  
46 oils containing higher proportions of polyphenolics. The waste generated from the mills is  
47 disposed on land but this can have a serious impact requiring soil remediation (Doula et al.,  
48 2017). In contrast, rapeseed meal is often used to supplement ruminant feeds at 20-30%,  
49 providing 50% of the animals' protein requirements, although significant variations in total  
50 protein content may be caused by many different factors (Dale, 1996). Protein variability will  
51 affect the market price of this material as an animal feed, but the presence of anti-nutritional  
52 factors has affected its uptake in the wider animal nutrition sector, because glucosinolates and  
53 phytates can make it unpalatable (Dale, 1996) although some cultivars have been selectively  
54 breed which contain much lower quantities of anti-nutrients (Ghodsvali, Khodaparast, Vosoughi,  
55 & Diosady, 2005). Citrus waste may also be used as animal feed after being dried and pelletized,  
56 although the majority is discharged to landfill due to the high cost of drying (Negro, Mancini,  
57 Ruggeri & Fino, 2016). However, new EU legislation requires that some attempt must be made  
58 to valorise the waste before landfill disposal, which could include limonene extraction and the  
59 production of biofuels. Likewise, only a small proportion of tomato waste may be used as  
60 animal feeds or as organic fertilizer, but much of it is discarded as landfill waste due to the short  
61 shelf-life of the tomatoes of less than one week (Fritsch et al., 2017). Consequently, methods are  
62 underway to develop the recovery of a range of bioactives from tomato waste.

63

## 64 **2. Basis for Chemical Extraction**

65

66       The extraction of proteins from plants using alkali or acid to degrade the cellular structure is  
67 an established approach which that has led to further refinements in order to recover high yields  
68 of intact proteins from each particular plant species. After the proteins have been extracted from  
69 the cells, they are precipitated using a salting-out technique, with reagents such as ammonium  
70 sulphate or a dewatering solvent such as ethanol. It is evident from many of these studies that  
71 most of the protein present in the plant cells can be recovered using these methods, but the  
72 technical challenge is obtaining a representative diversity of all the proteins present in the plant  
73 matrix, using techniques such as gel electrophoresis under denaturing conditions to disentangle  
74 the protein chains. The tertiary structure of the proteins, which confers functional properties, is  
75 often disrupted during extraction and separation, reducing their potential applications in the food  
76 industry. Furthermore, if these proteins are in a new disordered secondary structure under  
77 neutral pH conditions, they are likely to exhibit reduced bioavailability, which negatively  
78 impacts their potential value in the animal feed or functional food sectors.

79

### 80 **3. Basis for Enzyme-assisted Extraction**

81

82       Protein extraction using a chemical approach can degrade not only the polysaccharide  
83 fraction in the plant matrix, but also the proteins being extracted, with the concomitant loss of  
84 functionality and bioavailability. Many enzymes show optimal activity ranging from weak acidic  
85 to weak alkaline conditions depending on the type of enzyme. Generally, the optimal activities  
86 of most carbohydrases occurs under low acidic conditions whereas most proteases occur under  
87 weak alkaline conditions. For example, the activity of carbohydrases on the plant cell walls of

88 olive pomace would result in the release of more enzymes, albeit different types such as  
89 lipoxygenase, which is the predominant form of protein in olive pomace (Montealegre et al.,  
90 2014). The majority of protein in olive seeds, rapeseed and tomato seeds are storage proteins  
91 enclosed within protein storage vacuoles (Gillespie et al., 2005; Montealegre et al., 2014; Nietzel  
92 et al., 2013), whereas a minor proportion are eleosins (protein membrane bodies) which enclose  
93 and facilitate translocation of oil across the membrane (Montealegre et al., 2014). The protein  
94 contained within seeds would be released by the selective activity of carbohydrases and  
95 pectinases in degrading plant cell walls (Rommi et al., 2014). Once released, proteases partially  
96 degrade the large molecular weight proteins into smaller soluble proteins. In contrast, most of  
97 the proteins found in tomato peels and oranges are most likely to be associated with carotenoids  
98 thereby contributing to colour formation (Vishnevetsky, Ovadis, & Vainstein, 1999).

99       Once the protein is released, enzymes can also limit the extent of complex formation of the  
100 extracted protein with other cell components such as carbohydrates and phytates under different  
101 physiological conditions (Serraino and Thompson, 1984; Zhan et al., 2019). However, the  
102 quantity of protein recovered using an enzyme-assisted process is often lower than with a  
103 comparable chemical process and many studies highlight this observation. Many reports  
104 described in this review use mechanical pre-treatment (e.g. sonication) alongside enzyme-  
105 assisted extraction to increase the yield. An in depth review describes many of these methods  
106 which include ultrasound, high pressure and microwave treatments (Nadar, Rao, & Rathod,  
107 2018). However, one current problem associated with commercial application using an  
108 enzymatic approach is the high prices of enzymes (Martínez-Maqueda et al., 2013) as well as  
109 some of the problems associated with the scale up caused by lower oxygen tension, difficulty in  
110 regulating the temperature and inconsistencies with nutrients as some will sediment (Puri,

111 Sharma, & Barrow, 2012). Nevertheless, as future developments continue, perhaps with the  
112 ability to reuse enzymes covalently linked to nanoparticles by magnetic capture methods, it is  
113 likely that enzyme costs will decrease.

114

## 115 **4. Olive Residues**

116

### 117 **4.1. Olive oil production and protein rich olive constituents**

118

119 The European market has the largest production of olive oil in the world where 10.4 million  
120 tonnes of olives are processed each year, yielding 2.3 million tonnes of olive pomace and an  
121 estimated 30 million m<sup>3</sup> olive mill waste-water (Fritsch et al., 2017). Consequently, 80% of the  
122 total mass of olives harvested, results in the production of waste pomace and waste-water (Fig. 2).  
123 Furthermore, 10% of additional olive waste is generated when leaves and twigs are accidentally  
124 collected in the olive mill and in-field during pruning of the branches from trees, which is required  
125 every two years (Niaounakis and Halvadakis, 2004).

126 The seeds located within the centre of olive stones are one particular fraction of olive waste  
127 containing the highest concentration of proteins and oils (Rodríguez et al., 2008). Currently, the  
128 olive stones, comprising 22% of the total dry biomass, are crushed to form meal cake, which is  
129 used as animal feed. The olive seeds form 4% of the total dry biomass of olives, and the protein  
130 content comprises all of the essential amino acids, making it a suitable supplement in the human  
131 diet and as an animal feed. The stones contain 3.2% protein (Rodríguez et al., 2008), but the  
132 majority of this is composed of a woody material (Bianchi, 2003), indicating that the protein  
133 content of the seed kernels to be 18%. It would be anticipated that most of this protein would be

134 globulins that would be stored in specialized organelles (Montealegre et al., 2014). In addition,  
135 fresh olive leaves, accounting for 10% of the total harvest weight, (Lafka, Lazou, Sinanoglou, &  
136 Lazos, 2013) contain 7.2% crude protein in undried leaves (Aydinglu & Sargin, 2013), which are  
137 most likely to be oleosins, proteins associated with the high oil content in the leaves. However,  
138 the high concentration of polyphenols in leaves could inhibit downstream protein recovery  
139 (Romero-García et al., 2014).

140

#### 141 4.2. Chemical Processing of Olive Leaves, Pomace and Stones

142

143 The separate recovery of proteins from olive pomace and milled olive stones can be achieved  
144 using a chloroform: methanol (2:1) solvent mixture (Montealegre, Marina, & García-Ruiz,  
145 2010). Usually, this method involves the recovery of lipids, but the association of lipids with  
146 proteins appeared selectively to assist in protein recovery. This protein isolation method was  
147 preferred to the conventional method that utilized detergents such as sodium dodecyl sulphate  
148 and 2-mercaptoethanol, because the solvents facilitated lipid extraction, which is detrimental to  
149 protein recovery. The use of two volumes of ice-cold acetone caused precipitation of protein,  
150 which co-incidentally also resulted in enzyme inactivation and maintaining polyphenol  
151 solubility. Analysis of recovered proteins from olive pomace using capillary electrophoresis  
152 indicated that seven of the major proteins were predominant throughout the different varieties of  
153 olive trees (Montealegre et al., 2012). Seeds were removed from the stones, milled under liquid  
154 nitrogen and then extracted using three separate extraction buffers, to yield a combined total of  
155 61 globular and histone proteins (Esteve et al., 2012). In the same study, 231 proteins were  
156 recovered from olive pulp showing diverse metabolic activities including proteins that induce

157 allergic responses. The focus of this research was to explore the complete diversity of proteins,  
158 especially those that were in minor proportions, rather than maximise protein recovery but the  
159 results did appear to show more intense protein bands on the SDS-PAGE gel using one particular  
160 buffer. It is possible that the presence of low EDTA concentrations in this buffer caused a  
161 disruption in the enzyme activity naturally associated with the olives, thereby leading to  
162 increased protein recovery. Furthermore, the use of different buffers did not appear to influence  
163 the protein profiles obtained on the SDS-PAGE gel.

164 A later review by the same authors recommended the use of Tris-HCl buffer along with the  
165 detergents SDS and 2-mercaptoethanol (Montealegre et al., 2014). The presence of  
166 mercaptoethanol acts to inhibit the nascent activity of proteases naturally present in the olives.  
167 The co-extracted polyphenols were removed by repeated washing with trichloroacetone, acetone  
168 and methanol. It was reported that the protein profiles were similar to those obtained using the  
169 phenol and SDS extraction protocol, which confirms the results found in another independent  
170 study showing that different extraction buffers had little influence on the protein profiles that  
171 were obtained. The seed proteins were extracted using buffered sucrose at pH 7.5 containing  
172 salts, coordination complexes, and ascorbic acid. The protein recovered as determined using the  
173 Bradford protein assay from whole olives ranged from 0.3 to 1.2 mg/g whereas 11 mg/g was  
174 associated with stones. These results indicate that the protein concentrations associated with  
175 olives are low, but considering that the majority of the stone is devoid of protein and that only  
176 the seeds contain high quantities, it would seem prudent to develop a process to recover the more  
177 pliable seed material and leave the stone material behind.

178 The development of a method to extract purified proteins from olive leaves involved finely  
179 grinding the leaves and repeatedly washing them in 10% trichloroacetone in acetone to remove

180 polyphenols (Wang et al., 2003). The release of 2.49 mg proteins/g biomass after using phenol  
181 and SDS on the washed leaf particles, were precipitated by centrifugation after the addition of  
182 methanol to the lower phenol phase. The washing steps in trichloroacetone ensured the extracted  
183 protein was free from contaminating polyphenols and could be easily resuspended. It is evident  
184 that trichloroacetone is a useful solvent in reducing the high the polyphenol content associated  
185 with olive and olive leaves.

186

#### 187 **4.3. Enzyme-assisted Processing of Olive Leaves, Pomace and Stones**

188

189 There is only one report describing an enzymatic approach to recover protein from olive  
190 leaves and method optimization revealed that the following conditions were necessary: 30%  
191 acetonitrile, 5% Celluclast 1.5 L, pH 5, 55°C for 15 min (Vergara-Barberán, Lerma-García,  
192 Herrero-Martínez & Simó-Alfonso, 2015). The success of extraction was evaluated by  
193 quantifying the total protein yield using the Bradford assay and the molecular weight of the  
194 proteins were examined using SDS-PAGE to reveal two different proteins, which were  
195 consistently expressed in different genetic varieties of olive trees in addition to other proteins.

196 An examination of different enzymes revealed that the most effective protein extraction from  
197 olive pomace was achieved using 5% lipase (Palatase 20000 L) for 15 min at 30°C with  
198 sonication, resulting in the recovery of just over 1 mg protein/ g dry biomass (Vergara-Barberán  
199 et al., 2014). Longer incubation times appeared to affect protein recovery negatively, perhaps  
200 due to the release of proteases that would be involved in protein degradation or the growth of  
201 attached microorganisms. It would appear that similar concentrations were obtained compared  
202 with a chemical non-enzyme based extraction protocol. Much higher quantities of protein were

203 obtained when 10 g olive pomace was treated with a protease, 80 mg Alcalase 2.4 L, in 100 ml  
204 water at pH 5 and at 50°C revealing that 0.4 g protein could be extracted, compared with 0.1 g  
205 protein extracted using the same treatment where no enzyme was used (Vioque et al., 2000).  
206 Analysis indicated that the soluble fibre content had increased perhaps indicating that there was  
207 some side polysaccharide activity.

208 Protein extraction from olive stones was examined in two different studies. In one study, the  
209 protein was solubilised using milled stones in Tris-HCl buffer containing NaCl, EDTA,  
210 dithiothrietirol and a protease inhibitor cocktail (Esteve et al., 2012). The proteins were purified  
211 using a ProteoMiner (BioRad), analysed on SDS-PAGE, and sequenced after trypsin digestion  
212 by mass spectrometry. This analysis resulted in the identification of 63 different proteins that  
213 were mostly globular. These results appear to be very similar to those obtained using a chemical  
214 approach. In the second study, cellulase (Celluclast 1.5L) or phospholipase (Lecitase Ultra) were  
215 most effective enzymes when using a 15 min digestion at 40°C with sonication, to obtain a  
216 protein concentration of 1 mg protein/g dry biomass as determined using the Bradford assay  
217 (Vergara-Barberán et al., 2014). It would appear that the quantity of proteins recovered using  
218 this enzyme assisted approach is ten-fold lower compared with the chemical approach.

219 The use of various physical treatments to increase protein recovery has been reported.  
220 These include voltage electrical discharge or ultrasonication, which was shown to significantly  
221 increase protein yield from olive kernels immersed in water at pH 7 by at least two-fold  
222 (Roselló-Soto et al., 2015). It was also found that the levels of extracted protein increased when  
223 using voltage electrical discharge at up to pH 12 or with increasing ethanol concentrations  
224 (>25%). However, polyphenols were also co-extracted with the proteins.

225 A comparison of the total quantities of protein associated with different components of the  
226 olive fruit revealed that leaves contained the highest levels followed by stones and finally  
227 pomace (Table 1). When each of these components were hydrolysed by different carbohydrases,  
228 a slightly higher quantity of proteins was recovered from the leaves compared with the pomace  
229 and stones. However, it would appear that a protease was more effective than the carbohydrases  
230 in recovering protein, albeit as smaller peptides.

231

## 232 **5. Rapeseed Residues**

233

### 234 **5.1. Rapeseed Pressing and Composition of Rapeseed Meal**

235

236 Rapeseed is an important crop for the production of vegetable oil (Canola) throughout  
237 Europe, with France, Germany, Poland and the United Kingdom the major producers. Rapeseed  
238 meal produced following the removal of the oil accounts for 80% of the waste generated from this  
239 crop (Fig. 2). Rapeseed contains a high protein content of ~34%, making it a useful supplement in  
240 animal feed (Lomascolo, Uzan-Boukhris, Sigoillot, & Fine, 2012), although a limitation is the  
241 high quantities of phenolics, which associate with the proteins to impart unusual flavours and  
242 may also act as anti-feeding agents (Alu'datt et al., 2017). Rapeseed press cake is the residual  
243 material left after defatting rapeseed by mechanical-extraction methods such as screw pressing.  
244 The application of mechanical pre-processing prepares the rapeseed material for downstream  
245 solvent extraction, which conventionally uses hexane. In addition, cold-pressing is used for the  
246 production of niche-market native rapeseed oils, with the residual material, cold-pressed press  
247 cake having an oil content of approximately 15–18%. Cold and hot pressing are performed at

248 60°C and 90°C, respectively, with a 10°C variation for both presses (Siger, Józefiak, Górnaś,  
249 2017). If an additional solvent extraction step is applied to further extract oil from the press  
250 cake, then rapeseed meal is obtained, which contains approximately 35–40% protein (based on  
251 nitrogen content using a conversion factor of 5.7 for Kjeldahl analysis) and 1–2% fat (Mosenthin  
252 et al., 2016). The majority of protein in rapeseed is composed of two globular storage proteins;  
253 mostly cruciferin and smaller quantities of napin. Cruciferins have good emulsifying properties  
254 whereas napins have good foaming properties that are considerably better than egg albumin  
255 (Rehder et al., 2017). These proteins are stored within protein bodies found throughout most  
256 types of cell in rapeseed (Rommi et al., 2014; Yiu, Poon, Fulcher, Altosaar, 1982).

257 The main limitation in using rapeseed protein for commercial applications, both in the food  
258 and non-food sectors, is the limited protein solubility in the press cake and press meal. One  
259 factor affecting solubility is the high temperature processes used during oil extraction, e.g. screw  
260 pressing, and downstream solvent extraction and removal, resulting in protein denaturation  
261 (Kemper, 2005). Despite the high levels of protein in both rapeseed cake and meal, other  
262 limitations for commercial applications are due to the high fibre content and the presence of  
263 residual anti-nutrients, in particular phytic acid, glucosinolates and phenolic compounds. The  
264 main technical challenge is efficient separation of the proteins from the other components such  
265 as carbohydrates, lignin, phenolics, and many current processes generate large volumes of  
266 effluent resulting in inefficient separation of the meal constituents.

267

## 268 5.2. Chemical Processing of Rapeseed and Rapeseed Meal

269

270 The majority of studies relating to the processing of rapeseed, have focused on optimising oil  
271 extraction in order to minimise the levels of non-lipids, using different mixtures of aqueous and  
272 non-hexane solvents. Consequently, the majority of non-lipid components remain in the  
273 rapeseed meal. One report (Citeau, Regis, Carré, & Fine 2018) investigated the influence on oil  
274 extraction efficiency and rapeseed meal quality, of using ethanol and isopropanol at various  
275 water concentrations. Rapeseed oil extraction was carried out using ethanol (up to 96 wt.%),  
276 isopropanol (up to 88 wt.%), using hexane as a reference solvent. The results indicated that  
277 hydroalcoholic extraction increased meal protein content by 13% compared to hexane extraction,  
278 but the type of alcohol and proportion of water had no significance on protein yields. Therefore,  
279 replacing hexane extraction with hydroalcoholic extraction would ensure that a higher proportion  
280 of protein remains with the rapeseed meal rather than some of the protein being extracted in the  
281 hexane.

282 In addition, there may be environmental benefits in using isopropanol and ethanol, rather  
283 than hexane, despite the difference in polarity, which effects oil selectivity and miscibility during  
284 extraction (Breil et al., 2017). A previous study demonstrated that the extraction of de-hulled  
285 rapeseed flour with 60% ethanol or isopropanol not only increased protein concentration from 53  
286 to 63 g / 100 g of de-oiled dry matter but also removed up to 97% of polyphenols and 99% of  
287 glucosinolates (Berot & Biffaud, 1983). Application of methanolic extraction, results in  
288 removal of phenolics, including d sinapic acid, which has potential applications in stabilising  
289 refined oils (Thiyam, Kuhlmann, Stöckmann & Schwarz, 2004).

290 The extraction of anti-nutritional factors from proteins is a technical challenge that needs to  
291 be addressed if material is required for use in food or animal feed applications. An early  
292 report of glucosinolate extraction from rapeseed, used aqueous and ethanolic mixtures to process

293 both the seed and meal, and although efficient, highlighted several disadvantages, including long  
294 (15 h) extraction periods, slow drying of the meal slurry and the dark appearance of the product  
295 (Kozłowska, Sosulski & Youngs, 1972). The ISO norm (1992) method is now the most common  
296 procedure for extracting glucosinolates from plant material, although the method requires  
297 modification to recover optimal yields from each particular plant material. A freeze-drying step,  
298 although not explicitly required in this method, prevents myrosinase mediated glucosinolate  
299 hydrolysis from occurring, which would normally occur during mechanical processing of leaf,  
300 stem or root tissues. Myrosinase, an enzyme found in Brassicaceae and compartmentalised in  
301 cells in close proximity to glucosinolates, is responsible for the hydrolysis of these glycosides  
302 during plant tissue disruption and freeze drying is used to remove water in order to prevent  
303 hydrolysis through thermal inhibition. Following freeze-drying, extraction is carried out at 75°C  
304 in 70% methanol for 10 min, in order to denature any residual myrosinase at the higher  
305 temperature. The extracted glucosinolates are then desulphated by ion exchange  
306 chromatography, separated and identified using HPLC. A simplified method for extracting  
307 glucosinolates from plant tissues, which does not require the use of a freeze drier or boiling  
308 methanol, and is therefore shorter, less hazardous and more cost effective, has been reported  
309 recently (Doheny-Adams et al., 2017). However, the use of isopropanol resulted in  
310 glucosinolates yields that were 49–73% lower in protein extracts compared with the use of other  
311 alcohols and the proportion of water present in the extraction mixture showed a correlation with  
312 glucosinolate yields.

313 An interesting alternative approach to reduce anti-nutritional factors such as tannins, phytate  
314 and enzyme inhibitors is the use of extrusion (Nikmaram et al., 2017). These particular  
315 compounds are high in seeds and nuts, although the effect of extrusion was dependent on

316 particular cultivars. A soaking pre-treatment of the biomass appears to increase the effectiveness  
317 of the extrusion process. Therefore, this method may not only result in the removal of anti-  
318 nutritional factors but also could also eliminate enzyme inhibitors leading to higher protein  
319 yields.

320 An alternative approach to extracting glucosinolates from rapeseed meal has been  
321 reported, which involved the chemical conversion of myrosinase to allyl isothiocyanate  
322 (Hetherington, Hoffmann, & Lindenbaum, 2018). Isothiocyanate was removed using volatile  
323 extraction that involved mild heat and negative pressure, resulting in glucosinolate levels that  
324 were 80% lower compared with the original starting material.

325 The extraction of cruciferin-rich protein from rapeseed meal was achieved at pH 2, using  
326 a patented procedure to collect three fractions, the rapeseed hulls, an insoluble protein fraction  
327 and a soluble protein fraction through a process of decanting and membrane filtration. The  
328 rapeseed meal contained an initial protein content of 27% and 22  $\mu\text{g}/\text{kg}$  glucosinolates, but  
329 following extraction the protein content in the insoluble and soluble fractions increased to 42%  
330 and to 58%, with a reduction in glucosinolate concentration to 1  $\mu\text{g}/\text{kg}$  and 3  $\mu\text{g}/\text{kg}$ ,  
331 respectively (Rehder et al., 2017).

332 Only one pilot scale protein extraction study has been described, that involved a two-  
333 stage aqueous washing extraction of dehulled rapeseed meal (Fauduet et al., 1995), using 15 kg  
334 of meal and 90 kg of deionised water, which was stirred for 30 min at 18°C and filtered, leaving  
335 material with a lower glucosinolate content of ~7%. Higher quantities of glucosinolates were  
336 removed with increasing temperatures. Limitations in scaling up this process in order to upgrade  
337 the rapeseed meal were lower yields with increasing biomass used and increasing incubation  
338 periods, but modifications were proposed, including use of a countercurrent extraction system.

339 The use of physical pre-processing to assist with recovery of protein enriched fractions from  
340 rapeseed was investigated by Laguna et al (2018). The impact of particle size on the efficiency  
341 of dry fractionation processes, including the use ultrafine milling and electrostatic sorting/ turbo  
342 separation was reported. The milling step was designed to release the rapeseed components from  
343 the cellular matrix, whilst the electrostatic separation was used to fractionate the protein without  
344 any loss of functionality. It was noted that although high purity protein fractions were obtained  
345 using this approach, four additional recycling steps were necessary in order to increase the final  
346 yield to 30%.

347

### 348 **5.3. Enzyme-assisted Processing of Rapeseed Meal**

349

350 Sari, Mulder, Sanders, & Bruins, (2015) reviewed details of different combined physical pre-  
351 treatment and enzymatic fractionation processes used to separate proteins for a range of biomass  
352 feedstocks, including rapeseed. This review highlighted the use of both proteases and  
353 carbohydrases that were applied to assist in protein extraction, with proteases aiding the  
354 fractionation process through proteolysis, while carbohydrases assisted by degrading component  
355 parts of the cell wall. It was noted that conventional alkaline extraction can be improved by  
356 protease addition, due to the reduction in protein size through proteolysis which facilitates easier  
357 extraction. In addition, the use of proteases can also be used to enable lower processing pH, thus  
358 avoiding the severe conditions that denature protein, with a resultant loss of potential  
359 functionality. The use of proteases was used in one study to improve the release of oil from  
360 dehulled ground rapeseeds by comparing five different proteases and it was reported that  
361 Alcalase 2.4L was the most effective (Meng et al., 2018). The rapeseeds were boiled in water for

362 15 min, extracted under alkaline conditions at pH 9, treated with 1.5% Alcalase 2.4L at pH 8.5,  
363 55°C for 4 h and then inactivated at 90°C. The extracted proteins after this treatment had become  
364 structurally disordered with a reduction in the proportion of  $\alpha$ -helix chains by 30%.

365 In a separate study, protein extraction from milled rapeseed, was examined using a variety of  
366 different Protex proteases. Higher reported protein extraction yields of 60-80% were obtained,  
367 using alkaline proteases rather than acidic proteases at 5% loading and pH ranging from 9.5-11,  
368 at 60°C and for 3 h (Sari, Bruins, & Sanders, 2013). Another study investigated protein  
369 extraction from pre-pressed (PPM) and cold-pressed rapeseed meal (CPM) under different  
370 parameters, that included variations in the solid to liquid ratio, extraction time, temperature, pH  
371 value, the number of extraction cycles and the employment of a protease- Protease A-01  
372 (Subtilisin, EC 3.4.21.62) (Fetzer et al., 2018). The highest protein yields achieved were 60.6%  
373 from PPM and 59.5% from CPM using protease activity in the presence of strong alkaline  
374 conditions, pH 11-12, during a single step process. In a triple washing-step process, 78.3% and  
375 80.7% was recovered from PPM and CPM, respectively.

376 In another report highlighting the use of proteases to increase protein recovery, casein was  
377 immobilized onto the surface of magnetic nanoparticles, resulting in the hydrolysis of 47% of the  
378 protein into amino acid and oligopeptides (Jin et al., 2010). However, the hydrolysis of rapeseed  
379 meal using these nanoparticles indicated that only 10% of the total amount present was  
380 hydrolysed, although this occurred at similar rate to the free enzyme. The advantage of using this  
381 system was that the protease retained activity up to 60 days at 4°C and could be easily recycled.  
382 In another variation, the protease was used after the proteins had been recovered by alkaline  
383 extraction, with the aim of increasing the purity of the extracted protein. High purity protein  
384 (92%) was obtained from ground rapeseed meal after washing with ethanol, extracting in an

385 alkaline NaCl solution and collecting the fraction <10k Da by ultrafiltration (Zinchenko et al.,  
386 2018). The proteins were successfully degraded into amino acids and oligopeptides when the  
387 extracted proteins were incubated with protosubtilin at a ratio of 20:1.

388 The use of carbohydrases to degrade cell wall components that retain the protein, rather than  
389 extracting the proteins directly from the plant substrate is an alternative approach. The highest  
390 yield of proteins of 50 mg/ g meal was obtained when phenolic acids and proteins were  
391 recovered in a sequential reaction. This involved the addition of sodium hydroxide and methanol  
392 to form phenolic acid esters, which were evaporated and the protein was extracted under alkaline  
393 conditions followed by precipitation under acidic conditions (Li & Guo, 2017). Cellic Ctec3 was  
394 used at a later stage to purify the extracted protein when incubated at 50°C. The recovery from  
395 rapeseed meal of sinapine (the ester form of sinapic acid – a dominant phenolic acid) was 7 mg/  
396 g and of protein was 0.5 g/ g. This protein had an enrichment content of 77%. In another study,  
397 the effect of carbohydrases on rapeseeds were determined by fluorescence microscopy when  
398 stained with Calcofluor to view remaining glucans and with Acid Fuchsin to view the protein  
399 distribution (Rommi et al., 2014). In addition, pectins were examined by microscopy after  
400 staining with Ruthenium red. Pectinex Ultra SP-1 showed the highest activity compared with  
401 Celluclast 1.5 L and Depol 740L, resulting in the complete disintegration of the cell walls, which  
402 contained the protein and the release of protein bound to pectins. Higher levels of protein were  
403 recovered from the dehulled seeds compared to the intact material, and SDS-PAGE revealed that  
404 napins were present at higher concentrations in protein extracts from the dehulled seeds. High  
405 yields of proteins can be recovered from cold pressed rapeseed meal using alkaline methods but  
406 these require large volumes of water and it was found using Pectinex Ultra SP-L resulted in  
407 higher yields under low moisture conditions (Rommi et al., 2015). It was also determined that

408 particle size had no effect on the protein recovery but enzyme activity increased protein recovery  
409 by 29-42% when extracted at 20% solid content.

410 There are a few reports citing the combined use of carbohydrases and proteinases to improve  
411 the extraction of protein of oil from rapeseeds. In one such study, a multi-enzyme approach using  
412 pectinase/ cellulase/ betaglucanase, Alcalase 2.4L, at pH 5-10, and a temperature range of 48-  
413 60°C, yielded 41-67% protein depending on the hydrolysis time (Zhang, Wang, & Xu, 2007a).  
414 Sari, Mulder, Sanders, & Bruins (2015) concluded that the application of carbohydrases, as part  
415 of the hydrolysis process, does not appear to result in increased yields of extracted protein,  
416 although their use may have a positive impact on protein extraction in a different way. Their  
417 capability to degrade the cell wall can be used to release components that otherwise buffer the  
418 reaction mixture, which would result in lower alkali consumption during subsequent protein  
419 extraction and a reduction in process costs. However, another study reported the sequential use  
420 of carbohydrases and proteases to successfully isolate a protein fraction from rapeseed. This  
421 study examined the effect of 2.5% pectinase, cellulase and  $\beta$ -glucanase at the optimised ratio of  
422 4:1:1 on wet, milled dehulled rapeseeds for 4 h (Zhang, Wang, & Xu, 2007b). This was followed  
423 with alkaline extraction at 60°C for 1 h and 200 rpm, and then protease treatment, Alcalase 2.4L,  
424 by adjusting to pH 9 at 60°C and 50 rpm at enzyme concentrations ranging from 0.5% to 1.5%  
425 and solid to liquid ratio ranging from 1:3 to 1:8. It was found that the optimum conditions were  
426 1.25-1.5% Alcalase 2.4L at 50°C for 3 h to recover about 80% of the protein with a molecular  
427 weight of less than 1500. The proteins were analysed after centrifugation by collecting the liquid  
428 fraction between the remaining seed pellet and extracted oil forming an upper surface layer.

429 Another approach that overcomes the anti-nutritional factors associated with rapeseed meal  
430 involves the use of 0.8 U/g of phytase at 55°C, pH 5, which reduced phytic acid content by 25%

431 phytase (Rodrigues, Carvalho, & Rocha, 2017). The protein yield obtained, as determined by  
432 Bradford assay, was optimum at 75°C, under alkaline conditions at pH 12.5, and then re-  
433 precipitating at pH 4. The phytic acid contents of rapeseed meal, defatted rapeseed meal and  
434 protein extract were 14 g/ kg, 10 g/ kg and 1 g/ kg, respectively.

435 A much higher quantity of protein was associated with rapeseed compared with other crops,  
436 especially with the cold pressed rapeseed meal, which contains lower quantity of oil and is  
437 pressed under low temperatures that would limit protein denaturation (Table 1). Studies where  
438 carbohydrases were deployed do not appear to describe the protein yields, except the study by Li  
439 & Guo (2017), where the enzyme was used after alkaline extraction in order to remove co-  
440 extracted carbohydrates. It would appear that proteases were very effective in recovering the  
441 majority of protein from rapeseed meal.

442

## 443 **6. Tomato Residues**

444

### 445 **6.1. Production and Tomato Constituents**

446

447 Tomato farming occurs throughout Europe, generating about 17% of waste. This is lower  
448 compared with the levels of waste generated from the production of olives and rapeseed (Fig. 2),  
449 although larger quantities of waste accumulate in Italy and Spain reflecting the larger extent of  
450 tomato farming in these countries. The dietary fibre associated with tomato waste is the most  
451 important constituent, forming 80% of the biomass, which is recovered using a patented process  
452 where the peels are ground after being separated from the seeds and then dried (Herrera,  
453 Sánchez-Mata, & Cámara, 2010). The proportions of total dry biomass and seeds in tomato

454 waste account for 15.8% and 3.5%, respectively (Zuorro, Lavecchia, Medici, & Piga, 2014).  
455 Most of the protein is associated with the tomato seeds at 35-40% and this protein contains most  
456 of the essential amino acids, except tryptophan that was present at lower amounts (Sarkar &  
457 Kaul, 2014; Zuorro, Lavecchia, Medici, & Piga, 2014). Globulins, storage proteins, comprise  
458 70% of the total proteins in tomatoes (Sogi, Arora, Garg, Bawa, 2002a), which are most probably  
459 associated with the seeds. Another study reported that while tomato seeds have a quite high  
460 protein content, the predominant amino acids present were those with lower levels of  
461 digestibility e.g. arginine and asparagine (Persia, Parsons, Schang, & Azcona, 2003). Feeding  
462 experiments to chicks revealed that tomato seeds could substitute soyabean meal, although the  
463 weight gain of the chicks was lower, but higher compared with using a non-nitrogen feed. The  
464 same study also revealed that the tomato seed composition showed disparity between different  
465 cultivars but did show consistency within different samples collected from the same farm (Persia,  
466 Parsons, Schang, & Azcona, 2003). The tomato seeds have quite a high content of anti-  
467 nutritional factors in the form of phytate (26 µg/g) and trypsin inhibitors (12.5 U/mg), but these  
468 inhibitors can readily be reduced >80% with the removal of the bran from the seed to recover  
469 protein (Sarkar & Kaul, 2014).

470

## 471 6.2. Chemical Processing of Tomato Seeds

472

473 The majority of studies have focused on protein recovery from tomato seeds and a number of  
474 similar methods have been described using alkaline extraction. In one of the first described  
475 methods using standard alkaline processing, proteins were extracted from different fractions of  
476 tomato waste that was solubilised at pH 8, pressed and then the pH was sequentially reduced to

477 pH 4.8, pH 4 and finally pH 3.5, in order to collect different protein concentrates as the proteins  
478 precipitated (Kramer & Kwee, 1977). The proportion of soluble protein increased from 35% to  
479 56% as the pH changed from pH 4.5 to pH 3.5. A further development in another study,  
480 examined the purity level of proteins recovered when the proteins were precipitated at pH 3.9  
481 (Liadakis, Tzia, Oreopoulou, & Thomopoulos, 1995). In this study, proteins were extracted from  
482 tomato seed meal using water at a liquid to solid ratio of 30:1 at 50°C and pH 11.5 for 20 min.  
483 The solids were removed using centrifugation, the pH of the supernatant was adjusted to 3.9, and  
484 the precipitated proteins were collected by centrifugation. The final product after vacuum drying  
485 contained 72% protein. In another shorter method using weak alkaline conditions, the tomato  
486 seeds were separated from the skins using sedimentation which were then subjected to sodium  
487 hydroxide treatment for 5 min, centrifuged and the supernatant was adjusted to pH 7.5  
488 (Savadkoohi & Farahnaky, 2012). The tomato seed protein was centrifuged and structural  
489 chemical analysis revealed that the globular protein exhibited weak gelling properties. In another  
490 method, the use of different solvents for extraction was investigated after using hexane to  
491 remove oil from the tomato seed meal and recovering the proteins using alkaline conditions with  
492 1.2% sodium hydroxide (Sogi, Arora, Garg, & Bawa, 2002a). Extraction with water, ethanol or  
493 acetic acid resulted in the recovery of different molecular weight proteins under each of the  
494 extraction strategies ranging from 67-310 kDa.

495 The emulsifying properties of the extracted proteins were evaluated to determine their  
496 potential functionality for applications as food ingredients. In one of these studies, the emulsion  
497 properties were evaluated against water and peanut oil, after the proteins were extracted from  
498 sedimented, hammer-milled tomato seeds, using 1% NaOH at ambient temperature for 10 min  
499 (Sogi, Garg, & Bawa, 2002b). The protein concentrates and isolates from the seed meal showed

500 improved emulsifying properties and much lower absorption of water compared with peanut oil.  
501 In another study, the emulsifying properties of the proteins extracted from tomato seed protein  
502 were found to be stable in high sodium chloride concentrations and thermally stable to 80°C,  
503 whereupon the proteins aggregated and were stable within the pH range 6-8 (Sarkar,  
504 Kamaruddin, Bentley, & Wang, 2016). The proteins were extracted from hammer-milled seeds,  
505 soaked for 1 h in 1 M sodium chloride at 50°C, adjusting to pH 8 with sodium hydroxide,  
506 centrifuging to remove non-proteins and then readjusting the pH to 3.5 and centrifuging the  
507 proteins.

508 Tomato waste was pulped and the seeds were separated from the peels by sedimentation  
509 (Sarkar & Kaul, 2014). The seeds were then hexane extracted to lower the fat content and the  
510 seed protein was extracted using 1 M NaCl, which was maintained at pH 8 for 1 h at 50°C. The  
511 remaining biomass was centrifuged and the extracted protein was precipitated with the addition  
512 of HCl to form a protein isolate of 92%.

513

### 514 **6.3. Enzyme-assisted Processing of Tomatoes and Tomato Seeds**

515

516 The ripening of tomatoes may provide some guidance as to the type of enzymes that could be  
517 involved in softening the fruit. Tomassen, Barrett, van der Valk, & Woltering (2007) described  
518 an activating enzyme that was found to modify a pectin-degrading enzyme, polygalacturonase,  
519 into an active isoenzyme state. The protein was recovered from ripe tomatoes after gentle heating  
520 of the extracted enzyme to separate the combined enzymes.

521 Only one study has been reported involving the enzyme mediated extraction of proteins from  
522 either whole tomatoes or a specific component of tomatoes, although the use of enzymes have

523 been successfully employed in the recovery of other carotenoids and lycopene. The extraction of  
524 umami acids from defatted tomato seed meal was achieved using papain and it was found that at  
525 pH 3, high enzyme activity and a long incubation period of 5 h resulted in extract containing  
526 86% of protein (Zhang et al., 2015). The tomato seeds were milled and the resultant particle size  
527 was 0.43- 0.85 mm. About 50% of the protein was extracted from this material, but decreasing  
528 the particle size further to <0.25 mm resulted in an increase in yield to 90%.

529 The tomato seeds appear to contain a high protein content although there is some variation  
530 depending on the cultivar being assessed (Table 1). It would appear that all of the protein was  
531 recovered from the seeds when a protease was used.

532

## 533 **7. Citrus Residues**

534

### 535 **7.1. Production and Citrus Constituents**

536

537 The cultivation of citrus fruits, comprising mostly of oranges, tangerines, lemons, limes and  
538 grapefruit, occurs only in southern Europe. It is estimated that 3.2 million tonnes are deemed  
539 unsuitable and processing of these fruits generates a significant proportion of waste products after  
540 juicing, which is composed of the peel, pulp, rag and seeds at 1.6 million tonnes (Fig. 2). The  
541 waste could be useful in bioethanol production especially considering the high cellulosic content  
542 (cellulose and hemicelluloses), with a particularly low lignin content that can vary from being  
543 undetectable to 7.5% in orange peels (Mamma & Christakopoulos, 2014). Citrus waste has a  
544 low protein content, between 6.6-9.1% in both the peels and pulp, and proposals have suggested  
545 increasing the protein content by using microbial fermentation to form single cell protein.

546

## 547 **7.2. Chemical Processing of Citrus**

548

549 An extrusion process was developed whereby equal proportions of whey proteins and citrus  
550 pectins were covalently linked to produce compounds that showed improved emulsifying  
551 properties at 120-140°C (Koch, Emin, & Schuchmann, 2017). It was determined that during  
552 heat treatment of these whey proteins, their solubility decreased, whereas viscosity increased due  
553 to the increase in molecular weight of the protein-polysaccharide conjugants and then gradually  
554 decreased due the degradation of polysaccharides. The emulsifying properties as determined by  
555 smaller droplet sizes improved after 2 min of extrusion at 140°C , but longer incubation times  
556 did not result in any further improvements. The soluble protein content of citrus juices  
557 originating from the flavedo (the outer orange coloured peel) showed a significant decrease with  
558 increasing temperatures up to 100°C, resulting in insoluble precipitates causing increased  
559 cloudiness of the fruit juice (Shomer, 1991). However, protein insolubility was also influenced  
560 by enzymatic degradation of pectins at pH 4.5 into neutral sugars and galacturonic acid. It was  
561 found that the protein coagulants particularly contained arabinose and galacturonic acid.

562

## 563 **7.3. Enzyme-assisted Processing of Citrus Pulp and Peel**

564

565 An enzymatic extraction of orange peel using a buffer containing Celluclast 1.5L from  
566 different cultivars resulted in the recovery of 5.45 mg proteins/ g peel, as determined by the  
567 Bradford assay (Vergara-Barberán et al., 2017). Protein separation was achieved on the basis of  
568 molecular weight, using capillary gel electrophoresis and assigned to particular roles based on

569 previous published research. Many of the 14 common proteins were either allergens or enzymes,  
570 while other proteins were unique to particular cultivars.

571 Only one study investigated protein extraction from citrus pulp, which revealed that Palatase  
572 20,000 L was more effective, albeit at lower yields, in recovering 1.7 mg protein/ g pulp  
573 (Vergara-Barberán et al., 2017). The results obtained were similar to those found using citrus  
574 peels where eight of the proteins were common within citrus fruits while other proteins were  
575 unique to particular cultivars.

576 The quantity of protein associated with the citrus peels is comparable to the quantity that can  
577 be recovered from the olive leaves (Table 1). It would be expected that carbohydrases would be  
578 effective in releasing protein from the peels, considering the high cellulose content of the peels.  
579 However, only a small proportion of the protein was recovered and no study has appeared to  
580 determine whether higher quantity of protein could be recovered using a protease.

581

## 582 **8. Conclusions**

583

584 It is apparent that the optimum recovery of proteins from each of these crop residues using  
585 the chemical methods rely on organic solvents, alkalis or acids, which may be environmentally  
586 hazardous. In contrast, there are many studies showing the development of methods to  
587 incorporate an enzymatic approach to recovering proteins from different components of crop  
588 residues. At this stage, it would appear that proteases operating under low alkaline conditions  
589 are more effective than carbohydrases in recovering plant protein, although the hydrolytic  
590 activity of proteases results in the generation of low molecular weight peptides. It is most likely  
591 that intact functional proteins would be recovered using carbohydrases and that these intact

592 proteins would be useful in human and animal feed, because of the potential to impart additional  
593 functionality through partial hydrolysis. Nevertheless, it is clear that methods are being  
594 developed for the recovery of protein using enzymatic assisted extraction and this approach is  
595 being investigated using a wide range of agri-food residues.

596

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598

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602

## 603 **References**

604

605 Anon. (2019) The fight against food waste: Where are we now? Questions and Answers.

606 European Commission Memo.

607 [https://ec.europa.eu/food/sites/food/files/safety/docs/fs\\_eu-actions\\_fwm\\_qa-fight-food-](https://ec.europa.eu/food/sites/food/files/safety/docs/fs_eu-actions_fwm_qa-fight-food-waste.pdf)  
608 [waste.pdf](https://ec.europa.eu/food/sites/food/files/safety/docs/fs_eu-actions_fwm_qa-fight-food-waste.pdf)

609 Alu'datt, M. H., Rababah, T., Alhamad, M. N., Al-Mahasneh, M. A., Almajwal, A., Gammoh,  
610 S., & Alli, I. (2017). A review of phenolic compounds in oil-bearing plants: Distribution,  
611 identification and occurrence of phenolic compounds. *Food Chemistry*, 218(1), 99-106.

612 Aydınoglu, T., & Sargin, S. (2013). Production of laccase from *Trametes versicolor* by solid-  
613 state fermentation using olive leaves as a phenolic substrate. *Bioprocess and biosystems*  
614 *engineering*, 36(2), 215-222.

615 Baiano, A. (2014). Recovery of biomolecules from food wastes—a review. *Molecules*, 19(9).

616 Bianchi, G. (2003). Lipids and phenols in table olives. *European Journal of Lipid Science and*  
617 *Technology*, 105(5), 229-242.

618 Berot, S., & Briffaud. J. (1983). Parameters for obtaining concentrates from rapeseed and  
619 sunflower meal. *Plant Foods for Human Nutrition* 33(2-3), 237–242.

620 Breil C, Vian M, Zemb T, Kunz, W., & Chemat F. (2017). “Bligh and Dyer” and Folch methods  
621 for solid-liquid-liquid extraction of lipids from microorganisms. Comprehension of  
622 solvation mechanisms and towards substitution with alternative solvents. *International*  
623 *Journal of Molecular Sciences*. 18(4), 708.

624 Citeau, M., Regis, J., Carré, P., & Fine, F. (2018). Value of hydroalcoholic treatment of rapeseed  
625 for oil extraction and protein enrichment. *Oilseeds and fats, Crops and Lipids*.  
626 <https://doi.org/10.1051/ocl/2018035>.

627 Dale, N. (1996). Variation in feed ingredient quality: oilseed meals. *Animal Feed Science and*  
628 *Technology*, 59(1-3), 129-135.

629 Doheny-Adams, T., Redeker, K., Kittipol, V., Bancroft, I. & Hartley, S.E. (2017). Development  
630 of an efficient glucosinolate extraction method. *Plant Methods* 13(1), 17.

631 Esteve, C., D'Amato, A., Marina, M. L., García, M. C., Citterio, A., & Righetti, P. G. (2012).  
632 Identification of olive (*Olea europaea*) seed and pulp proteins by nLC-MS/MS via  
633 combinatorial peptide ligand libraries. *Journal of Proteomics*, 75(8), 2396-2403.

634 Fauduet, H., Coic, J. P., Lessire, M., Quinsac, A., Ribailier, D., & Rollin, P. (1995). Rapeseed  
635 meal upgrading—pilot scale preparation of rapeseed meal materials with high or low  
636 glucosinolate contents. *Animal Feed Science and Technology*, 56(1-2), 99-109.

637 Ferreira-Leitao, V., Gottschalk, L. M. F., Ferrara, M. A., Nepomuceno, A. L., Molinari, H. B. C.,  
638 & Bon, E. P. (2010). Biomass residues in Brazil: availability and potential uses. *Waste*  
639 *and Biomass Valorization*, 1(1), 65-76.

640 Fetzer, A., Herfellner, T., Stäbler, A., Menner, M., & Eisner, P. (2018). Influence of process  
641 conditions during aqueous protein extraction upon yield from pre-pressed and cold-  
642 pressed rapeseed press cake. *Industrial Crops and Products*. 112, 236-246.

643 Fritsch, C., Staebler, A., Happel, A., Cubero Márquez, M. A., Aguiló-Aguayo, I., Abadias, M., &  
644 Suárez-Estrella, F. (2017). Processing, valorization and application of bio-waste derived  
645 compounds from potato, tomato, olive and cereals: a review. *Sustainability* 9(8), 1492.

646 Ghodsvali, A., Khodaparast, M. H., Vosoughi, M., & Diosady, L. L. (2005). Preparation of  
647 canola protein materials using membrane technology and evaluation of meals functional  
648 properties. *Food Research International*, 38(2), 223-231.

649 Gillespie, J., Rogers, S. W., Deery, M., Dupree, P., & Rogers, J. C. (2005). A unique family of  
650 proteins associated with internalized membranes in protein storage vacuoles of the  
651 Brassicaceae. *The Plant Journal*, 41(3), 429-441.

652 Herrera, P. G., Sánchez-Mata, M. C., & Cámara, M. (2010). Nutritional characterization of  
653 tomato fiber as a useful ingredient for food industry. *Innovative Food Science and*  
654 *Emerging Technologies*, 11(4), 707-711.

655 Hetherington, M., Hoffmann, T., & Lindenbaum, M. (2018). Method for removing  
656 glucosinolates from oilseed meals. United States Patent Application, Publication number:  
657 US 2018 / 0042266 A1, Pub. Date: Feb. 15, 2018.

658 ISO norm. (1992). Rapeseed–Determination of glucosinolates content–Part 1: Method using  
659 high-performance liquid chromatography. *ISO 9167-1*, 1-9.

660 Kemper, T.G. (2005). Oil Extraction, Bailey's Industrial Oil and Fat Products. John Wiley Sons  
661 Inc. Chapter 2, pp 57-98.

662 Kozłowska, H., Sosulski F.W., & Youngs, C.G. (1972). Extraction of Glucosinolates from  
663 Rapeseed. *Canadian Institute of Food Science and Technology Journal*, 5(3), 149-154.

664 Kramer, A., & Kwee, W. H. (1977). Functional and nutritional properties of tomato protein  
665 concentrates. *Journal of Food Science*, 42(1), 207-211

666 Jin, X., Li, J. F., Huang, P. Y., Dong, X. Y., Guo, L. L., Yang, L., & Chen, H. (2010).  
667 Immobilized protease on the magnetic nanoparticles used for the hydrolysis of rapeseed  
668 meals. *Journal of Magnetism and Magnetic Materials*, 322(14), 2031-2037.

669 Koch, L., Emin, M. A., & Schuchmann, H. P. (2017). Influence of processing conditions on the  
670 formation of whey protein-citrus pectin conjugates in extrusion. *Journal of Food*  
671 *Engineering*, 193, 1-9.

672 Laguna, O., Barakat, A., Alhamada, H., Durand, E., Baréa, B., Fine, F., Villeneuve, P. Citeau,  
673 M., Dauguet, S., & Lecomte, J. (2018). Production of proteins and phenolic compounds  
674 enriched fractions from rapeseed and sunflower meals by dry fractionation processes.  
675 *Industrial Crops and Products*, 118, 160-172.

676 Lafka, T-I., Lazou, A.E., Sinanoglou, V.J., & Lazos, E.S. (2013). Phenolic Extracts from Wild  
677 Olive Leaves and Their Potential as Edible Oils Antioxidants. *Foods*, 2(1), 18-31.

678 Li, J., & Guo, Z. (2017). Complete Utilization of Rapeseed Meal to Produce Lipophilic  
679 Antioxidants, Protein, and Monosugars in a Concordant Manner. *ACS Sustainable*  
680 *Chemistry & Engineering*, 5(7), 6218-6226.

681 Liadakis, G. N., Tzia, C., Oreopoulou, V., & Thomopoulos, C.D. (1995). Protein isolation from  
682 tomato seed meal, extraction optimization. *Journal of Food Science*, 60(3), 477-482.

683 Lomascolo, A., Uzan-Boukhris, E., Sigoillot, J. C., & Fine, F. (2012). Rapeseed and sunflower  
684 meal: a review on biotechnology status and challenges. *Applied Microbiology and*  
685 *Biotechnology*, 95(5), 1105-1114.

686 Mamma, D., & Christakopoulos, P. (2014). Biotransformation of citrus by-products into value  
687 added products. *Waste and Biomass Valorization*, 5(4), 529-549.

688 Martínez-Maqueda, D., Hernández-Ledesma, B., Amigo, L., Miralles, B., & Gómez-Ruiz, J. Á.  
689 (2013). Extraction/fractionation techniques for proteins and peptides, and protein  
690 digestion. In *Proteomics in Foods* (pp. 21-50). Springer, Boston, MA.

691 Meng, Z., Wei, S., Qi, K., Guo, Y., Wang, Y., & Liu, Y. (2018). Secondary structure of proteins  
692 on oil release in aqueous enzymatic extraction of rapeseed oil as affected hydrolysis state.  
693 *International Journal of Food Properties*, 21(1), 119-127.

694 Mosenthin, R., Messerschmidt, U., Sauer, N., Carre, P., Quinsac, A., & Schone, F. (2016). Effect  
695 of the desolventizing/toasting process on chemical composition and protein quality of  
696 rapeseed meal. *Journal of Animal Science and Biotechnology*. 7(1), 36.

697 Montealegre, C., Marina, M. L., & García-Ruiz, C. (2010). Separation of olive proteins  
698 combining a simple extraction method and a selective capillary electrophoresis (CE)  
699 approach: application to raw and table olive samples. *Journal of Agriculture and Food*  
700 *Chemistry*, 58(22), 11808-11813.

701 Montealegre, C., García, M. C., del Río, C., Marina, M. L., & García-Ruiz, C. (2012). Separation  
702 of olive proteins by capillary gel electrophoresis. *Talanta*, 97, 420-424.

703 Montealegre, C., Esteve, C., García, M. C., García-Ruiz, C., & Marina, M. L. (2014). Proteins in  
704 olive fruit and oil. *Critical Reviews in Food Science and Technology*, 54(5), 611-624.

705 Nadar, S. S., Rao, P., & Rathod, V. K. (2018). Enzyme assisted extraction of biomolecules as an  
706 approach to novel extraction technology: A review. *Food Research International*, 102,  
707 7239-7255.

708 Negro, V., Mancini, G., Ruggeri, B., & Fino, D. (2016). Citrus waste as feedstock for bio-based  
709 products recovery: Review on limonene case study and energy valorization. *Bioresource*  
710 *Technology*, 214, 806-815.

711 Niaounakis, M & Halvadakis, C.P. (2004) Olive Mill Waste Management – Literature Review  
712 and Patent Survey Typothito, George Dardanos Publications, Athens (2004)

713 Nietzel, T., Dudkina, N.V., Haase, C., Denolf, P., Semchonok, D.A., Boekema, E.J., Braun, H.P.,  
714 Sunderhaus, S. (2013). The native structure and composition of the cruciferin complex in  
715 *Brassica napus*. *Journal of Biological Chemistry*, 288(4), 2238-2245.

716 Nikmaram, N., Leong, S.Y., Koubaa, M., Zhu, Z., Barba, F.J., Greiner, R., Oey, I., &  
717 Roohinejad, S. (2017). Effect of extrusion on the anti-nutritional factors of food products:  
718 An overview. *Food Control*, 79, 62-73.

719 Persia, M. E., Parsons, C. M., Schang, M., & Azcona, J. (2003). Nutritional evaluation of dried  
720 tomato seeds. *Poultry Science*, 82(1), 141-146.

721 Puri, M., Sharma, D., & Barrow, C. J. (2012). Enzyme-assisted extraction of bioactives from  
722 plants. *Trends in Biotechnology*, 30(1), 37-44.

723 Rehder, A., Sulewska, A. M., Markedal, K. E., Sørensen, S., & Sørensen, J. C. (2017). Solubility  
724 of a cruciferin-rich protein product purified from rapeseed pressed cake (*Brassica napus*  
725 L.) by an aqueous processing method. *International Journal of Food Science and*  
726 *Technology-Mysore.*, 52(7), 1653-1659.

727 Rezzadori, K., Benedetti, S., & Amante, E. R. (2012). Proposals for the residues recovery:  
728 orange waste as raw material for new products. *Food and Bioproducts Processing*, 90(4),  
729 606-614.

730 Rodrigues, I. M., Carvalho, M. G. V., & Rocha, J. M. (2017). Increase of protein extraction yield  
731 from rapeseed meal through a pretreatment with phytase. *Journal of the Science of Food*  
732 *and Agriculture*, 97(8), 2641-2646.

733 Rodríguez, G., Lama, A., Rodríguez, R., Jiménez, A., Guillén, R., & Fernández-Bolaños, J.  
734 (2008). Olive stone an attractive source of bioactive and valuable  
735 compounds. *Bioresource Technology*, 99(13), 5261-5269.

736 Romero-García, J. M., Niño, L., Martínez-Patiño, C., Álvarez, C., Castro, E., & Negro, M. J.  
737 (2014). Biorefinery based on olive biomass. State of the art and future  
738 trends. *Bioresource Technology*, 159, 421-432.

739 Rommi, K., Hakala, T. K., Holopainen, U., Nordlund, E., Poutanen, K., & Lantto, R. (2014).  
740 Effect of enzyme-aided cell wall disintegration on protein extractability from intact and  
741 dehulled rapeseed (*Brassica rapa* L. and *Brassica napus* L.) press cakes. *Journal of*  
742 *Agriculture and Food Chemistry*, 62(32), 7989-7997.

743 Rommi, K., Holopainen, U., Pohjola, S., Hakala, T. K., Lantto, R., Poutanen, K., & Nordlund, E.  
744 (2015). Impact of particle size reduction and carbohydrate-hydrolyzing enzyme treatment  
745 on protein recovery from rapeseed (*Brassica rapa* L.) press cake. *Food and Bioprocess*  
746 *Technology*, 8(12), 2392-2399.

747 Roselló-Soto, E., Barba, F. J., Parniakov, O., Galanakis, C. M., Lebovka, N., Grimi, N., &  
748 Vorobiev, E. (2015). High voltage electrical discharges, pulsed electric field, and

749 ultrasound assisted extraction of protein and phenolic compounds from olive  
750 kernel. *Food and Bioprocess Technology*, 8(4), 885-894.

751 Sari, Y.W., Bruins, M.E., & Sanders, J.P.M. (2013). Enzyme assisted protein extraction from  
752 rapeseed, soybean, and microalgae meals. *Industrial Crops and Products* 43, 78–83.

753 Sari, Y.W., Mulder, W.J., Sanders, J.P., & Bruins, M.E. (2015). Towards plant protein refinery:  
754 review on protein extraction using alkali and potential enzymatic assistance.  
755 *Biotechnology Journal*, 10(8), 1138-1157.

756 Savadkoochi, S., & Farahnaky, A. (2012). Dynamic rheological and thermal study of the heat-  
757 induced gelation of tomato-seed proteins. *Journal of Food Engineering*, 113(3), 479-485.

758 Sarkar, A., & Kaul, P. (2014). Evaluation of Tomato Processing By-Products: A Comparative  
759 Study in a Pilot Scale Setup. *Journal of Food Process Engineering*, 37(3), 299-307.

760 Sarkar, A., Kamaruddin, H., Bentley, A., & Wang, S. (2016). Emulsion stabilization by tomato  
761 seed protein isolate: Influence of pH, ionic strength and thermal treatment. *Food*  
762 *Hydrocolloids*, 57, 160-168.

763 Scherhauser, S., Moates, G., Hartikainen, H., Waldron, K., & Obersteiner, G. (2018).  
764 Environmental impacts of food waste in Europe. *Waste management*, 77, 98-113.

765 Searle, S., & Malins, C. (2013). Availability of cellulosic residues and wastes in the EU. *ICCT:*  
766 *Washington, DC, USA*.

767 Serraino, M. R., & Thompson, L. U. (1984). Removal of phytic acid and protein-phytic acid  
768 interactions in rapeseed. *Journal of Agricultural and Food Chemistry*, 32(1), 38-40.

769 Shomer, I. (1991). Protein coagulation cloud in citrus fruit extract. 1. Formation of coagulates  
770 and their bound pectin and neutral sugars. *Journal of Agricultural Food*  
771 *Chemistry*, 39(12), 2263-2266.

772 Siger, A., Józefiak, M., & Górnaś, P. (2017). Cold-pressed and hot-pressed rapeseed oil: The  
773 effects of roasting and seed moisture on the antioxi-dant activity, canolol, and tocopherol  
774 level. *Acta Scientiarum Polonorum Technologia Alimentaria*, 16(1), 69-81.

775 Sogi, D. S., Arora, M. S., Garg, S. K., & Bawa, A. S. (2002a). Fractionation and electrophoresis  
776 of tomato waste seed proteins. *Food Chemistry*, 76(4), 449-454.

777 Sogi, D. S., Garg, S. K., & Bawa, A. S. (2002b). Functional properties of seed meals and protein  
778 concentrates from tomato-processing waste. *Journal of Food Science*, 67(8), 2997-3001.

779 Souilem, S., El-Abbassi, A., Kiai, H., Hafidi, A., Sayadi, S., & Galanakis, C. M. (2017). Olive  
780 oil production sector: environmental effects and sustainability challenges. In *Olive Mill*  
781 *Waste* (pp. 1-28). Academic Press.

782 Thiyam, U., Kuhlmann, A., Stöckmann, H., Schwarz, K. (2004). Prospects of rapeseed oil by-  
783 products with respect to antioxidative potential. *Comptes Rendus Chimie*, 7(6-7), 611-  
784 616.

785 Tomassen, M. M., Barrett, D. M., van der Valk, H. C., & Woltering, E. J. (2007). Isolation and  
786 characterization of a tomato non-specific lipid transfer protein involved in  
787 polygalacturonase-mediated pectin degradation. *Journal of Experimental Botany*, 58(5),  
788 1151-1160.

789 Union européenne. Commission européenne, & EUROSTAT. (2015). *Agriculture, forestry and*  
790 *fishery statistics*. Publications office of the European Union.

791 Vergara-Barberán, M., Lerma-García, M. J., Herrero-Martínez, J. M., & Simó-Alfonso, E. F.  
792 (2014). Efficient Extraction of Olive Pulp and Stone Proteins by using an Enzyme-  
793 Assisted Method. *Journal of Food Science*, 79(7), C1298-C1304.

794 Vergara-Barberán, M., Lerma-García, M., Herrero-Martínez, J., & Simó-Alfonso, E. F. (2015).  
795 Use of an enzyme-assisted method to improve protein extraction from olive leaves. *Food*  
796 *Chemistry*, *169*, 28-33.

797 Vergara-Barberán, M., Mompó-Roselló, Ó., Lerma-García, M. J., Herrero-Martínez, J. M., &  
798 Simó-Alfonso, E. F. (2017). Enzyme-assisted extraction of proteins from Citrus fruits and  
799 prediction of their cultivar using protein profiles obtained by capillary gel  
800 electrophoresis. *Food Control*, *72(A)*, 14-19.

801 Vioque, J., Clemente, A., Sánchez-Vioque, R., Pedroche, J., & Millán, F. (2000). Effect of  
802 Alcalase™ on olive pomace protein extraction. *Journal of the American Oil Chemists'*  
803 *Society*, *77(2)*, 181-185.

804 Vishnevetsky, M., Ovadis, M., & Vainstein, A. (1999). Carotenoid sequestration in plants: the  
805 role of carotenoid-associated proteins. *Trends in plant science*, *4(6)*, 232-235.

806 Wang, W., Scali, M., Vignani, R., Spadafora, A., Sensi, E., Mazzuca, S., & Cresti, M. (2003).  
807 Protein extraction for two-dimensional electrophoresis from olive leaf, a plant tissue  
808 containing high levels of interfering compounds. *Electrophoresis*, *24(14)*, 2369-2375.

809 Yiu, S. H., Poon, H., Fulcher, R. G., & Altosaar, I. (1982). The microscopic structure and  
810 chemistry of rapeseed and its products. *Food Structure*, *1(2)*, 4.

811 Zhang, S. B., Wang, Z., & Xu, S. Y. (2007a). Downstream processes for aqueous enzymatic  
812 extraction of rapeseed oil and protein hydrolysates. *Journal of the American Oil*  
813 *Chemists' Society*, *84(7)*, 693–700.

814 Zhang, S. B., Wang, Z., & Xu, S. Y. (2007b). Optimization of the aqueous enzymatic extraction  
815 of rapeseed oil and protein hydrolysates. *Journal of the American Oil Chemists' Society*,  
816 *84(1)*, 97-105.

817 Zhang, Y., Pan, Z., Venkitasamy, C., Ma, H., & Li, Y. (2015). Umami taste amino acids  
818 produced by hydrolyzing extracted protein from tomato seed meal. *LWT-Food Science  
819 and Technology*, 62(2), 1154-1161.

820 Zhan, F., Shi, M., Wang, Y., Li, B., & Chen, Y. (2019). Effect of freeze-drying on interaction  
821 and functional properties of pea protein isolate/soy soluble polysaccharides complexes.  
822 *Journal of Molecular Liquids*, 285, 658-667.

823 Zinchenko, D. V., Muranova, T. A., Melanyina, L. A., Belova, N. A., & Miroshnikov, A. I.  
824 (2018). Soy and Rapeseed Protein Hydrolysis by the Enzyme Preparation Protosubtilin.  
825 *Applied Biochemistry and Microbiology*, 54(3), 294-300.

826 Zuorro, A., Lavecchia, R., Medici, F., & Piga, L. (2014). Use of cell wall degrading enzymes for  
827 the production of high-quality functional products from tomato processing  
828 waste. *Chemical Engineering*, 38, 355-360.

829 **List of Figures**

830 Fig. 1 The percentages of the total quantities of crops and different categories of crops grown in  
831 Europe. Compiled using data (Union européenne, 2018). The total production of all crops,  
832 vegetables, fruits and oilseeds amounts to 988.8 mT, 64.8 mT, 32.6 mT and 35.0 mT,  
833 respectively.

834 Fig. 2 The percentages of crop waste from processing of 16.3 mT of tomatoes (Scherhauser et al.,  
835 2018), 6.2 mT of oranges (Ferreira-Leitao et al., 2010; Rezzadori et al., 2012), and 10.3 mT of  
836 olives and 21.9 mT of rapeseed (Searle and Malins, 2013). Most of the olives and rapeseeds  
837 remain once the oils have been extracted, while the waste from tomatoes is seemingly low  
838 although 82% of the total weight is composed of moisture and the squeezing of oranges for juice  
839 leaves behind peel, pith and seeds.

840

841 Table 1 The determination of total protein associated with each of the crops (no enzyme) and  
842 protein extracted using different enzymes. A chemical approach was used when the entry is  
843 described as none in the column labelled as enzyme.

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846

Table 1

<b>Sample</b>	<b>Enzyme</b>	<b>Protein</b>	<b>Reference</b>
Olive leaves	None	7.2%	Aydinglu & Sargin, 2013
Olive pomace	None	0.1-1.2%	Montealegre et al., 2014
Olive stones	None	3.2%	Rodríguezb et al., 2008
Olive leaves	Celluclast 1.5L	0.2-0.7%	Vergara-Barberán, Lerma-García, Herrero-Martínez & Simó-Alfonso, 2015
Olive pomace	Palatase 20000	0.1%	Vergara-Barberán et al., 2014
Olive pomace	Alcalase	4%	Vioque et al., 2000
Olive stones	Celluclast 1.5L	0.1%	Vergara-Barberán et al., 2014
Rapeseed	None	33.9%	Lomascolo, Uzan-Boukhris, Sigoillot, & Fine, 2012
Rapeseed meal	None	35-40%	Mosenthin et al., 2016
CPRM	None	40.6%	Fetzer et al., 2018
PPRM	None	34.4%	Fetzer et al., 2018
Rapeseed	Protex proteases	15.8-21.0%	Sari, Bruins, & Sanders, 2013
CPRM	Protease A-01	24.2%	Fetzer et al., 2018
PPRM	Protease A-01	20.8%	Fetzer et al., 2018
Rapeseed meal	Cellic Ctec3	50%	Li & Guo, 2017
Tomato seeds	None	35-58.7%	Sarkar & Kaul, 2014; Zuorro, Lavecchia, Medici, & Piga, 2014; Zhang et al., 2015
Tomato seeds	Papain	50.3%	Zhang et al., 2015
Citrus peel and pulp	None	6.6-9.1%	Mamma & Christakopoulos, 2014
Citrus peel	Celluclast 1.5L	0.5%	Vergara-Barberán et al., 2017

Abbreviations: CPRM cold pressed rapeseed meal; PPRM pre-pressed rapeseed meal